Acupuncture and exercise restore adipose tissue expression of sympathetic markers and improve ovarian morphology in rats with dihydrotestosterone-induced PCOS

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Mannerås L, Cajander S, Lön M, Stener-Victorin E. Acupuncture and exercise restore adipose tissue expression of sympathetic markers and improve ovarian morphology in female rats that received dihydrotestosterone (DHT) continuously, starting before puberty, to induce PCOS. At age 11 wk, rats with DHT-induced PCOS were randomly divided into three groups: PCOS, PCOS plus EA, and PCOS plus exercise. The latter two groups received 2-Hz EA (evoking muscle twitches) three times/week or had free access to a running wheel for 4–5 wk. In mesenteric adipose tissue, expression of β3-adrenergic receptor (ADRB3), nerve growth factor (NGF), and neuropeptide Y (NPY) mRNA was higher in untreated PCOS rats than in controls. Low-frequency EA and exercise downregulated expression of ADRB3, compared with untreated rats with DHT-induced PCOS. EA and exercise improved ovarian morphology, as reflected in a higher proportion of healthy antral follicles and a thinner theca interna cell layer than in untreated PCOS rats. These findings support the theory that increased sympathetic activity contributes to the development and maintenance of PCOS and that the effects of EA and exercise may be mediated by modulation of sympathetic outflow to the adipose tissue and ovaries.

sympathetic activity; β3-adrenergic receptor; androgen receptor; nerve growth factor; neuropeptide Y

THE AUTONOMIC NERVOUS SYSTEM has been suggested to contribute to polycystic ovary syndrome (PCOS) (15, 19, 20, 61). Features of PCOS and the related metabolic syndromes, such as hyperandrogenemia, hyperinsulinemia, insulin resistance, and abdominal obesity, are associated with disturbed activity of the sympathetic nervous system (13, 33). Women with PCOS have increased sympathetic and decreased parasympathetic components of heart rate variability, an indirect measure of cardiac autonomic control (61), and abnormal heart rate recovery, another measure of autonomic function (17). Furthermore, increased ovarian sympathetic nerve activity stimulates androgen secretion in rats (19, 47), and PCOS is associated with an increase in ovarian catecholaminergic nerve fibers (20, 48) and altered catecholamine metabolism (15, 49), suggesting increased sympathetic nervous system activity. Increased sympathetic nerve activity in the ovaries might contribute to PCOS by stimulating androgen secretion (19, 47). Ovarian surgeries reduce the amount of androgen-producing theca cells and the regulation of the hypothalamic- pituitary-ovarian axis is changed, which increases ovulatory response in women with PCOS (21). Additionally, ovarian surgeries may improve ovulatory dysfunction via disruption of ovarian sympathetic innervation.

Recently, we performed direct intra-neural recordings of sympathetic nerve activity in PCOS patients (58). This novel study showed that PCOS is associated with increased sympathetic nervous system activity, which correlated with the elevated testosterone levels that characterize this syndrome. Increased sympathetic outflow might explain the increased prevalence of vascular disease in women with PCOS and might contribute to its etiology.

Obesity and metabolic disturbances exacerbate many of the typical symptoms in women with PCOS and increase the risk of long-term health consequences. Adipose tissue participates in the integrative physiology of whole body glucose and fat metabolism and is innervated by the autonomic nervous system, mainly the sympathetic nervous system, which modulates its metabolic and endocrine functions (6). Alterations in sympathetic activity in adipose tissue can affect the metabolic and endocrine functions of other tissues by altering the secretion of fatty acids and adipokines. Indeed, in obese women with metabolic syndrome, circulatory concentrations and gene expression of nerve growth factor (NGF), a marker of sympathetic activity, are increased in subcutaneous adipose tissue (7). However, the expression of genes encoding sympathetic markers and the influence of therapy on those markers has not been studied in PCOS.

Since PCOS appears to be associated with increased sympathetic nerve activity, interventions thought to modulate sympathetic nerve activity, such as physical exercise (43) and low-frequency electro-acupuncture (EA) (39, 51), might be beneficial. In uncontrolled studies of women with well-defined PCOS and women with undefined ovulatory dysfunction, acu-

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puncture exerted long-lasting beneficial effects on endocrine parameters and anovulation without negative side effects (8, 40, 57). In overweight women with PCOS, exercise together with other lifestyle modification decreases central fat and increases insulin sensitivity and ovulatory function (23, 27). However, those studies were not focused on parameters related to the autonomic nervous system or the mechanisms underlying the effects of EA.

