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Innervation pattern of polycystic ovaries in the women

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ABSTRACT

The aim of the present study was to determine the changes in both the distribution pattern and density of nerve fibers containing dopamine β-hydroxylase (DβH), vesicular acetylcholine transporter (VAChT), neuronal nitric oxide synthase (nNOS), substance P (SP), calcitonin gene related peptide (CGRP), neuropeptide Y (NPY), vasoactive intestinal peptide (VIP), somatostatin (SOM), galanin (GAL) and pituitary adenylate cyclase-activating polypeptide (PACAP) in the human polycystic ovaries. In the polycystic ovaries, when compared to the immunoreactions pattern observed in the control gonads, following changes were revealed: (1) an increase in the number of DβH-, VAChT-, VIP- or GAL-immunoreactive (IR) nerve fibers within the stroma as well as in the number of DβH-IR fibers near primordial follicles and medullar veins and venules; (2) a reduction in the number of nerve fibers containing nNOS, CGRP, SOM, PACAP within the stroma and in the numbers of CGRP-IR fibers around arteries; (3) an appearance of SP- and GAL-IR fibers around medullar and cortical arteries, arterioles, veins and venules, with except of GAL-IR fibers supplying medullar veins; and (4) the lack of nNOS-IR nerve fibers near primordial follicles and VIP-IR nerves around medullar arteries and arterioles. In conclusion, our results suggest that the changes in the innervation pattern of the polycystic ovaries in human may play an important role in the pathogenesis and/or course of this disorder.

Introduction

Polycystic ovary syndrome (PCOS) is a common endocrinopathy in women of reproductive age (for a review see (Shannon and Wang, 2012)). The major clinical symptoms are excessive hair growth (hyperandrogenism), menstrual irregularities (anovulation), and polycystic ovaries. This triad of symptoms is commonly accompanied by obesity, insulin resistance and infertility. Patients with PCOS are also at increased risk for endometrial carcinoma, diabetes mellitus and cardiovascular disease. Reproductive–metabolic abnormalities include the overproduction of ovarian androgens, increased pituitary luteinizing hormone (LH) secretion, incomplete maturation of ovarian follicle development, and insulin resistance with compensatory hyperinsulinemia. The etiology of PCOS remains uncertain, despite the recognized abnormalities of hypothalamic–pituitary–ovarian function and a disordered metabolic processes (Shayya and Chang, 2010).

It has been reported that not only the density of sympathetic nerve fibers increased in cystic ovaries of women (Heider et al., 2001) rats (Stener-Victorin et al., 2005) and pigs (Jana et al., 2005; Kozlowska et al., 2013) but also the cholinergic (Kozlowska et al., 2009) and sensory (Kozlowska et al., 2011) divisions of ovarian innervation undergo a profound remodeling. Moreover porcine ovaries, in which cyst formation was induced by dexamethasone phosphate disodium salt treatment, also show similar remodeling and increase in nerve fiber density. Furthermore, it has been found that ovulation can be induced following an iatrogenic injury, such as the resection (Krishna et al., 2001) or laparascopic laser cauterization of the ovarian medulla fragment that contains nerves innervating of the ovary (Donesky and Adashi, 1995). This has been shown to be effective in women with PCOS in which hormonal therapy was ineffective. A similar effect was also observed in rats with PCOS after

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unilateral sectioning of the superior ovarian nerves (Balen and Jacobs, 1994). Furthermore, the increased activity of ovarian sympathetic nerves observed in estradiol valerate (EV)-induced polycystic ovaries in rats is related to an overproduction of nerve growth factor (NGF) and its low affinity receptor in the gonads (Manni et al., 2006). Intraovarian fibers are localized around follicles, corpora lutea, blood vessels and interstitial gland as well as within ground plexus in all stages of development. Under physiological conditions, noradrenaline (NA), acetylcholine (ACh), substance P (SP), CGRP, nitric oxide (NO), neuropeptide Y (NPY), vasoactive intestinal peptide (VIP), somatostatin (SOM), galanin (GAL) and pituitary adenylate cyclase-activating polypeptide (PACAP) play important roles in the regulation of ovarian function in women, rodents, cows and pigs. These neurotransmitters participate in follicular development, ovulation and steroidogenesis as well as take part in the ovary blood supply and blood flow through the gonad (Debeljuk and Lasaga, 2006; Morelli et al., 2008; Latini et al., 2010). It has also earlier been indicated that ovarian-related afferent nerves are involved in the transmission of sensory modalities from the ovary to the spinal cord. It is worth mentioning that adrenergic (Liang et al., 2000) and muscarinic (Bodis et al., 2002) receptors along with receptors for SP (Lasaga and Debeljuk, 2011), PACAP (Morelli et al., 2008), NPY (Korner et al., 2004), VIP (Barberi et al., 2007) and GAL (Pang et al., 1999) are expressed in the ovarian cells.

Although many studies have examined the innervation pattern of the ovaries and the importance of neurotransmitters in many physiological processes, little information is available concerning the distribution and density of nerve fibers in the polycystic ovaries of women. Based on the above-mentioned findings, we hypothesize that changes in the innervation pattern of the polycystic ovaries can be important for the course and/or outcomes of this pathological condition. Therefore, the aim of the present study was to determine the changes in the distribution and density of noradrenergic, cholinergic, nitrergic and peptidergic nerve fibers in the ovaries of the women with PCOS, in order to provide better insight into the eventual neuronal mechanism(s) leading to/underlying the PCOS.

