Effect of anti-NGF on ovarian expression of α₁- and β₂-adrenoceptors, TrkA, p75NTR, and tyrosine hydroxylase in rats with steroid-induced polycystic ovaries

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Submitted 4 February 2005; accepted in final form 26 September 2005

Manni, Luigi, Agneta Holmäng, Stefan Cajander, Thomas Lundeborg, Luigi Aloe, and Elisabet Stener-Victorin. Effect of anti-NGF on ovarian expression of α₁- and β₂-adrenoceptors, TrkA, p75NTR, and tyrosine hydroxylase in rats with steroid-induced polycystic ovaries. Am J Physiol Regul Integr Comp Physiol 290: R826–R835, 2006. First published September 29, 2005; doi:10.1152/ajpregu.00078.2005.—Estradiol valerate (EV)-induced polycystic ovaries (PCO) in rats are associated with higher ovarian release and content of norepinephrine, decreased β₂-adrenoceptors (ARs), and dysregulated expression of α₁-AR subtypes, all preceded by an increase in the production of ovarian NGF. The aim of this study was to further elucidate the role of NGF in the ovaries by blocking the action of NGF during development of EV-induced PCO in rats. Control and EV-injected rats were treated with intraperitoneal injections of IgG (control and PCO groups) or with anti-NGF antibodies (anti-NGF and PCO anti-NGF groups) every third day for 5 wk starting from the day of PCO induction. Rat weight, estrous cyclicity, ovarian morphology, ovarian mRNA, and protein expression of α₁-AR subtypes, β₂-AR, the NGF receptor tyrosine kinase A (TrkA), p75 neurotrophin receptor (p75NTR), and tyrosine hydroxylase (TH) were analyzed. Ovaries in both PCO and PCO anti-NGF groups decreased in size as well as in number and size of corpora lutea. mRNA expression of α₁a-AR and α₁d-AR in the ovaries was lower, whereas expression of α₁b- and α₁c-AR and TH was higher, in the PCO group than in controls. Protein quantities of α₁-ARs, TrkA, p75NTR, and TH were higher in the PCO group compared with controls, whereas the protein content of β₂-AR was lower. Anti-NGF treatment in the PCO group restored all changes in mRNA and protein content, except that of α₁b-AR and TrkA mRNAs, to control levels. The results indicate that the NGF/NGF receptor system plays a role in the pathogenesis of EV-induced PCO in rats.

nerve growth factors; sympathetic nervous system; ovarian innervation; tyrosine kinase A; p75 neurotrophin receptor

POLYCYSTIC OVARY SYNDROME (PCOS) is a heterogeneous condition of uncertain etiology that affects between 5% and 10% of women of reproductive age (41). PCOS is characterized by a myriad of symptoms and signs, which include ovulatory dysfunction, the presence of polycystic ovaries (PCO), abdominal obesity, hyperandrogenism, and in many cases hypertension and insulin resistance (41). Several theories have been proposed to explain the pathogenesis of PCOS, such as an enhanced production of ovarian androgens, an alteration in the metabolism of cortisol resulting in enhanced production of adrenal androgens, a defect in the action and secretion of insulin, and a neuroendocrine defect that causes an exaggeration in the pulse and frequency amplitude of LH (41).

The involvement of the sympathetic nervous system in the etiology and maintenance of PCOS is suggested by both clinical and experimental findings (5, 20, 22, 24, 25). Ovarian function is known to be regulated by both hormonal and intracrine signaling. It is also known that the sympathetic innervation of the ovary is involved in follicular development (23) and in ovarian steroidogenesis (1). That the sympathetic nervous system is involved in the pathophysiology of human PCOS is supported by the fact that the innervation of the catecholaminergic nerve fibers in the PCO of women with PCOS is more dense than in normal ovaries (18, 35).

