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Cryptotanshinone reverses reproductive and metabolic disturbances in prenatally androgenized rats via regulation of ovarian signaling mechanisms and androgen synthesis

Xinning Yang,1,2* Yuehui Zhang,1* Xiaoke Wu,1,3,4 Chun Sik Bae,5 Lihui Hou,1 Haixue Kuang,6 Yongyan Wang,7 and Elisabet Stener-Victorin1,8*

1Key Laboratory of Reproduction in Chinese Medicine, 2Division of Ultrasound, 3Department of Obstetrics and Gynecology, First Affiliated Hospital, Heilongjiang University of Chinese Medicine, Harbin; 4Jiangsu Key Laboratory of Molecular Medicine & Center for Public Health Research, Medical School of Nanjing University, Nanjing; 5Key Laboratory of Pharmacology, First Affiliated Hospital, Heilongjiang University of Chinese Medicine, Harbin; 6Institute of Basic Research in Clinical Medicine, China Academy of Chinese Medical Science, Beijing, China; 7College of Veterinary Medicine, Biotechnology Research Institute, Chonnam National University, Gwangju, Korea; and 8Department of Physiology, Institute of Neuroscience and Physiology, Sahlgrenska Academy, University of Gothenburg, Gothenburg, Sweden

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Yang X, Zhang Y, Wu X, Bae CS, Hou L, Kuang H, Wang Y, Stener-Victorin E. Cryptotanshinone reverses reproductive and metabolic disturbances in prenatally androgenized rats via regulation of ovarian signaling mechanisms and androgen synthesis. Am J Physiol Regul Integr Comp Physiol 300: R869–R875, 2011. First published January 12, 2011; doi:10.1152/ajpregu.00334.2010.—This trial explores 1) prenatally androgenized (PNA) rats as a model of polycystic ovary syndrome (PCOS) and 2) reproductive and metabolic effects of cryptotanshinone in PNA ovaries. On days 16–18 of pregnancy, 10 rats were injected with testosterone propionate (PNA mothers) and 10 with sesame oil (control mothers). At age 3 mo, 12 female offspring from each group were randomly assigned to receive saline and 12 cryptotanshinone treatment during 2 wk. Before treatment, compared with the 24 controls, the 24 PNA rats had 1) disrupted estrous cycles, 2) higher 17-hydroxyprogesterone (P = 0.030), androstenedione (P = 0.016), testosterone and insulin (P values = 0.000), and glucose (P = 0.047) levels, and 3) higher areas under the curve (AUC) for glucose (AUC-Glu, P = 0.025) and homeostatic model assessment for insulin resistance (HOMA-IR, P = 0.008). After treatment, compared with vehicle-treated PNA rats, cryptotanshinone-treated PNA rats had 1) improved estrous cycles (P = 0.045), 2) reduced 17-hydroxyprogesterone (P = 0.041), androstenedione (P = 0.038), testosterone (P = 0.003), glucose (P = 0.036), and insulin (P = 0.041) levels, and 3) lower AUC-Glu (P = 0.045) and HOMA-IR (P = 0.024). Western blot showed that cryptotanshinone reversed the altered protein expressions of insulin receptor substrate-1 and -2, phosphatidylinositol 3-kinase p85α, glucose transporter-4, ERK-1, and 17α-hydroxylase within PNA ovaries. We conclude that PNA model rats exhibit reproductive and metabolic phenotypes of a PCOS rat model, as previously described (8). The Androgen Excess & PCOS Society Position Statement, however, recently emphasized the androgenic component of PCOS, making hyperandrogenism fundamental to the syndrome (4). The effect was to exclude the phenotype of the nonhyperandrogenic woman with ovulatory dysfunction, which the Rotterdam criteria allow (4, 24a).

Insulin resistance and compensatory hyperinsulinemia are prominent features of PCOS that occur in 60–70% of affected patients and are believed to be a major factor in ovary hyperandrogenism (10). Insulin resistance in PCOS women resides mainly in muscle, adipose tissue, and the liver (10), which are generally accepted as classic targets of insulin action. Baillargeon and Carpenter (5) further demonstrated in vivo that insulin levels play a significant role in PCOS hyperandrogenemia, even in normoinsulinemic insulin-sensitive women with PCOS, suggesting altered insulin signaling in androgen-secreting tissues (7).

Recently, our laboratory confirmed (29, 30) that insulin resistance occurs within the PCOS ovary, as demonstrated by defects in glucose uptake, concomitant with altered expressions and phosphorylations of key insulin-signaling molecules. Thus, insulin resistance in a classic target tissue, such as skeletal muscle, contributes to overall metabolic abnormalities in PCOS patients while resistance in insulin-signaling pathways that regulate metabolic function in nonclassical tissues, such as the ovaries, may contribute to ovarian dysfunction. In this study, we hypothesized that a direct connection between insulin resistance and androgen excess may occur in the ovaries of a PCOS rat model.