In rats with polycystic ovaries induced with estradiol valerate (EV), we found that low-frequency (2 Hz) EA (3, 37, 53–56) and physical exercise (36) restored the ovarian expression of markers of sympathetic nervous system activity, consistent with the notion that EA and exercise inhibit sympathetic hyperactivity. In rats with PCOS induced with dihydrotestosterone (DHT), we showed that low-frequency EA and physical exercise each reduced insulin resistance and the expression of adipose tissue genes associated with insulin resistance, obesity, and inflammation (35). EA did so without affecting adiposity or adipose tissue cellularity.

Hyperandrogenism is the central feature of PCOS and may be a key factor in the excessive sympathetic tone associated with the syndrome. Our DHT-induced rat PCOS model develops obesity and displays ovarian dysfunction, both of which are associated with high sympathetic activity. Therefore, we aimed to investigate the mRNA expression of the β3-adrenergic receptor (Adrb3), Nfat, NGF receptor (Ngfr), and neuropeptide Y (Npy), all markers of sympathetic nerve activity, and of the androgen receptor (Ar) in adipose tissue in our rat model of DHT-induced PCOS (34). We also assessed the effects of low-frequency EA and physical exercise on the expression of those genes and on ovarian morphology.

**MATERIALS AND METHODS**

**Animals**

Six Wistar dams, each with 8–9 female pups, were purchased from Charles River (Frankfurt, Germany). Pups were raised with a lactating dam until 21 days of age and were then housed four to five per cage under controlled conditions (21–22°C, 55–65% humidity, 12:12-h light-dark cycle). Rats were fed commercial chow containing 18.7% protein, 4.7% fat, 63% carbohydrates, vitamins, minerals (B&K Universal, Sollentuna, Sweden), and tap water ad libitum. Animals were cared for in accordance with the principles of the Guide to the Care and Use of Experimental Animals (49a). The study was approved by the Animal Ethics Committee of the University of Gothenburg.

**Study Procedure**

The study procedure has been described (35). In brief, at 21 days of age, rats were randomly divided into two groups. The DHT-induced PCOS rats (n = 36) were implanted subcutaneously in the neck with 90-day continuous-release pellets (Innovative Research of America, Sarasota, FL) containing 7.5 mg of DHT (daily dose, 83 μg), which induces metabolic disturbances and other characteristics of PCOS in adulthood (34); controls (n = 13) received pellets containing 7.5 mg of vehicle. Forty days later, to compensate for weight gain, an additional pellet was implanted that released 3.5 mg of DHT or placebo for 60 days (i.e., an additional daily dose of 58 μg). A microchip (AVID, Norco, CA) with an identification number was inserted along with the pellets. After 7 wk of DHT exposure, rats in the PCOS group were randomly subdivided into three treatment groups: PCOS (n = 12), PCOS plus exercise (n = 13), and PCOS plus EA (n = 11). The treatments lasted 4–5 wk. The rats were weighed weekly. The study was concluded after ~12 wk of DHT exposure, when the rats were 15–16 wk of age.

**EA.** Low-frequency EA was administered to conscious rats every second weekday for 4–5 wk (12–14 treatments). The treatment procedure has been described in detail previously (35). Each treatment lasted 15 min during the first week, 20 min during weeks 2–3, and 25 min during weeks 4–5. Acupuncture needles were inserted bilaterally into the abdominal and hindlimb muscles, in somatic segments corresponding to the innervation of the ovaries. The intensity was adjusted to produce local muscle contractions and varied from 0.8–1.3 mA.

Before needle insertion, rats were lightly anesthetized with 2% isoflurane (Isoba Vet; Schering-Plough, Stockholm, Sweden) in a 1:1 mixture of oxygen and air for 2–3 min. After needle insertion, the rats were suspended in a fabric harness during EA treatment. To avoid possible acute effects of EA, rats were not treated for 24 h before the experiment was ended.