Chronic anovulation and PCO can be induced by a single intramuscular injection of estradiol valerate (EV) in female rats (6). We demonstrated previously in a dose-response study (38) that rats injected with a dose of 4 mg of EV dissolved in 0.2 ml of oil exhibit a progressive decrease in the number of primary and secondary follicles after 30 days and that 60 days after the EV injection true cystic follicles were found and the well-defined PCO was fully developed in accordance with previous reports by Brawer et al. (6). The EV-induced rat PCO model reflects some endocrinologic and morphological characteristics of human PCOS, and it is assumed that activity in the ovarian sympathetic nerves of the rats in this model is higher than in the nerves of normal rats (5, 22). This is evidenced by a higher release and content of norepinephrine (NE) in the ovaries, together with a lower expression of β₂-adrenoceptors (ARs) in the ovarian compartment (5, 22). Transection of the sympathetic nerves arriving at the ovary via the superior ovarian sympathetic nerves reduces steroid response, increases β₂-AR expression to more normal levels, and restores estrous cyclicity and ovulation in rats with EV-induced PCO to normal levels (5).

It appears that the higher activity of the ovarian sympathetic nerves of rats with EV-induced PCO is related to an increased synthesis of ovarian NGF—a target-derived neurotrophin—and its low-affinity receptor, p75 neurotrophin receptor (p75NTR) (20, 37, 38). Intravarian blockade of NGF/p75NTR action with an antiseraum to NGF and an antisense oligodeoxynucleotide to p75NTR restores estrous cyclicity and ovu-
tory capacity in PCO rats (20). In the ovary, NGF and both of its receptors, p75NTR and the high-affinity receptor tyrosine kinase A (TrkA), are synthesized in thecal cells (13, 15). It is also known that p75NTR facilitates transfer of NGF from its sites of production to NGF-sensitive fibers (8) as well as collaborating with the high-affinity Trk/A receptor to potentiate cellular response to neurotrophin (17). However, the expression of TrkA has not, to our knowledge, been investigated in the ovarian cellular response to neurotrophin (17). However, the expression of TrkA has not, to our knowledge, been investigated in the ovary of rats with steroid-induced PCO.

Recently, electrical stimulation of the splanchnic nerve has been shown to decrease ovarian blood flow (OBF) via the activation of α-AR in the ovarian blood vessels (42); this was confirmed by an injection of phenotamine, an α1-AR agonist, and indicates an involvement of the α1-AR in the regulation of ovarian function. In a recent study (27) we showed for the first time that all α1-AR subtypes are expressed in the ovaries of healthy rats and that the mRNA and protein contents of these α1-AR subtypes are dysregulated after induction of PCO in rats.

In the present study, the aim was to determine the role of NGF in the mRNA and protein expression of ovarian α1α-, α1β-, and α1γ-AR, β2-AR, p75NTR, TrkA, and tyrosine hydroxylase (TH) and in ovarian morphology by blocking ovarian NGF action with repeated intraperitoneal injections of antibodies against NGF in the development of EV-induced PCO.

MATERIALS AND METHODS

Animals. Forty virgin adult cycling female Wistar rats (Charles River Wiga, Uppsala, Sweden) weighing 205–230 g were housed four to each cage at a controlled temperature of 22°C with a 12:12-h light-dark cycle for at least 1 wk before and throughout the experimental periods. The rats had free access to pelleted food and tap water. The rats were divided into four experimental groups: 1) control group (n = 10), 2) anti-NGF group (n = 10), 3) PCO group (n = 10), and 4) PCO anti-NGF group (n = 10). Twenty rats, those in the PCO groups, were given a single intramuscular injection of 4 mg of EV (Riedeldehaen) in 0.2 ml of oil to induce well-defined PCO (6). Twenty rats, those in the control group and the anti-NGF group, received a single intramuscular injection of 0.2 ml of oil (arachidic oleum, Apoteket, Umeå, Sweden) only. Thirty to thirty-three days after intramuscular injection of EV, the rats were killed by decapitation. Before decapitation, the rats were anesthetized with 125 mg/kg body wt of thiobutarbarital sodium (Inactin, RBI, Natick, MA). The experiments were carried out according to the principles and procedures outlined in the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals and were approved by the local animal ethics committee at Göteborg University.

Vaginal smear. Estrous cyclicity was monitored by vaginal smear obtained between 8:00 AM and 12:00 PM for 10 consecutive days before the experiment to measure ovarian functionality. The different stages of the estrous cycle were determined according to the predominant cell type present in the vaginal smears. The control and anti-NGF groups were killed in estrus because the estradiol levels are low at that stage, and the PCO and PCO anti-NGF groups were killed in estrus or in a pseudopregnant stage as described previously (26, 40).