One difficulty in basic research on PCOS is construction of a satisfactory animal model; most models today only mimic aspects of PCOS phenotypes. Abbott and colleagues (1) recently reported progress in closely replicating PCOS in female rhesus monkeys with prenatal androgen exposure. Monkeys, however, are expensive to maintain in the laboratory, so we used a prenatally androgenized (PNA) rat model, as previously described (8). Cryptotanshinone was originally isolated from the dried roots of *Salvia miltiorrhiza Bunge* (14, 33), traditionally known

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POLYCYSTIC OVARY SYNDROME (PCOS) is a common endocrine disorder that affects ~7–8% of women in their reproductive years. At present, PCOS is most commonly defined according to the 2003 Rotterdam criteria, which requires two of three diagnostic features (hyperandrogenism, ovulatory dysfunction, and PCO morphology) for a diagnosis (24a). The Androgen Excess & PCOS Society Position Statement, however, recently emphasized the androgenic component of PCOS, making hyperandrogenism fundamental to the syndrome (4). The effect was to exclude the phenotype of the nonhyperandrogenic woman with ovulatory dysfunction, which the Rotterdam criteria allow (4, 24a).

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Cryptotanshinone was originally isolated from the dried roots of *Salvia miltiorrhiza Bunge* (14, 33), traditionally known
as tanshinone. In Chinese medicine, this herb has been widely prescribed for several pathologies, including diabetes, acne, cardiovascular disease, hematological abnormalities, hepatitis, and hyperlipidemia. More than 30 diterpene compounds, including tanshinone I, IIA, and IIB and cryptotanshinone, have been isolated from the herb and identified as major chemical constituents. This study explores effects and mechanisms whereby cryptotanshinone ameliorates insulin resistance and androgen excess in a PNA rat model. The results may contribute to the development of a novel therapeutic approach for the treatment of PCOS outside Chinese medicine.

MATERIALS AND METHODS

Animals. Twenty adult Wistar female rats (age 12–14 wk; body wt 250–300 g) were selected. On day 16, 17, and 18 of pregnancy, 10 of the females were injected subcutaneously with 2.5 mg/day testosterone propionate (fetal testosterone treatment) and 10 with sesame oil vehicle (fetal vehicle treatment); 24 female offspring from each group of mothers were studied as adults. The Institutional Animal Care Committee at Heilongjiang University of Chinese Medicine approved all animal experiments.

At age 3 mo, the 48 female offspring from each mother were randomly assigned to receive cryptotanshinone or saline treatment, thus forming four groups: PNA-cr (n = 12), fetal testosterone treatment and postnatal cryptotanshinone treatment; control-cr (n = 12), fetal vehicle treatment and postnatal cryptotanshinone treatment; PNA-v (n = 12), fetal testosterone treatment and postnatal vehicle treatment; and control-v (n = 12), fetal vehicle treatment and postnatal vehicle treatment.

At age ~3.5 mo, two groups (PNA-cr and control-cr) received 0.1% cryptotanshinone (isolated from dried roots of S. miltiorrhiza, 98% purity; Shanghai First Biochemical Pharmaceutical, Shanghai, China) in a vehicle of polysorbate 80 (Tween 80) and normal saline. The other two groups (PNA-v and control-v) received the vehicle. The cryptotanshinone solution (dose: 0.027 mg·g body wt−1·day−1, 1 mg cryptotanshinone was dissolved in 0.4 ml vehicle) and the vehicle (same dosage volume) were administered orally for 14 days between 9:00 and 10:00 A.M. Estrous cyclicity. Estrous phase was determined by microscopic analysis of the predominant cell type in vaginal smears obtained daily from age 3 mo to the end of the experiment. Cycle length was determined in all rats before treatment start and after treatment end on day 14.

Oral glucose tolerance test and blood sampling for hormone analyses. The oral glucose tolerance test (OGGT) and the hormone analyses were each done two times: once in the test period before treatment and once in the test period after treatment. In each test period, the blood sampling interval between OGGT and sampling for hormone analysis was at least 4 days. The OGGT was done with a glucose load of 3 g/kg wt (9). Blood samples from the tail vein were drawn immediately before (0 min) and 30, 60, and 120 min after oral glucose ingestion. For hormone analyses, blood was collected via puncture of the retro-orbital venous plexus when the rats were in diestrus and centrifuged; serum was stored at −20°C until analyses.