Materials and methods

Collection of gonads

Both the control ($n = 9$; patient's age: 31–53 years, mean 42.9 ± 4.1), and polycystic gonads ($n = 9$; patient's age: 34–46 years, mean 39.9 ± 2.2) were obtained from patients of Department of General and Oncological Gynecology (Military Medical Institute, Warsaw, Poland). Polycystic ovary syndrome was diagnosed in women with a history of oligo/amenorrhoea, hirsutism and typical morphological appearance of polycystic ovaries (normal or enlarged ovarian volume with multiple subcapsular cysts <8 mm in diameter) at laparotomy or laparoscopy. According to the patients' history, neither the coincident diabetes or obesity was diagnosed, nor a hormonal therapy was performed in the past. A light microscopic examination of haematoxylin and eosin-stained specimens revealed the presence of numerous immature follicles and a lack of Graafian follicles, as well as functioning corpora lutea. The control ovaries in the follicular phase of the menstrual cycle were obtained from six patients, who underwent hysterectomy for cancer of the uterine cervix. The collection of studied tissues was performed under approval and in accordance with the guidelines of the appropriate bioethical committee.

Single-labelling immunofluorescence

Routine single-immunofluorescence was performed on nine randomly chosen 10-μm-thick, cryostat (Reichert-Jung, Nuβloch, Germany) sections from each ovary. Sections were air-dried at room temperature (RT) for 45 min, rinsed (3×15 min) with phosphate buffered saline (PBS, pH 7.4) and blocked with a blocking mixture consisting of: 10% normal goat serum (MP Biomedicals, USA), 0.1 M in PBS, 1% Triton X-100 (Sigma–Aldrich, USA), 0.05% thimerosal (Sigma–Aldrich, USA) and 0.01% NaN_3 in other to saturate the unspecific binding sites. Sections were then incubated overnight (RT) in the humid chamber, with primary rabbit polyclonal antibodies against DβH, VAChT, SP, CGRP, nNOS, NPY, VIP, SOM, GAL or PACAP (for details see Table 1). Afterwards, sections were rinsed ($3 \text{ min} \times 15 \text{ min}$) and incubated (1 h, RT) with either CY3- or FITC-conjugated F (ab') fragment of a donkey anti-rabbit IgG (diluted 1:5000 or 1:1000, respectively, both from Jackson Immunoreach, USA), rinsed again and mounted with carbonate-buffered glycerol (pH 8.6). The specificity of the primary antisera was tested as follows: (1) sections were incubated with antibodies that had been preabsorbed with a synthetic antigen (10 μg of antigen per milliliter diluted antiserum), (2) the primary antibody was omitted from the incubation, or (3) normal rabbit serum was substituted for the primary antibody. Immunolabelled nerve fibers were analyzed under Olympus BX51 microscope equipped with epi-fluorescence and appropriate filter sets. The sections were examined under the 40× objective and localization and density of the immunoreactive nerve fibres were estimated under the epithelium, within the tunica albuginea and stroma as well as around the follicles and blood vessels. In order to evaluate the differences in the pattern of perifollicular nerve fibres, follicles were, depending on their stage of development, microscopically classified according to McGee and Hsueh (McGee and Hsueh, 2000) into following classes: primordial, primary, secondary and tertiary. The pattern of the perivesicular innervation was determined for arteries, arterioles, veins and venues. Density of nerve fibers was estimated by the use of Merz grid on photographs showing immunostained nerve fibers. The grid printed on the transparency was superimposed on the photographs and the points of intersection between nerve fibers and the grid lines were counted on each photograph to give a score. The nerve fibers supplying the particular ovarian structures were counted on nine sections from each ovary (on fifty sections for each group).

Statistical analysis

To calculate the statistical significance in the mean (±SD) numbers of nerve fibers supplying the particular ovarian structures in the CON and PCOS groups, one-way analysis of variance (ANOVA) followed Bonferroni test was chosen (InStat GraphPad, San Diego, CA). Differences with probability of $P < 0.05$ were considered significant.

Table 1
List of primary and secondary antibody used in this study.

Primary antibody				
Antisera	Code	Host species	Dilution	Supplier
DβH	AB1536	Rabbit	1:4000	Merk Milipore; http://www.merckmillipore.pl
NPY	NA 1233	Rabbit	1:4000	Biomol Research Laboratories Inc., US
SOM	8330-0154	Rabbit	1:4000	Biogenesis Inc., UK; www.biogenesis.co.uk
VIP	11428	Rabbit	1:4000	PROGEN Biotechnik GmbH
NOS	AB5380	Rabbit	1:4000	Merk Milipore; http://www.merckmillipore.pl
LENK	EA 1149-0025	Rabbit	1:4000	Biomol Research Laboratories Inc., US
SP	8450-0505	Rabbit	1:4000	Biogenesis Inc., UK; www.biogenesis.co.uk
VAChT	H-V006	Rabbit	1:4000	Phoenix Europe www.phoenixpeptide.com
GAL	4600-5004	Rabbit	1:4000	Biogenesis Inc., UK; www.biogenesis.co.uk
PACAP	H-052002	Rabbit	1:4000	Phoenix Europe; www.phoenixpeptide
CGRP	AB5920	Rabbit	1:4000	Merk Milipore; http://www.merckmillipore.pl
PHI-27	3668	Rabbit	1:4000	Gift from Department of Anatomy, University of Copenhagen, DK

Secondary antibodies		
Reagent	Dilution	Supplier
CY3-conjugated F(ab') fragment donkey anti-rabbit IgG (H + L)	1:5000	Jackson Immunoreschach, USA; 711-166-152
FITC-conjugated F(ab') fragment donkey anti-rabbit IgG (H + L)	1:1000	Jackson Immunoreschach, USA; 711-096-152