**Physical exercise.** Rats in the PCOS exercise group were placed individually in cages with an exercise wheel and allowed to exercise voluntarily for 4–5 wk. The exercise wheels were locked 24 h before the experiment was ended to avoid possible acute effects of physical exercise.

Three times per week, rats in the PCOS group and the PCOS exercise group were anesthetized, suspended in a harness, and handled in the same way as rats in the PCOS EA group but without needle insertion or EA stimulation. All rats were conscious during the handling/treatment procedure.

**Vaginal smears.** The stage of cyclicity was determined by microscopic analysis of the predominant cell type in vaginal smears obtained daily from rats at 11 wk of age to the end of the experiment (38).

**Tissue collection, RNA isolation and cDNA synthesis and real-time RT-PCR.** Rats were decapitated at 15–16 wk of age (i.e., after 4–5 wk of treatment and 11–12 wk after pellet implantation). The mesenteric fat depot was dissected, snap frozen in liquid nitrogen, and stored at ~80°C for mRNA analyses. mRNA isolation and real-time RT-PCR were performed as previously described in detail by using a low-density array card (35). Primers and probes for rat genes corresponding to the TaqMan Gene Expression Assay numbers and GenBank accession numbers (Table 1). Gene expression values were calculated with the 2−ΔΔCt method (31), where Ct is cycle threshold. The ovaries were excised, fixed in neutral buffered 4% formaldehyde for 24 h, placed in 70% ethanol, dehydrated, and embedded in paraffin.

**Ovarian morphology.** The ovaries were longitudinally and serially sectioned at 4 μm; every 20th section (n = 5 per ovary) was mounted on a glass slide, stained with hematoxylin and eosin, and analyzed under a conventional bright-field microscope by two persons blinded to the origin of the sections. The slides were scanned with ScanScope (Aperio Technologies, Vista, CA) and analyzed with ImageScope virtual microscopy software (Aperio Technologies). The diameter of the ovaries in the section with the largest ovarian cross section was measured with a calibrated scale tool in the virtual microscope. Antral follicles, distinguished by an antrum within the granulosa cell layers enclosing the oocyte, were counted by two persons (to avoid duplicate counting) and classified as atretic or healthy. Follicles were considered atretic if at least two pyknotic granulosa cells were observed or if the oocyte showed obvious signs of degeneration (22). The thickness of theca interna cell layer was measured in the largest healthy and atretic antral follicles. Corpora lutea were noted but not counted.

**Statistical Analyses**

All statistical evaluations were performed with SPSS software (version 13.0; SPSS, Chicago, IL). Values are reported as means ± SE. The Kruskal-Wallis test was used for comparisons of all groups,
and if significant, a Mann-Whitney U-test was performed between individual groups. $P < 0.05$ was considered significant.

**RESULTS**

**Exercise and EA Influence Ovarian Morphology**

All control rats had a normal estrus cycle of 4 days. Rats in the PCOS group were acyclic, while those in the PCOS exercise and PCOS EA groups exhibited irregular cycles. Ovarian weight and area were lower in the PCOS group than in controls, and neither variable was affected by exercise or low-frequency EA (Table 2).

Morphologically, ovaries from control rats exhibited follicles in various stages of development, ranging from primordial follicles to mature preovulatory follicles, as well as several corpora lutea, many resulting from recent ovulations (Fig. 1, A–B). Some antral follicles contained pyknotic granulosa cells.

In rats with DHT-induced PCOS, small follicles in early development were observed, as were antral follicles with various degrees of atresia: some follicles had nuclear pyknosis in few cells, and others exhibited massive degeneration and granulosa cells in the antrum, as well as degenerated cells containing pyknotic nuclei scattered throughout the membrane granulosa (Fig. 1, C–D). Corpora lutea were absent in all PCOS rats. Although the number of antral follicles was similar in DHT-induced PCOS and control rats, DHT-induced PCOS rats had a higher proportion of atretic antral follicles (Fig. 2) in which the thickness of theca interna was increased (Table 2).