Anti-NGF and IgG treatment. NGF plays a crucial role in the maintenance of sympathetic neuron phenotype and metabolic functions and in the regulation of sympathetic innervation and plasticity (33). We previously described (26, 40) the use of anti-NGF antibodies to block NGF action and/or induce biological effects opposite to those elicited by NGF. In the present study, the control groups (control and PCO groups) were given 0.5-ml intraperitoneal injections of premune goat IgG (500 μg/rat) produced and purified in our laboratory. The experimental groups (anti-NGF and PCO anti-NGF groups) were given on 0.5-ml intraperitoneal injection of goat anti-NGF IgG (500 μg/rat) produced and purified as previously described (3). Injection was given every third day, beginning on the day of PCO induction, in total 8–10 treatments/rat, and the experiment was finalized within 3 days after the last immunization.

Tissue collection. At the completion of the experiment, the rats were decapitated in estrus or pseudoestrus, as described under Vaginal smear, and the ovaries were quickly dissected on dry ice. One ovary was divided in two pieces, weighed, snap frozen in liquid nitrogen, and stored at −80°C until extraction. The second ovary was weighed and fixed in buffered 4% formaldehyde for at least 24 h for morphological analyses. Ovarian morphology. The ovaries were dehydrated after 24 h of formaldehyde fixation and imbedded in paraffin. They were partially serially sectioned (4 μm, every 10th section mounted on the glass slide) and stained with hematoxylin and eosin. The sections, blinded to grouping, were analyzed under a conventional brightfield light microscope. There was no intention to quantify the number of corpora lutea or growing or atretic follicles but rather to establish whether ovulation with corpora lutea formation had occurred within the given time frame. According to morphometric (stereological) and statistical principles, there is no need to perform a statistical analysis in this situation.

Real-time PCR for AR, TH, and TrkA. Total RNA from the ovary was extracted with RNeasy Mini kits (Qiagen, Hilden, Germany). PCR analysis was performed with the ABI Prism 7700 Sequence Detection System (PE Applied Biosystems, Stockholm, Sweden) and FAM-labeled probes specific for the α1α-AR (Rn00567876_m1), the α1β-AR (ADRA1B-EX 152027A02), the α1γ-AR (Rn00577931_m1), the β2-AR (Rn00560650_s1), TH (NM_012740), and TrkA (NM_021589) (PE Applied Biosystems). Designed primers and a VIC-labeled probe for GAPDH (NM_031144) were included in the reactions as an internal standard. The cDNA was amplified under the following conditions: 1 cycle at 50°C for 2 min and 95°C for 10 min, followed by 40 cycles at 95°C for 15 s and 60°C for 1 min. The amount of mRNA of each gene was calculated with the standard curve method (following the instructions in PE Applied Biosystems User Bulletin no. 2) and adjusted for the expression of GAPDH.

RT-PCR-ELISA for p75NTR. The expression of p75NTR mRNA was evaluated with a RT-PCR ELISA protocol exactly as described by Tirassa and coworkers (39). Total RNA was extracted from the ovaries by the method of Chomczynski and Sacchi (9) as modified in the TRizol kit (Invitrogen, Lidingö, Sweden). Complementary DNA was synthesized from 1 μg of total RNA, using 200 U of Moloney murine leukemia virus reverse transcriptase (Promega Italia, Milan, Italy) in 20 μl of total volume reaction. p75NTR and GAPDH genes were amplified in a single-tube PCR reaction (35 cycles; 1 min at 95°C, 1 min at 55°C, 2 min at 72°C), using 5'-biotinylated specific primers to generate biotinylated PCR products detectable by digoxigenin-labeled probes in an immunoenzymatic assay. Primer/probe sequences are as follows: p75NTR biotinylated forward: 5'-CCGTGT-CTCCATGGGAGGACCA-3'; p75NTR reverse: 5'-GAGATGGCCTGCT-CTGCGTGTTG3'; p75NTR digoxigenin-labeled probe: 5'-ACAG-CAGCCCAAGTGGAGCCTAACAGCAGG-3' (GenBank M14764); relative positions of forward and reverse primers of the probe: 518–537, 1020–1039, 873–901, respectively; predicted size of the fragment: 521 bp); GAPDH biotinylated forward: 5'-CACCCACGTAAGCCACCA-3'; GAPDH reverse: 5'-GATGATGTGCTTGGCCAGG-3'; GAPDH digoxigenin-labeled probe: 5'-AAATCTTCTGATTCTGCTCTATATTTCCG-3' (GenBank M23599); relative positions of forward and reverse primers and of the probe: 346–365, 517–536, 451–480, respectively; predicted size of the fragment: 290 bp). The amount of amplified products was measured at an optical density (OD) of 450/690 nm (OD 450/690), using a Dynatech ELISA Reader 5000. A GAPDH level of OD 450/690 was used to normalize the relative differences in sample size, differences in integrity of the
individual RNA, and variations in reverse transcription efficiency. For exact methodological details see Tirassa et al. (39).