Ten days before treatment start (~10 days), at age ~3 mo, all 48 rats were fasted overnight for 10–12 h, and OGGT was done the following morning, independent of cycle day. At least 4 days after the OGGT, i.e., between ~6 days and ~1 day before treatment start, when the rat was in diestrus, the rat was again fasted overnight for 10–12 h, and blood sampling for hormone analyses was drawn the following morning. All rats began treatment on day 1.

On day 14, the last day of treatment, all 48 rats were fasted overnight for 10–12 h, and OGGT was repeated on the morning of day 15. Between 4 and 10 days after the last treatment (days 18–24 of the study), when the rat was in diestrus or (in the case of the acyclic PNA rats treated with vehicle) 10 days following day 14 (day 24 of the study), the rat was again fasted overnight for 10–12 h. Blood sampling for hormone analyses was drawn the next morning. Immediately following blood sampling for hormone analyses, rats were decapitated. The ovaries were dissected, cleaned of fat, and weighed. One ovary from each rat (n = 48) was snap-frozen for Western blot analysis, and the other was immediately fixed for immunohistochemical (n = 24) or for light microscopic (n = 24) analyses.

Biochemical assessments. Serum glucose was determined with a glucose blood test meter (Roche, Germany) (19). Fasting serum insulin concentrations, estradiol (E2), luteinizing hormone (LH), follicle-stimulating hormone (FSH), and androstenedione were assessed with Chemiluminescent immunoassay kits (insulin kit, LKIN10310; E2 kit, LKE210324; LH kit, LKLIH01924; FSH kit, LKFSI0298; androstenedione kit, LKA010313; Siemens Medical Solutions Diagnostics, Los Angeles, CA) and requires 50 μl serum/assay (20, 25). Serum concentrations of testosterone and 17-hydroxyprogesterone (17-OH) were determined with commercial double-antibody RIA kits (testosterone RIA kit, DSL-5400; 17-hydroxyprogesterone RIA kit, DSL-8800; Diagnostic Systems Laboratories, Webster, TX) and requires 100 μl serum/assay (21). The area under the curve (AUC) was calculated for glucose (AUC-Glu) using the trapezoidal rule (18).

Homeostatic model assessment (HOMA-IR). The intra-assay and interassay coefficients of variation and sensitivity were 7.4 and 6.8%, 0.6 nmol/l (glucose); 5.8 and 4.7%, 2 μIU/ml (insulin); 6.7 and 6.5%, 15 pg/ml (E2); 6.0 and 3.3%, 0.1 mlU/ml (LH); 1.9 and 2.2%, 0.1 mlU/ml (FSH); 3.6 and 4.8%, 0.3 ng/ml (androstenedione); 2.5 and 3.2%, 0.05 ng/ml (testosterone); and 2.3 and 1.9%, 0.03 ng/ml (17-OH).

Immunohistochemistry. Six ovaries from each group were immediately fixed in Bouin’s solution for 24 h, dehydrated, embedded in paraffin, and sliced into 7-μm sections. The ovary sections were deparaffinized and rehydrated. After blocking of nonspecific binding in 10% (vol/vol) normal horse serum in PBS at 37°C for 1 h, the sections were incubated overnight in 10% (vol/vol) horse serum at 4°C with one of the following: rabbit anti-human insulin receptor substrate-1 (IRS-1), rabbit anti-human ERK-1, goat anti-mouse IRS-2, goat anti-mouse 17α-hydroxylase (CYP17), mouse anti-human phosphatidylinositol 3-kinase (PI3K) p85α, or goat anti-human glucose transporter (GLUT4) (Santa Cruz Biotechnology, Santa Cruz, CA).

The next morning, the sections were incubated with biotinylated goat anti-rabbit IgG, biotinylated rabbit anti-goat IgG, or biotinylated goat anti-mouse IgG, followed by a streptavidin-alkaline phosphatase complex and Vector Red according to the manufacturer’s instructions (Vectastain ABC-AP kit; Vector Laboratories, Burlingame, CA). Vector Red was visualized as a red color; 1 mM levamisole (Sigma) was added to the Vector Red substrate solution to inhibit endogenous alkaline phosphatase activity. As a negative control, the same concentration of normal rabbit IgG or goat IgG was used in place of the corresponding primary antibody. Sections were counterstained with hematoxylin and mounted. Two investigators assessed degree of immunostaining by blinded examination.

Light microscopic analysis. Six ovaries from each group were longitudinally and serially sectioned in 4-μm slices; every 10th section (n = 6/ovary) was mounted on a glass slide and stained with hematoxylin and eosin. Two investigators, blinded to the sections’ origin, independently analyzed the sections under a conventional birefringence microscope.