Rats in the PCOS exercise and PCOS EA groups had a lower proportion of atretic antral follicles (Fig. 2), and the theca interna cell layer in those follicles was thinner than in untreated DHT-induced PCOS rats (Table 2). However, both the PCOS exercise and PCOS EA rats had still a significantly higher proportion of atretic antral follicles compared with controls (Fig. 2). In the PCOS EA group, fresh corpora lutea were observed in five of eleven rats (45%) (Fig. 1, E–F), indicating ovulation. In the PCOS exercise group, healthy follicles were observed, but no fresh corpora lutea (Fig. 1, G–H).

**PCOS Increases Gene Expression of Sympathetic Markers in Mesenteric Adipose Tissue**

In mesenteric adipose tissue, expression of Adbrb3, Ngf, and Npy mRNA was higher in rats with DHT-induced PCOS than controls (Fig. 3). Expression of Ar and Ngfr mRNA did not differ in DHT-induced PCOS rats and controls (Fig. 3).

**EA and Physical Exercise Influence the Expression of Sympathetic Markers and AR in Mesenteric Adipose Tissue**

Repeated low-frequency EA and physical exercise for 4–5 wk each lowered DHT-induced changes in the expression of Ngf and Npy mRNA in mesenteric adipose tissue; EA also lowered the expression of Adbrb3 and Ar mRNA (Fig. 3). Neither exercise nor EA influenced Ngfr mRNA expression (Fig. 3). Furthermore, the expression of selected genes after low-frequency EA and physical exercise were not significantly different from the corresponding level in control rats.

**DISCUSSION**

Increased sympathetic activity may be an important etiological factor in PCOS (15, 17, 19, 20, 48, 49, 58, 61). Like the
metabolic syndrome, PCOS is characterized by insulin resistance and obesity, which are associated with enhanced activity of the sympathetic nervous system (28, 59). Recently, it was demonstrated that sympathetic activation is greater in subjects with central obesity, a feature of PCOS, than in those with peripheral obesity (18). These findings underscore the importance of evaluating new treatment strategies that attenuate sympathetic activity in patients with PCOS.

Using a rat model of DHT-induced PCOS, we show here that the expression of several markers of sympathetic activity is increased in mesenteric adipose tissue, a fat depot that affects metabolic status by releasing free fatty acids to the liver via the portal vein. This finding supports the notion that autonomic nervous system is involved in the pathogenesis of PCOS. Low-frequency EA and physical exercise each influenced ovarian morphology and downregulated the expression of several markers of sympathetic nervous activity in mesenteric adipose tissue, indicating that these interventions exert their effects by modulating sympathetic outflow to the adipose tissue and ovaries.

Fig. 1. A: ovary from a normal cycling control rat. B: high-power view shows a recently ovulated collapsed follicle with the apical rupture hole (broken arrow) closed by granulosa cells and perifollicular edema (thin arrow) typical of ovulation and (on the left) an antral follicle containing macrophages (thick arrow) and a trapped oocyte (long thick arrow). C: ovary from a rat with dihydrotestosterone (DHT)-induced polycystic ovary syndrome (PCOS) with small early atretic follicles (EAF) and antral follicles with various degrees of atresia. D: high-power view shows degenerating pyknotic granulosa cells in the antral part of the membrane granulosa in EAF. E: ovary from a rat in PCOS EA group with healthy follicles (HF), atretic follicles (AF), and fresh corpora lutea (FCL). F: high-power view shows FCL and a HF. G: ovary from a rat in the PCOS exercise group with HF, EAF, and old AF (arrows). H: high-power view shows a HF and an EAF.
Mechanisms Behind the Effects of Low-Frequency EA and Physical Exercise