Western blotting analysis for ARs, TH, TrkA, and p75NTR proteins. Commercially available antibodies were used for detection by Western blotting of the $\alpha_{1a}$-AR ($\alpha_{1a}$-AR [C-19]; sc-1477, Santa Cruz), the $\alpha_{1b}$-AR ($\alpha_{1b}$-AR [C-18]; sc-1476, Santa Cruz), the $\alpha_{1d}$-AR ($\alpha_{1d}$-AR [H-142]; sc-10721, Santa Cruz), the $\beta_{2}$-AR ($\beta_{2}$-AR [M-20]; sc-1570, Santa Cruz), TH (AB5986, Chemicon International), TrkA (sc-118, Santa Cruz), and $\beta$-actin (sc-8432, Santa Cruz). The Western blotting detection of $p75^{NTR}$ was carried out with monoclonal anti-$p75$ antibody (clone 192; Ref. 7) purified in our laboratory. Tissue samples were homogenized in lysis buffer (0.1 M Tris-HCl buffer pH 7.6, containing 0.1 M NaCl, 1 mM EDTA, 1 mM EGTA, 2 mM PMSF, 50 $\mu$M leupeptin, 100 $\mu$g/ml pepstatin, and 100 $\mu$g/ml aprotinin) at 4°C. After centrifugation at 8,000 g for 20 min, the supernatants underwent Western blotting. Samples (30 $\mu$g total protein) were dissolved with loading buffer (0.1 M Tris-HCl buffer pH 6.8, containing 0.2 M DTT, 4% SDS, 20% glycerol, and 0.1% bromophenol blue), separated by 12.5% SDS-PAGE, and electro-photographically transferred to polyvinylidene difluoride membranes for 3 h. The membranes were incubated for 40 min at room temperature with blocking buffer (10% nonfat dry milk, 10 mM Tris pH 7.5, 100 mM NaCl, 0.1% Tween 20). Membranes were washed three times for 10 min each at room temperature in 10 mM Tris pH 7.5, 100 mM NaCl, 0.1% Tween 20 (TTBS), followed by an incubation for 1 h at room temperature with primary antibodies. Membranes were washed three times for 10 min each at room temperature in TTBS and incubated for 1 h with horseradish peroxidase-conjugated anti-rabbit IgG, horseradish peroxidase-conjugated anti-goat IgG, or horseradish peroxidase-conjugated anti-mouse IgG as the secondary antibody. The blots were developed with enhanced chemiluminescence (Amersham Bioscience) as the chromophore. Similar results were obtained in five independent Western blot runs. Band densitometry evaluation, expressed as arbitrary units of gray level, of five different gel runs/blot, was made on a Macintosh computer with the public domain NIH Image program (developed at NIH, Bethesda, MD; available at http://rsb.info.nih.gov/NIH-image/), which determines the OD of the bands with a gray scale thresholding operation. The OD of $\beta$-actin bands was used as a normalizing factor.

Statistical analyses. All data were analyzed with the software package StatView for Macintosh (Abacus Concepts, Berkeley, CA). The effect of EV injection and/or anti-NGF treatment on the weight of the rats was analyzed with ANOVA considering the repeated measures (5 tests) and the treatments (4 levels: control, anti-NGF, PCO, and PCO anti-NGF). mRNA expression, OD data for Western blot analysis, and ELISA of $p75^{NTR}$ in the ovaries were evaluated by two-way ANOVA and Fisher’s paired least significant difference post hoc test. All results are reported as means ± SE. A $P$ value <0.05 was considered significant.