Western blot analysis. Twelve ovaries from each group (one per animal) were homogenized in lysis buffer [20 mM Tris, pH 7.5, 150 mM NaCl, 1% Triton X-100, and a protease inhibitor cocktail (1:100 vol/vol), sodium pyrophosphate, β-glycerophosphate, EDTA, Na3VO4, and leupeptin], incubated for 30 min on ice, and centrifuged for 30 min at 16,000 g (4°C). The supernatant was saved as a whole protein fraction. Total protein was assayed using the bichinchoninic acid method (Pierce Biotechnology, Rockport, IL). After SDS-PAGE electrophoresis (200 V, 35 min), the protein (50 μg) was transferred to nitrocellulose filters.
Table 1. Body weight and food intake during treatment and ovarian weight and volume after treatment end in the four treatment groups

<table>
<thead>
<tr>
<th>Group</th>
<th>Day 0</th>
<th>Day 7</th>
<th>Day 14</th>
<th>Food Intake, g · day⁻¹ · rat⁻¹</th>
<th>Weight, g</th>
<th>Ovarian Volume, ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control-cr</td>
<td>244 ± 1.77</td>
<td>234.0 ± 1.12b</td>
<td>234.0 ± 1.90b</td>
<td>16.5 ± 0.11</td>
<td>69.12 ± 0.65</td>
<td>0.032 ± 0.002</td>
</tr>
<tr>
<td>Control-v</td>
<td>253.8 ± 1.04</td>
<td>266.2 ± 1.38</td>
<td>265.0 ± 1.56</td>
<td>16.7 ± 0.18</td>
<td>69.42 ± 1.45</td>
<td>0.031 ± 0.001</td>
</tr>
<tr>
<td>PNA-cr</td>
<td>270.0 ± 1.59</td>
<td>262.0 ± 1.99</td>
<td>255.0 ± 1.79a</td>
<td>16.2 ± 0.12</td>
<td>65.97 ± 0.91*</td>
<td>0.030 ± 0.001</td>
</tr>
<tr>
<td>PNA-v</td>
<td>269.0 ± 2.44</td>
<td>270.0 ± 2.21</td>
<td>275.0 ± 1.97</td>
<td>16.1 ± 0.13</td>
<td>80.43 ± 0.84</td>
<td>0.026 ± 0.000</td>
</tr>
<tr>
<td>P value</td>
<td>0.475</td>
<td>0.039</td>
<td>0.043</td>
<td>0.513</td>
<td>0.027</td>
<td>0.264</td>
</tr>
</tbody>
</table>

Values are means ± SE. PNA, prenatally androgenized; Control-cr, cryptotanshinone-treated; Control-v, vehicle-treated; PNA-cr, cryptotanshinone-treated; PNA-v, vehicle-treated. *P < 0.05 vs. Control-cr and Control-v (one-way ANOVA followed by Bonferroni t-test). **P < 0.05 vs. Control-cr and Control-v (one-way ANOVA followed by Bonferroni t-test).

(Pall-Gelman, Lawrence, KS) by electrophoresis (25 mM Bicine, 25 mM Bis-tris, 1 mM ethylenediaminetetraacetic acid, 0.05 mM chlorobutanol, and 20% methanol, pH 7.2).

The membrane was blocked for 2 h at room temperature in TBS (100 mM Tris and 0.9% NaCl, pH 7.5) containing 5% low-fat milk powder. Membranes were then incubated for 1 h at room temperature with the primary antibodies of one of the following: IRS-1, IRS-2, PI3K p85, GLUT4, ERK-1, or CYP17 in TBS (1:200 dilution). Membranes were washed three times in TBS for 15 min and then incubated for 1 h with secondary antibodies (1:1,000 dilution in TBS) marked by one of the following: a horseradish peroxidase-linked rabbit anti-goat IgG, goat anti-rabbit IgG, or goat anti-mouse IgG (Dako Denmark, Glostrup, Denmark). Diaminobenzidine (Dako) was then used to detect immunoreactive proteins.

Statistical analyses. Statistical evaluations were done using the Statistical Package for the Social Sciences (SPSS version 13.0; SPSS, Chicago, IL). Values are expressed as means ± SE. Independent t-tests assessed differences between the PNA and control groups before treatment start. After treatment, the one-way ANOVA with Bonferroni post hoc test analyzed differences among groups. P < 0.05 was considered statistically significant.