Most likely, low-frequency EA and physical exercise exerted their effects in rats with DHT-induced PCOS by stimulating ergoreceptors and somatic afferents in the muscles, which results in modulation of spinal reflexes and central sympathetic outflow. Previously, we showed that low-frequency EA increases ovarian blood flow and that this effect is mediated as a reflex response via ovarian sympathetic nerves, which in turn is controlled via central nervous system pathways (50). Furthermore, reduction in central sympathetic activity, as reflected by a drop in blood pressure, after low-frequency electrical muscle stimulation is mediated by the release of β-endorphin (24, 25). Neurons that express proopiomelanocortin (the precursor of α-MSH and β-endorphin) and other neuropeptides reside in the hypothalamic arcuate nucleus, a site for the regulation of metabolism and reproduction (9). Hypothetically, low-frequency EA and physical exercise could exert their effects in rats with DHT-induced PCOS by stimulating ergoreceptors and somatic afferents in the muscles, which results in modulation of spinal reflexes and central sympathetic outflow. Previously, we showed that low-frequency EA increases ovarian blood flow and that this effect is mediated as a reflex response via ovarian sympathetic nerves, which in turn is controlled via central nervous system pathways (50). Furthermore, reduction in central sympathetic activity, as reflected by a drop in blood pressure, after low-frequency electrical muscle stimulation is mediated by the release of β-endorphin (24, 25). Neurons that express proopiomelanocortin (the precursor of α-MSH and β-endorphin) and other neuropeptides reside in the hypothalamic arcuate nucleus, a site for the regulation of metabolism and reproduction (9). Hypothetically, low-frequency EA and physical exercise could exert their effects in rats with DHT-induced PCOS by stimulating ergoreceptors and somatic afferents in the muscles, which results in modulation of spinal reflexes and central sympathetic outflow. Previously, we showed that low-frequency EA increases ovarian blood flow and that this effect is mediated as a reflex response via ovarian sympathetic nerves, which in turn is controlled via central nervous system pathways (50). Furthermore, reduction in central sympathetic activity, as reflected by a drop in blood pressure, after low-frequency electrical muscle stimulation is mediated by the release of β-endorphin (24, 25). Neurons that express proopiomelanocortin (the precursor of α-MSH and β-endorphin) and other neuropeptides reside in the hypothalamic arcuate nucleus, a site for the regulation of metabolism and reproduction (9). Hypothetically, low-frequency EA and physical exercise could exert their effects in rats with DHT-induced PCOS by stimulating ergoreceptors and somatic afferents in the muscles, which results in modulation of spinal reflexes and central sympathetic outflow. Previously, we showed that low-frequency EA increases ovarian blood flow and that this effect is mediated as a reflex response via ovarian sympathetic nerves, which in turn is controlled via central nervous system pathways (50).

Furthermore, in women with PCOS, circulating levels of NPY are elevated independently of body mass index (4). Both low-frequency EA and physical exercise resulted in pronounced downregulation of DHT-induced increase of mesenteric Npy mRNA expression, indicating decreased sympathetic activity. Moreover, the mesenteric Npy expression after low-frequency EA and physical exercise were not significantly different from the corresponding level in control rats.

NGF and NGFR. The development and maintenance of sympathetic nerves are facilitated by the target-derived neurotrophin NGF and its low-affinity receptor (NGFR). NGF is secreted from adipocytes and can therefore be considered to be an adipokine (46), which may be involved in communication between adipocytes and sympathetic neurons. NGF expression is closely linked to inflammatory conditions, as TNF-α stimulates NGF production by adipocyte (46). Overweight women and women with metabolic syndrome have high levels of circulating NGF (7), which are associated with insulin resistance, as well as increased levels of IL-6 and leptin. The high circulatory levels of NGF may reflect the increased levels of NGF mRNA in adipose tissue in obese women (7). NGF production in adipose tissue is also increased in genetic models of obesity in animals (44).
**Ngf** expression in mesenteric adipose tissue was higher in rats with DHT-induced PCOS than in controls and was reduced by both low-frequency EA and exercise. This result is in line with the decrease we observed in the high levels of ovarian NGF expression after 4 wk of low-frequency EA and exercise in rats with EV-induced polycystic ovaries (36, 54, 56). Furthermore, the *Ngf* expression level after low-frequency EA and exercise was different from the *Ngf* expression level in control rats.

**ADRB3.** Lipolysis is under control of stimulatory β-ADR and inhibitory α-ADR. Increased release of free fatty acids from visceral fat cells, due to enhanced lipolysis, to the portal venous system has been hypothesized to cause insulin resistance and other metabolic disturbances. In lean women with PCOS, lipolytic activity in visceral adipose tissue is higher than in lean controls (12). This finding might partly explain the increased prevalence of insulin resistance in lean women with PCOS, although obesity has an additive role to insulin resistance in PCOS (11, 41, 58). Moreover, a relationship between upper-body obesity, its associated metabolic complications, and increased visceral fat ADRB3 sensitivity has been reported (26, 32).