Fig. 2. A: partial restoration of estrous cyclicity in PCO rats injected with anti-NGF IgG. Left: estrous cycle of 4 representative control rats receiving goat IgG. Most of the rats displayed a normal 4-day cycle. The injection of anti-NGF in control animals had no affect on estrous cyclicity. The estrous cycle of PCO rats, however, was disrupted, with persistent proestrous/estrous phases. The normal estrous cycle was partially restored in the PCO anti-NGF group, with most of the rats showing the occurrence of diestrous phases of the cycle. The different stages of the estrous cycle (depicted on vertical axis) were determined according to the predominant cell type present in vaginal smears (P, proestrus; E, estrus; D1, diestrus day 1; D2, diestrus day 2). B–D: ovarian morphology: a typical rat ovary from the control group with sequentially higher magnifications in B through D. Boxes indicate where the next higher power view was taken. In this ovary there are growing nonatric follicles and fresh corpora lutea (CL) with vasculization and luteal cell maturation still going on as demonstrated by the incomplete central development. In B a group of oocytes are observed in the dilated tube (only seen under higher magnification) at top left (arrow). E–G: a typical ovary from the PCO group. When compared with the corresponding photos from the control group (magnifications similar in the vertical columns), it is obvious that this ovary is smaller in size. Antral follicles are mainly atretic and often cystic, CL and luteal cells are smaller than in control ovaries, and signs of regression with foam cells can be observed. No young CL are observed. H–J: a typical ovary from the PCO anti-NGF group. CL are larger than in the PCO group, and cystic fluid-filled atretic follicles are missing. CL appear younger than in the PCO group, with larger lipid-containing lutein cells; sometimes these CL were as large as in the oil control group, but fresh CL with eggs in the tube or ampullae were not found.
As shown in Fig. 2, B–D, the ovaries in the oil-treated groups were normal in appearance, with large corpora lutea and follicles in different stages of growth and regression. The ovaries in both the PCO (Fig. 2, E–G) and the PCO anti-NGF (Fig. 2, H–J) groups displayed decreased ovarian size as well as a decrease in number and size of the corpora lutea compared with the ovaries in the oil-treated groups. The corpora lutea in the PCO anti-NGF group were slightly larger than in the PCO group, and there were more “healthy” nonatretic follicles. As illustrated in Fig. 2, there were more cystically dilated atretic follicles in the PCO group than in the PCO anti-NGF group. Compared with the control ovaries, we never observed fresh corpora lutea or signs of recent ovulations with eggs in the tube in either of the PCO groups.

Low \( \alpha_{1a} \)-AR mRNA expression and high protein levels in ovaries of rats with EV-induced PCO are restored to control levels by anti-NGF treatments. As seen in Fig. 3A, the mRNA expression of \( \alpha_{1a} \)-AR was lower in the PCO group than in the control group. \( \alpha_{1a} \)-AR mRNA expression in the ovaries of PCO rats that had undergone repeated anti-NGF treatment was similar to the values in the control group.

High \( \alpha_{1b} \)-AR mRNA expression and protein levels are modulated by anti-NGF treatments in rats with EV-induced PCO. mRNA expression of \( \alpha_{1b} \)-AR is shown in Fig. 4. The expression of \( \alpha_{1b} \)-AR was significantly higher in the PCO group compared with the control group. Anti-NGF treatment influenced the expression of \( \alpha_{1b} \)-AR mRNA in neither the anti-NGF nor the PCO anti-NGF group compared with the control group and the PCO group, respectively.

As shown in Fig. 4B, the amount of ovarian \( \alpha_{1b} \)-AR protein was higher in the PCO group than in the control group. The amount of \( \alpha_{1b} \)-AR protein in the ovaries of PCO rats that had undergone repeated anti-NGF treatment, however, was similar to levels in the control groups.