RESULTS

Metabolic effects of cryptotanshinone on PNA rats. Mean body weights of the 24 PNA and 24 control rats (age ~3.5 mo) differed nonsignificantly (268.75 ± 24.51 vs. 248.33 ± 17.85 g, P = 0.078) at baseline (day 0). On days 7 and 14, mean body weight in the control-cr was significantly lower than in the PNA-cr group; however, there were no differences compared with the control-v group; however, there were no differences compared with the PNA-cr group; however, there were no differences compared with the control-cr and control-v groups (Table 2).

After treatment, there were no differences in serum glucose levels during OGTT, serum insulin levels, AUC-Glu, and HOMA-IR between the control-cr and control-v groups (Table 2). In the PNA-cr-treated group, serum glucose levels at 30 and 120 min during OGTT, serum insulin levels, AUC-Glu, and HOMA-IR were significantly lower compared with the PNA-v group; however, there were no differences compared with control-cr and control-v groups (Table 2).

Reproductive effects of cryptotanshinone on PNA rats. Differences in mean ovarian volume and weight between cryptotanshinone-treated rats and vehicle-treated control rats (PNA-cr, control-cr, and control-v) were nonsignificant. Mean ovarian weight in the PNA rats who had received no active treatment (PNA-v), however, was significantly higher than in the cryptotanshinone-treated PNA rats (P = 0.027) (Table 1).

Before treatment, the control rats (control-cr and -v) had normal estrous cycles of 4.42 ± 0.5 days, whereas the PNA females (PNA-cr and -v) were completely acyclic or exhibited an extended estrous cycle of 10.25 ± 0.97 days. This difference between control and PNA rats was significant (P = 0.036). Following treatment with cryptotanshinone, estrous

Table 2. Results of OGTT, HOMA-IR, insulin, and AUC for glucose in offspring of oil-injected and testosterone propionate-injected mothers before and after experimental treatment with cryptotanshinone and control treatment with vehicle

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>0</th>
<th>30</th>
<th>60</th>
<th>120</th>
<th>AUC-Glu</th>
<th>Insulin, mL/μl</th>
<th>HOMA-IR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Before</td>
<td></td>
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<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>treatment</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PNA</td>
<td>24</td>
<td>6.42 ± 0.06</td>
<td>9.85 ± 0.05a</td>
<td>10.03 ± 0.09</td>
<td>8.16 ± 0.07a</td>
<td>27.27 ± 0.10a</td>
<td>41.87 ± 0.17a</td>
<td>12.01 ± 0.14a</td>
</tr>
<tr>
<td>Control</td>
<td>24</td>
<td>6.31 ± 0.02</td>
<td>8.95 ± 0.05</td>
<td>8.74 ± 0.03</td>
<td>6.92 ± 0.02</td>
<td>20.31 ± 0.07</td>
<td>24.31 ± 0.26</td>
<td>7.01 ± 0.07</td>
</tr>
<tr>
<td>After</td>
<td></td>
<td></td>
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<td>treatment</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>PNA-cr</td>
<td>12</td>
<td>5.64 ± 0.05</td>
<td>8.30 ± 0.03b</td>
<td>10.18 ± 0.21</td>
<td>8.63 ± 0.04b</td>
<td>22.46 ± 0.27b</td>
<td>32.99 ± 0.49b</td>
<td>7.21 ± 0.13b</td>
</tr>
<tr>
<td>PNA-v</td>
<td>12</td>
<td>7.68 ± 0.01</td>
<td>10.58 ± 0.13</td>
<td>12.53 ± 0.35</td>
<td>11.60 ± 0.30</td>
<td>28.80 ± 0.62</td>
<td>38.90 ± 0.35</td>
<td>12.70 ± 0.03</td>
</tr>
<tr>
<td>Control-cr</td>
<td>12</td>
<td>6.08 ± 0.07</td>
<td>8.16 ± 0.09</td>
<td>9.16 ± 0.15</td>
<td>8.40 ± 0.08</td>
<td>20.56 ± 0.27</td>
<td>30.14 ± 0.35</td>
<td>7.94 ± 0.16</td>
</tr>
<tr>
<td>Control-v</td>
<td>12</td>
<td>6.77 ± 0.11</td>
<td>8.47 ± 0.12</td>
<td>10.80 ± 0.3</td>
<td>10.30 ± 0.23</td>
<td>22.74 ± 0.75</td>
<td>31.88 ± 0.64</td>
<td>9.75 ± 0.20</td>
</tr>
</tbody>
</table>

Values are means ± SE; n, no. of animals. OGTT, oral glucose tolerance test; Glu, glucose; AUC, area under the curve; HOMA-IR, homeostatic model assessment of insulin resistance. *P < 0.05 vs. control. **P < 0.01 vs. control (independent t-test). bP < 0.05 vs. PNA-v (one-way ANOVA followed by Bonferroni t-test).