In the present study, expression of Adrb3 mRNA in mesenteric adipose tissue was higher in rats with DHT-induced PCOS than in controls, supporting the notion that androgens enhance lipolytic activity in rodents (2). Low-frequency EA downregulated Adrb3 gene expression compared with untreated PCOS rats, and the Adrb3 expression after low-frequency EA was not different from the corresponding level in control rats. Exercise had no effect on Adrb3 gene expression. Lower Adrb3 expression in visceral adipose tissue might decrease lipolytic activity in this depot, a potential explanation for the increased insulin sensitivity induced by EA in our previous study (35).

**AR.** The presence of ARs in preadipocytes and adipocytes suggests that androgens contribute to the control of adipose development and regulation (10). The induction of PCOS in our rat model by continuous administration of DHT did not influence the expression of Ar mRNA in adipose tissue. However, after EA treatment, Ar gene expression in adipose tissue was lower than in untreated rats with DHT-induced PCOS, and did not differ from Ar gene expression in controls.

**Exercise and EA Improve Ovarian Morphology**

Ovarian morphology in DHT-induced PCOS rats has been extensively described elsewhere (34). In evaluating the effects of different treatment modalities, it is important to be aware that the rat is a multiovulatory species. Therefore, the ovarian morphology of our DHT-induced rat PCOS model cannot be directly compared with human polycystic ovaries. For example, the decreased ovarian size, as well as increased proportion of atretic follicles, seen in this rat PCOS model, are not in line with human polycystic ovaries. Nevertheless, both exercise and low-frequency EA positively influenced ovarian morphology. In the PCOS EA groups, we observed fresh corpora lutea in 45% of rats, indicating ovulation, and a decreased proportion of atretic antral follicles, both novel findings. A lower proportion of atretic antral follicles was also observed in the PCOS exercise group. Previously, we showed that ovarian morphological changes are partly reversed by exercise (36) but not by EA (54, 56) in rats with EV-induced polycystic ovaries. A reasonable explanation for this discrepancy might be that rats in the present study were conscious during EA but were anesthetized in our previous studies. Regarding the exercise, rats with EV-induced polycystic ovaries ran longer distances than rats with DHT-induced PCOS in the present study. We found no differences in ovarian weight or area after EA or exercise treatment. Since corpora lutea were observed in the EA group, one would expect increases in ovarian weight and area. However, the EA group had fewer antral follicles than untreated rats with DHT-induced PCOS, which might explain why weight and area were unaffected.

Furthermore, the theca interna cell layer was thinner in both the PCOS EA and PCOS exercise groups than in PCOS rats. Since the theca interna is innervated by sympathetic neurons and is important in steroidogenesis (47), the reduced thickness might reflect inhibition of ovarian sympathetic neurons. Previously, we showed that low-frequency EA increases ovarian blood flow (50, 52). Transection of the ovarian sympathetic nerves eliminated this response and also reversed the changes in ovarian morphology induced by EV in rats with polycystic ovaries (5). These findings confirm the role of the sympathetic nervous system in the control of ovarian function. Further supporting the involvement of sympathetic nervous system in PCOS, women with the syndrome have significantly higher NGF levels in the follicular fluid than controls (16).

**Perspectives and Significance**

Previously, we found that women with PCOS had increased sympathetic nerve activity related to hormonal and metabolic features and that both testosterone and cholesterol were independent predictors of such activity, with testosterone having the stronger impact (60). It is not yet clear whether increased sympathetic activity is an etiologic factor in PCOS or a consequence of hyperandrogenism. However, our findings support the theory that increased sympathetic activity contributes to the development and maintenance of PCOS. Thus, therapies aimed at reducing sympathetic nerve activity might alleviate the signs and symptoms of PCOS.

**Conclusion**

In rats with DHT-induced PCOS, low-frequency EA and exercise each reduced the expression of genes encoding markers of sympathetic activity in adipose tissue and had beneficial effects on ovarian morphology, reflecting modulation of sympathetic outflow to the adipose tissue and ovaries.

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