High \( \alpha_{1c} \)-AR mRNA expression and protein levels are modulated by anti-NGF treatments in rats with EV-induced PCO. The expression of \( \alpha_{1c} \)-AR mRNA (see Fig. 5A) was significantly higher in the PCO group compared with the control group. Anti-NGF treatment influenced the expression of \( \alpha_{1c} \)-AR mRNA in neither control nor PCO rats. The amount of \( \alpha_{1c} \)-AR protein in the ovaries of PCO rats that had undergone repeated anti-NGF treatment was similar to levels in the control groups.

High \( \alpha_{1d} \)-AR mRNA expression and protein levels are modulated by anti-NGF treatments in rats with EV-induced PCO.

The amount of ovarian \( \alpha_{1a} \)-AR protein (Fig. 3B) was higher in the PCO group than in the control group. The amount of \( \alpha_{1a} \)-AR protein in the PCO rats that had undergone repeated anti-NGF treatment was similar to the values in the control group.
group. mRNA expression of α₁d-AR in the ovaries of PCO rats that had undergone repeated anti-NGF treatment, however, was significantly lower than in the control group.

The ovarian protein amount of α₁d-AR in the PCO group was significantly higher compared with that in the control group (Fig. 5B). A reduction in α₁d-AR content to control values was found in the PCO anti-NGF group.

Low levels of β₂-AR protein are modulated by anti-NGF treatments in rats with EV-induced PCO. The mRNA expression of β₂-AR (Fig. 6A) was unchanged in the PCO group compared with the control group. Anti-NGF treatment did not alter the mRNA expression of β₂-AR in PCO rats.

The ovarian amount of β₂-AR protein (see Fig. 6, B and C) was lower in the PCO group compared with the control group. Anti-NGF treatment restored levels of β₂-AR protein in the PCO anti-NGF group to those in the control group.

High levels of ovarian p75<sup>NTR</sup> protein are lower after anti-NGF treatment in rats with EV-induced PCO. The mRNA expression of TrkA (Fig. 8A) was significantly lower in the PCO group compared with the control group. The mRNA expression of TrkA in the ovaries of PCO rats that had undergone repeated anti-NGF treatment (the PCO anti-NGF group) was still significantly lower than in the control group and differed nonsignificantly from that in the PCO group.

The ovarian amount of TrkA protein (Fig. 8B) was significantly higher in the PCO group compared with the control group. PCO rats who had undergone anti-NGF treatment had a
lower amount of TrkA protein than PCO rats who had not (the PCO group) but the same protein level as rats in the control group.

High mRNA expression and protein levels of TH are down-regulated by anti-NGF treatment in rats with and without EV-induced PCO. Expression of TH mRNA (see Fig. 9A) was significantly higher in the PCO group than in the control group. Although the effect of repeated anti-NGF treatments on the mRNA expression of TH in PCO rats (the PCO anti-NGF group) was not significant compared with in PCO rats who had not undergone anti-NGF treatment (the PCO group), the difference in TH mRNA expression between the PCO anti-NGF group and the control group was no longer significant.

We found that the ovarian protein amount of TH (Fig. 9B) was significantly higher in the PCO group compared with the control group. Although the TH protein level in PCO rats that had undergone anti-NGF treatment was lower than in PCO rats, the TH protein level in the PCO anti-NGF group was still significantly higher than in the control group.

DISCUSSION

The present study aimed to investigate the role of endogenous NGF in the ovarian expression of the sympathetic markers α1-AR, β2-AR, TH, p75NTR and TrkA. This was achieved by repeated intraperitoneal treatments with neutralizing anti-NGF antibodies in control and EV-treated rats. The novel findings obtained in the present study were that repeated systemic anti-NGF treatments in EV-injected rats modulate most of the measured sympathetic markers. Furthermore, the estrous cycle, which had been disrupted by EV injection, was also partially restored as well as the ovarian morphology of PCO rats that underwent anti-NGF treatment.

It has been reported that rats with EV-induced PCO develop ovarian follicular cysts that are preceded by an increased synthesis of NGF in the ovary (20). In the present study, we found a difference in ovarian morphology between the control and PCO groups, where the latter displayed a decrease both in ovarian size and in the number and size of corpora lutea. Interestingly, ovarian morphology was partially restored after repeated anti-NGF treatments in the PCO anti-NGF group.
compared with the PCO group. No difference was found between the groups regarding ovarian weight. However, there were more atretic follicles in the PCO group, and many of them were dilated or even cystic compared with the PCO anti-NGF group. This, together with the observation of larger corpora lutea in the PCO anti-NGF group than in the PCO group, means that there is more cellular and active (according to our other data) tissue in the anti-NGF-treated PCO ovaries because the ovarian weights were equal in the two groups. The fluid-filled, dilated atretic follicles in the PCO group might be the reason for the lack of ovarian weight reduction found in previous studies (19).

