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Plasma level of calcitonin gene-related peptide in patients with polycystic ovary syndrome and its relationship to hormonal and metabolic parameters

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ABSTRACT

The aim of the study was to evaluate the plasma level of calcitonin gene-related peptide (CGRP) in patients with polycystic ovary syndrome (PCOS) and its relationship to hormonal and metabolic parameters. We also observed the effect of CGRP on testosterone (T) and estradiol (E_2) release in cultured human granulosa cells. PCOS subjects (n = 215) and matched healthy control women (n = 103) at age of 22–38 years were enrolled in this study. We analyzed plasma CGRP concentrations, relationship of plasma CGRP with insulin resistance (IR), body mass index (BMI), luteinizing hormone/follicle-stimulating hormone (LH/FSH) ratio and T. The T and E_2 release levels of cultured human granulosa cells treated by CGRP were also measured. The results showed that plasma CGRP concentrations were significantly higher in women with PCOS than those of control subjects. In women with PCOS, there was a strong positive correlation between the plasma CGRP level with HOMA-IR, AUC-insulin, AUC-glucose, the ratio of LH/FSH and plasma T concentration. Human granulosa cells expressed CGRP receptor. Exogenous CGRP caused an elevation of T and E_2 released from the human granulosa cells. These findings suggest that CGRP may participate in the pathophysiological process of PCOS.

1. Introduction

Polycystic ovary syndrome (PCOS) is an endocrine disorder of unknown etiology that affects 5–10% of women of childbearing age [11]. It is characterized by hyperandrogenism, chronic anovulation and polycystic ovaries [13,17]. In addition, women with PCOS frequently exhibit insulin resistance (IR), obesity and inverted ratio of luteinizing hormone (LH) to follicle stimulating hormone (FSH) [13].

Calcitonin gene-related peptide (CGRP) is a 37-amino acid regulatory neuropeptide that is encoded by an alternative processing of calcitonin gene and mainly synthesized in dorsal root ganglia [19]. CGRP mediates its effects through receptor composed of a G protein-coupled receptor called calcitonin receptor-like receptor (CRLR) and receptor activity-modifying protein 1 ($RAMP_1$) [31]. It has been well documented that CGRP exerts complex beneficial cardiovascular effects including potent vasorelaxation and protective effects on cardiomyocytes and endothelial cells [24], and contributes to pain transmission and inflammation responsible for the mechanisms of migraine and skin pain [9,18]. CGRP has also been linked to metabolic disorder including IR and obesity [1,16,21,27,32].

The role of CGRP in the female reproductive system has been reported. The increased mRNA expression of CGRP-receptor component protein in gravid myometrium supports the possibility of involvement of CGRP in the control of myometrial contractility [15]. In addition, CGRP inhibits myometrial contractility at the background of the nonselective nitric oxide synthase inhibitor N^G-nitro-L-arginine methyl ester action [20]. However, the regulating effect of CGRP on ovary function remains unclear. It has been reported that CGRP immunoreactive fibers enter ovary via plexus nerves [6]. Interestingly, it has been demonstrated that by immunolabeling more CGRP-positive nerve fibers exist in dihydroepiandrosterone-induced polycystic rat ovaries than in controls [22]. These findings suggest that CGRP may play a role in the genesis and development of PCOS.

The aim of the present study was therefore to evaluate the plasma level of CGRP in patients with PCOS and its relationship to hormonal and metabolic parameters. We further observed the effect of CGRP on testosterone (T) and estradiol (E_2) levels in cultured human granulosa cells.

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2. Materials and methods

2.1. Subjects and protocols

Two hundred and fifteen women patients diagnosed with PCOS in reproductive and genetic hospital of CITIC-Xiangya admitted between August, 2009 and August 2011 were included in this study. One hundred and three age-matched healthy childbearing age women with husband of azoospermatism or severe oligoasthenospermia were admitted as controls. The PCOS diagnosis was established according to the revised Rotterdam criteria [17]. PCOS was defined as the existence of at least two of the following three features: oligomenorrhea or amenorrhea (<6 menstrual cycles for each year), hyperandrogenism, and polycystic ovaries (PCO) [17]. Hyperandrogenism was defined by clinical hirsutism (Ferriman–Gallwey score ≥7), acne or male pattern alopecia, or elevated androgen levels. The PCO was diagnosed by transvaginal ultrasound, defined as the presence of at least one ovary more than 10 ml or with at least 12 follicles of 2–9-mm diameter. The study was approved by the Institute Review Board of the reproductive and genetic hospital of CITIC-Xiangya and conformed to the principles outlined in the Declaration of Helsinki. All subjects had not taken hormonal contraceptives or steroids and taken laparoscopic ovarian drilling within the previous six months. Enrolled subjects underwent a medical history and physical examination. Clinical features, including menstrual cycle, anthropometric variables, as well as endocrine and biochemical parameters were recorded.

Blood samples were obtained in the early morning in menstrual cycle (days 2–5). The blood containing 10% Na_2 ethylenediaminetetraacetic acid (v/v: 1 ml/20 μl) was centrifuged at 1300 × *g* for 20 min at 4 °C, and plasma was stored at −70 °C until analysis. The plasma concentrations of FSH, LH, E_2 and T were detected by Abbott AxSym (Abbott, USA) based on microparticle enzyme immunoassay. Reagent list numbers for these assays were: FSH, 7A60-20; LH, 7A61-22; E_2, 7A63-20; and T, 3C85-20. Plasma levels of CGRP were assessed by double antibody enzyme linked immunosorbend assay (ELISA, Cusabio, USA) according to the manufacturer's specifications (Catalog No. CSB-E08210h). The plasma was added a protease inhibitor aprotinin 400 mU/L, and CGRP concentrations (ng/L) were extrapolated from a best-fit line calculated from serial dilutions of a CGRP standard. Each sample was run in duplicate and analyzed at a 450 nm wavelength with a microplate reader. This assay recognizes recombinant and natural human CGRP. No significant cross-reactivity or interference was observed. The minimum detectable concentration of human CGRP is typically less than 0.39 pg/ml. The sensitivity of this assay, or lower limit of detection was defined as the lowest protein concentration that could be differentiated from zero.