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cycle determinations found that percentage of time spent in estrus and mean cycle length (4.45 ± 0.32 days) in most PNA-cr females (9/12, 75%) no longer differed from in control females.

Light microscopic analysis showed no structural abnormalities in control rats (control-cr and -v); follicles and corpora lutea (CL) were in varying stages of development and regression, there were no cystic follicles (a large fluid-filled cyst), and theca and granulosa cell layers were normal (the nos. of theca and granulosa cell layer were, respectively, 2–3 and 6–9), neither were significant differences between these groups observed in the numbers of CL or cystic follicles.

Differences in mean numbers of CL and atretic follicles between cryptotanshinone-treated rats and vehicle-treated control rats (PNA-cr, control-cr, and control-v) were nonsignificant. However, when PNA rats not treated with cryptotanshinone (PNA-v) were compared with these three groups, the mean number of atretic follicles in PNA-v was significantly higher compared with the two control groups pooled (31.56 ± 3.01 vs. 18.86 ± 2.85, \( P = 0.026 \)) or with PNA-cr (31.56 ± 3.01 vs. 19.38 ± 2.78, \( P = 0.031 \)). Likewise, the mean number of CL in PNA-v was significantly lower compared with the two control groups pooled (3.50 ± 0.76 vs. 10.71 ± 1.80, \( P = 0.012 \)) or with PNA-cr (3.50 ± 0.76 vs. 8.89 ± 0.43, \( P = 0.045 \)).

Before treatment, comparisons between the 24 PNA females and the 24 control females found that 1) differences in mean serum concentrations of LH, FSH, and E2 were nonsignificant and 2) concentrations of 17-OH, androstenedione, and testosterone (\( P = 0.000 \)) were significantly higher in PNA rats. After treatment, differences in serum concentrations of FSH, LH, and E2 between the four groups (PNA-cr, PNA-v, control-cr, control-v) were nonsignificant, but concentrations of 17-OH, androstenedione, and testosterone were significantly lower in cryptotanshinone-treated PNA rats compared with vehicle-treated PNA rats (\( P = 0.041, 0.038, \) and 0.003, respectively, Table 3) and did not differ from the control-cr or the control-v group.

Cryptotanshinone effects on ovarian insulin signaling and androgen synthesis. The immunohistochemical analysis of vehicle-treated ovaries (control-v and PNA-v) showed that 1) ERK-1 was expressed in theca and granulosa cells, 2) PI3K p85α, IRS-1, and IRS-2 were primarily expressed within the ovarian stroma and theca, and 3) GLUT4 was primarily expressed in the ovarian theca, stroma, and CL (Fig. 1).

Table 3. Hormone analyses in offspring of oil-injected and testosterone propionate-injected mothers before and after experimental treatment with cryptotanshinone and control treatment with vehicle

<table>
<thead>
<tr>
<th>Group</th>
<th>( n )</th>
<th>LH, mIU/ml</th>
<th>FSH, mIU/ml</th>
<th>E2, pg/ml</th>
<th>17-OH, ng/ml</th>
<th>Androstenedione, ng/ml</th>
<th>Testosterone, nM/l</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baseline (diestrus)</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>PNA</td>
<td>24</td>
<td>6.83 ± 0.06</td>
<td>5.73 ± 0.02</td>
<td>36.18 ± 0.32</td>
<td>41.97 ± 0.52</td>
<td>0.73 ± 0.01**</td>
<td>131.97 ± 0.85**</td>
</tr>
<tr>
<td>Control</td>
<td>24</td>
<td>6.91 ± 0.11</td>
<td>5.86 ± 0.03</td>
<td>37.77 ± 0.37</td>
<td>26.96 ± 0.48</td>
<td>0.54 ± 0.004</td>
<td>62.43 ± 0.60</td>
</tr>
<tr>
<td>After treatment (diestrus)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PNA-cr</td>
<td>12</td>
<td>4.75 ± 0.01</td>
<td>7.71 ± 0.03</td>
<td>34.26 ± 0.72</td>
<td>31.29 ± 0.78b</td>
<td>0.49 ± 0.01b</td>
<td>47.91 ± 2.39**</td>
</tr>
<tr>
<td>PNA-v</td>
<td>12</td>
<td>4.69 ± 0.01</td>
<td>7.65 ± 0.10</td>
<td>25.87 ± 0.24</td>
<td>45.17 ± 1.36</td>
<td>0.73 ± 0.02</td>
<td>131.75 ± 1.48</td>
</tr>
<tr>
<td>Control-cr</td>
<td>12</td>
<td>5.09 ± 0.12</td>
<td>7.99 ± 0.09</td>
<td>30.03 ± 0.52</td>
<td>26.78 ± 1.09</td>
<td>0.53 ± 0.01</td>
<td>65.97 ± 0.53</td>
</tr>
<tr>
<td>Control-v</td>
<td>12</td>
<td>4.70 ± 0.01</td>
<td>8.02 ± 0.09</td>
<td>33.77 ± 0.09</td>
<td>24.70 ± 0.80</td>
<td>0.56 ± 0.01</td>
<td>73.09 ± 1.55</td>
</tr>
</tbody>
</table>