It has been shown that an ovary-specific increase in NGF availability leads to disruption of the estrous cycle and to changes in follicular dynamics similar to some of the alterations produced by EV treatment (14). Furthermore, estrous cyclicity in EV-treated rats was restored by the ovarian application of anti-NGF antibodies and a p75NTR antisense mRNA oligodeoxynucleotide. The present data on the partial restoration of estrous cyclicity in PCO rats by intraperitoneal anti-NGF treatment confirm the importance of NGF in the regulation of follicle development and indicate the pathogenetic role of the neurotrophin in rat PCO. It is worth mentioning that we obtained results similar to those achieved by Lara’s group (20), although they administered anti-NGF treatment locally instead of systemically. This implies that both a local and a systemic component of the NGF/NGF receptor system may be active in the regulation of ovarian pathophysiology. It is known that NGF can directly affect ovarian function through the activation of both the p75NTR and TrkA receptors expressed by several types of ovarian cells (13, 20). Nevertheless, it cannot be excluded that intraperitoneal injections of NGF antibodies trigger a systemic response, modulating both central sympathetic outflow and the sympathetic drive to the ovaries, which in turn affects estrous cyclicity (16, 22).

PCO induction in adult rats by EV injection causes a decrease in body weight gain. A variety of studies have demonstrated that activation of the sympathetic nervous system can increase metabolic rate and fat consumption, leading to a reduction in body weight (30, 32). The NGF/NGF receptor system could participate in this response to EV injection. The evidence that NGF is overexpressed in the brain of PCO rats (4) and that intracerebroventricular administration of NGF has an anorectic effect (44) provides reasonable support for the hypothesis that the NGF/NGF receptor system expressed in the central nervous system, in particular by hypothalamic neurons, participates in this behavioral/metabolic response. The finding that the systemic blockade of endogenous NGF counteracts the anorectic effect of EV injection in the present study supports this hypothesis.

A novel result of the present study is that intraperitoneal injection of highly purified anti-NGF IgG in rats with EV-induced PCO causes significant changes toward normal levels in the α1-AR protein subtypes and gene expression in the ovaries. In a recent study (27) we found that the ovarian expression of the three α1-AR subtypes was affected by EV injection in the PCO model used in this study. The present study confirms and extends our previous data. Here we describe—for the first time to the best of our knowledge—evidence of control exerted by endogenous NGF on the ovarian expression of α1-ARs. Thus a reasonable hypothesis is that the sympathetic nervous system regulates the expression and content of the α1-AR subtypes because systemic blockade of the NGF action normalizes the EV-induced changes. The increased expression of ovarian α1a-AR protein content in PCO rats is in line with the finding that spontaneously hypertensive rats exhibit increased central α1a-AR expression and decreased α2a-AR expression that are correlated with elevated blood pressure and increased sympathetic activity (31). The functional significance of the α1-ARs in the ovaries of PCO rats has not been clearly identified. The function of these
types of ARs has been characterized in ovarian physiology as being primarily related to the regulation of OBF (45). Electrical stimulation of the splanchnic nerve has been shown to decrease OBF via activation of α-adrenergic receptors in the ovarian blood vessels (42). We have also demonstrated that modulation of the sympathetic drive to the ovaries differentially affects OBF in normal and PCO rats. Our present data suggest that PCO-induced alterations in the expression of the α1-ARs could, at least in part, be responsible for these effects and possibly for the dysregulation of OBF described in human PCOS (2). It was also demonstrated recently that α1-ARs could participate in the control of progesterone secretion by granulosa cells in vitro (43). Thus α1-ARs could most probably be involved in the upregulation of progesterone release that has been described in the EV-induced PCO ovary and related to β2-AR activation (5). Moreover, our present data demonstrate that both α1- and β2-AR expression are under NGF control, indicating that the involvement of the neurotrophin in the steroidogenic activity of the ovaries could be exerted both through a direct activation of neurotrophin receptors (23, 29) and through the modulation of ovarian responsiveness to sympathetic inputs. Further studies are necessary to investigate this hypothesis.