Blood samples for measurement of fasting plasma glucose and insulin were drawn after overnight fasting after a 3-day normal carbohydrate diet. The PCOS subjects underwent a standard 75 g oral glucose tolerance test and blood samples for measurement of plasma glucose and insulin were drawn before and at 60, 120 and 180 min after glucose ingestion. The plasma glucose was measured by Roche cobas c501 automatic biochemical analyzer based on spectrophotometry (the sensitivity was 0.11 mmol/L). The plasma insulin was measured by Roche cobas e170 automatic biochemical analyzer based on electrochemiluminescence immunoassay (The sensitivity was 0.2 IU/L). Homeostasis model assessment-insulin resistance (HOMA-IR) and the area under the curve (AUC) for insulin and glucose were used to assess IR. HOMA-IR score was calculated according to the following formula [26]: HOMA-IR = [fasting plasma insulin (IU/L) × fasting plasma glucose (mmol/L)]/22.5. The AUC for insulin or glucose was calculated using the trapezoid method as follows [5]: $AUC_{\text{-insulin}} = (0'_{\text{-insulin}} + 180'_{\text{-insulin}})/2 + 60'_{\text{-insulin}} + 120'_{\text{-insulin}}$; $AUC_{\text{-glucose}} = (0'_{\text{-glucose}} + 180'_{\text{-glucose}})/2 + 60'_{\text{-glucose}} + 120'_{\text{-glucose}}$. The control subjects did not undergo a standard 75 g oral glucose tolerance test just because of ethical relativism.

Body mass index (BMI) was calculated as weight (kg) divided by height (m) squared.

2.2. Collection and culture of human granulosa cells

Human granulosa cells were obtained from healthy childbearing age women who received in vitro fertilization treatment because of male factor at the reproductive and genetic hospital of CITIC-Xiangya. The subjects underwent a routine medical checkup to rule out reproductive system inflammation or tumor as well as other organic disease. All subjects received a GnRH analog 1 month before FSH therapy was started; the GnRH analog (1.5 mg, Triptorelin or Leuprorelin) was commenced on the preceding middle luteal phase. Superovulation was induced by daily administration of recombinant human FSH (Gonal-F, Merch Serono, Switzerland) from menstrual cycle days 2–4 and individualized thereafter, depending on plasma E_2, LH, progesterone, FSH levels and measurements of follicular growth by transvaginal ultrasound examination. When leading follicle reached 18 mm and at least three follicles were >16 mm in diameter, a single dose of 5000–10,000 IU human chorionic gonadotrophin was administrated, and the human granulosa cells were aspirated 36 h later, at the time of oocyte retrieval. Granulosa cells were centrifuged out of follicular fluid with a condition of 300 × *g* for 10 min at 37 °C, the underlayer cell pellet and a portion of follicular fluid were collected and then mixed. In order to separate the granulosa cells from blood cells, the cell suspension was layered on Percoll gradient (1:1 on volume, Sigma, USA) carefully and centrifuged at 300 × *g* for 20 min at 37 °C. Granulosa cells precipitated at the Percoll-phosphate buffered saline interface were aspirated and resuspended in phosphate buffered saline. This step was repeated two times for pure granulosa cells and followed with trypsogen treatment for 10–20 min in 37 °C. The cells pellet were then washed with phosphate buffered saline twice and resuspended in TCM-199 medium for culture.

Purified granulosa cells were plated at a density of 10×10^5 cells/well in 24-well dishes and cultured in 500 μl TCM-199 medium supplemented with 10% human serum albumin, 100 U/ml penicillin, and 100 μg/ml streptomycin under the conditions of 37 °C and 95% air–5% CO_2 humidified environment. The granulosa cells were attached to the plastic and separated from red blood cells after overnight incubation. Then the culture medium was replaced and the cells were cultured for 24 h before treatment. Three different concentrations of CGRP (10^{-10}, 10^{-9}, 10^{-8} M) (Sigma, USA. Dissolved in TCM-199 medium) were added into the culture medium, respectively. The control group was given same volume vehicle (TCM-199 medium). After incubation with CGRP for 24 h, the supernatants were collected for determining T and E_2 levels by Abbott AxSym.

2.3. RT-PCR analysis for CGRP receptors

CGRP receptors are composed of two subunits: CRLR and $RAMP_1$. PCR primers were derived from the published sequences of CRLR and $RAMP_1$ [10]. Primer sets were: CRLR (497 bp), 5′-TGCTCTGTGAAGGCATTTAC-3′ (forward), 5′-CAGAATTGCTTGAACCTCTC-3′ (reverse); $RAMP_1$ (447 bp), 5′-GAGACGCTGTGGTGTGACTG-3′ (forward), 5′-TCGGCTACTCTGGACTCCTG-3′ (reverse); β-actin (621 bp), 5′-ACACTGTGCCCATCTACGAGG-3′ (forward), 5′-AGGGGCCGGACTCGTCATACT-3′ (reverse).

Total RNA was isolated from human granulosa cells by use of 1 ml cooled Trizol and 0.2 ml chloroform and precipitated with 0.5 ml isopropanol. The suspension was centrifuged at 12,000 × *g*