Values are means ± SE; \( n \), no. of animals. LH, luteinizing hormone; FSH, follicle-stimulating hormone; E2, estradiol; 17-OH, 17-hydroxyprogesterone. 

*\( P < 0.05 \) vs. control, **\( P < 0.01 \) vs. control (independent t-test), b\( P < 0.05 \) vs. PNA-v, bP < 0.05 vs. PNA-v (one-way ANOVA followed by Bonferroni t-test).

DISCUSSION

In this study, compared with control rats whose mothers had been injected with a sesame oil vehicle, the PNA female rats had abnormal estrous cycles and polycystic ovaries (characterized by cysts formed from atretic follicles and diminished granulose layer) (21) and significantly higher 1) 17-OH, androstenedione, and testosterone levels, 2) 30- and 120-min OGTT glucose levels, 3) AUC-Glu, and 4) serum insulin concentrations and HOMA-IR. All of these parameters in PNA rats were coincident with previous reports of primate, sheep, and rat PNA models for PCOS (2, 8). In general, the PNA rats in this study recapitulated the reproductive and metabolic features of human PCOS, including polycystic ovaries (and irregular cycles, hyperandrogenism, impaired glucose tolerance, hyperinsulinemia, and insulin resistance) and thus the Rotterdam criteria and the criteria proposed by the Androgen Excess & PCOS Society in their position statement.

Theca cells are the source of androgen biosynthesis in the human ovary. In PCOS, theca cells overexpress mRNA for key genes involved in androgen biosynthesis, including LH receptor, steroidogenic acute regulatory protein, CYP17, and CYP11A1 (13). Many PCOS follicles have an excessive number of theca cells, and these theca cells have increased capacity to synthesize androgens on a per cell basis (32). In this study, one of the most important immunohistochemical findings is that, in

Figure 2 presents Western blot results of key molecules in the insulin-signaling pathway from whole ovarian extract. Protein expression of IRS-1, IRS-2, PI3K p85α, and GLUT4 was significantly lower in PNA-v rats compared with the control groups (control-cr and -v) and also with the cryptotanshinone-treated PNA rats (PNA-cr): in the PNA-cr group, the expression pattern for these parameters was partially reversed compared with in the PNA-v group and in line with expression in control-cr and control-v. No significant differences between control-cr, control-v, and PNA-cr were found (Fig. 2, A and B).

Expression of ERK-1 and CYP17 was increased in PNA-v compared with control ovaries. A partial reversal of the expression pattern for ERK-1 and CYP17 proteins was also found. In PNA-cr, expression was significantly lower than in PNA-v and in line with ERK-1 and CYP17 expression in the control groups (control-cr and -v). No significant differences between control-cr, control-v, and PNA-cr were found (Fig. 2, C and D).
PNA and control ovaries, both key insulin-signaling proteins and CYP17 protein are located in the theca and stroma of antral follicles. In a recent study, a specific inhibitor of PI3-kinase, LY-294002, inhibited insulin-induced 17α-hydroxylase activity in theca cells, indicating that the PI3-kinase pathway is a mediator of the insulin signal involved in regulating androgen production in human theca cells (23). These data suggest the direct participation of insulin signaling in androgen synthesis within theca.

A generally accepted paradigm is that insulin receptors, acting through insulin receptor substrates, stimulate the lipid kinase activity of PI3K (16, 30). Activation of PI3K propagates the signal to regulate several insulin-mediated metabolic functions, such as glucose transport and glycogen synthesis (the PI3K pathway). Another pathway proceeds through the activation of the mitogen-activated protein kinase (MAPK) isoforms of ERK-1 and -2, thus mediating mitogenic and other gene-regulatory actions of insulin (26). Defects in either pathway have close relationships with insulin resistance.