Unaltered β2-AR mRNA expression and reduced β2-AR protein levels after EV injection were also found in PCO rats, whereas inhibition of the biological activity of endogenous NGF counteracts the EV-induced reduction of ovarian β2-AR protein. The downregulation of ovarian α2-AR mRNA in PCO rats has been reported previously (21, 22), as has β2-AR hyperresponsiveness to challenges by β2 agonists (5). It is also worth mentioning that the activity of the sympathetic nervous system and the ovarian NE content regulate the expression of β2-AR after the induction of PCO (22). Thus the present observations point to the hypothesis of an involvement of NGF in the regulation of β2-AR in this PCO model. Studies by others report that challenging β2-AR with the selective agonist clenbuterol induces NGF synthesis in vitro (34) and that β2-AR agonists exert neuroproteective effects in rat models of focal cerebral ischemia through the induction of NGF synthesis (10). These findings, together with the data presented here, are consistent with the hypothesis of a regulatory feedback loop between β2-AR and NGF in physiological and physiopathological conditions.

The finding that the TrkA mRNA expression decreased after EV treatment appears to be in line with previous studies demonstrating that ovarian TrkA is under LH control (15) and that LH levels are lower than normal in EV-treated rats (6). Nevertheless, it has been demonstrated that NGF is able to regulate TrkA expression at several levels (46) and that the early increase of ovarian NGF is a cardinal feature of the steroid-induced PCO model (20, 37, 38). In the present study, we demonstrate that TrkA protein increased in EV-treated rats and both TrkA mRNA and protein were downregulated by anti-NGF treatment in EV-treated rats, indicating that NGF itself participates in the regulation of its receptor expression in the ovary. The activation of ovarian p75NTR synthesis after EV injection and the knowledge that p75NTR can collaborate with TrkA receptors in the formation of high-affinity binding sites and in enhancing cellular responses to NGF (17) are in line with the present findings of elevated TrkA protein levels that are counteracted by repeated anti-NGF treatment.

The observation that systemic injections of NGF antibodies counteract the EV-induced increase not only of ovarian p75NTR but also of ovarian TH indicates that NGF regulates p75NTR and TH synthesis during the development of PCO. This interpretation is supported by previous experiments that showed that the expression of both p75NTR and TH in mature sympathetic neurons is under NGF control (12, 28). Indeed, the enhanced activity of TH in the ovaries of PCO rats (22) and the higher content of TH mRNA in catecholaminergic cells of the celiac ganglion that selectively project to the ovaries (20) are considered reliable biochemical and molecular markers of activation of the sympathetic nervous system after EV injection. As for the mechanism responsible for the effects of NGF, our findings indicate that the action of NGF exerted on ovarian tissues expressing TH is most probably due to sympathetic nerve fibers and intrinsic catecholaminergic cells (11), both of which express p75NTR (20). As stated above, the hyperactivation of ovarian sympathetic nerves in EV-induced PCO are most likely related to an overproduction of NGF, which in turn regulates the expression of ovarian sympathetic markers such as α1- and β2-ARs and TH.

In conclusion, the data obtained with this animal model suggest a major role for NGF in the pathogenesis of PCO in rats. Whether NGF plays a similar role in analogous processes in humans remains to be investigated.

ACKNOWLEDGMENTS

The laboratory assistance provided by Britt-Marie Larsson is gratefully acknowledged.

GRANTS

This study was supported by grants from Wilhelm and Martina Lundgren’s Science Fund, the Hjalmar Svensson Foundation, The Royal Society of Art and Sciences in Göteborg and Magnus Bergwall’s stiftelse, the Novo Nordisk Foundation, The Göteborg Medical Society, the Medical Research Council (Project Nos. 12206, 2004-6399, and 2004-6827), and the Swedish Heart Lung Foundation. The contribution of L. Aloe and L. Manni is supported by Progetti Strategici Fondo Integrativo Speciale per la Ricerca/Neurobiotecnologie and by Fondazione Cass di Risparmio di Bologna, Bologna, Italy.

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