In this study, IRS-1, IRS-2, PI3K p85α, and GLUT4 ovarian protein expressions were significantly lower and ERK-1 protein expression was significantly higher in the ovaries of PNA rats after no active treatment (PNA-v) compared with in control (control-v and -cr) and cryptotanshinone-treated PNA (PNA-cr) rats. This result indicates that altered insulin signaling and insulin resistance occur within PNA rat ovaries. Because IRS-1 and -2, PI3K p85α, and GLUT4 were primarily expressed in ovarian theca cells, insulin resistance may affect PNA theca and contribute to its excessive androgens. Our recent study demonstrated that dexamethasone-induced insulin resistance directly exaggerated the theca cell androgenic potential (24). In addition, it has been reported that the insulin-sensitizing agent metformin directly inhibits androgen synthesis in ovary theca cells cultured in vitro (3).

In a previous study in human subjects, Yen et al. (32) found that IRS-1 and -2 were increased in PCOS theca cells but not in granulosa cells with no changes in the PI3K catalytic subunits p110α or p110β in either theca or granulosa cells. Although there are discrepancies between our model rats and Yen’s PCOS subjects, both studies indicate that altered insulin signaling may occur within polycystic ovary theca. In this study, we further found that ERK-1 was expressed in both theca cells and granulosa cells, indicating that the MAPK pathway is enhanced within the PNA ovary. Taken together, these data suggest a novel interaction between insulin resistance and androgen synthesis within PNA ovary theca (31).

In China, tanshinone and its major active ingredient, cryptotanshinone, are commonly used empirically for the treatment of acne because of their anti-androgenic properties (15, 28). Tanshinone’s therapeutic effect in acne treatment is based on a reduction of testosterone levels (11). We found that treatment with cryptotanshinone restored normal estrous cyclicity in PNA females and decreased testosterone without altering estrogen levels. It is conceivable that cryptotanshinone may directly suppress the androgenic activity of theca cells in PNA ovaries. These findings lead us to propose that the positive cryptotanshinone effect on reproduction in PNA rats is mainly associated with a decrease in excessive ovarian androgens.

Gong et al. (11) showed that tanshinone reduces adipose mass and body weight and improves glucose tolerance without affecting food intake in a high-fat diet-induced obese animal model. The molecular mechanisms behind the potent antidia-

Fig. 1. Immunohistochemical staining of rat ovaries for insulin receptor substrate (IRS)-1, IRS-2, phosphatidylinositol 3-kinase (PI3K) p85α, glucose transporter 4 (GLUT4), ERK-1, and 17α-hydroxylase (CYP17) in control vehicle ovaries. 1: IRS-1 was primarily localized within the ovarian stroma ant theca (arrows); 2: IRS-2 was primarily localized within the ovarian stroma ant theca (arrows); 3: PI3K p85α was primarily localized within the ovarian stroma ant theca (arrows); 4: GLUT4 was primarily expressed in the ovarian stroma (short thick arrows) and corpus luteum (long arrow in top left corner); 5: ERK-1 was expressed in theca cells (short thick arrows) and granulosa cells (long arrows); and 6: CYP17 was primarily expressed in theca cells (short thick arrow) and granulosa cells (long arrows).
and antiobesity effects of cryptotanshinone have been ascribed to activation of the MAPK pathway (17) and mediation through its characteristic as a natural antagonist of peroxisome proliferator-activated receptor-γ (15). In our experiment, body weight and ovarian weight in cryptotanshinone-treated PNA rats (PNA-cr) were significantly lower compared with PNA rats that had received no active treatment (PNA-v). After treatment, OGTT serum glucose levels at 30 and 120 min, AUC-Glu, insulin levels, and HOMA-IR in PNA-cr rats were lower than in PNA rats treated with vehicle. These data support that cryptotanshinone could improve peripheral insulin resistance in PNA rats.

In this study, CYP17 protein expression in the ovaries of vehicle-treated PNA rats was significantly lower than in the control group (control-v and -cr) or PNA-cr ovaries; CYP17 expression in these three groups (control-v, control-cr, and PNA-cr) was similar. Protein expression of ERK-1 was lower and protein expressions of IRS-1, IRS-2, PI3K p85α, and GLUT4 were higher in PNA ovaries after cryptotanshinone treatment compared with vehicle treatment. The coordinated alterations of CYP17 protein and insulin-signaling proteins by cryptotanshinone in PNA ovaries further support that a direct connection between insulin resistance and androgen excess occurs within PNA ovaries, and cryptotanshinone intervention appears to regulate both simultaneously.

Perspectives and Significance

PNA rats exhibited hyperandrogenism, anovulation, and insulin resistance, similar to the human phenotypes of PCOS. The therapeutic benefit of cryptotanshinone on PNA rats may be mediated by its dual regulation of key molecules during both insulin signaling and androgen synthesis within PNA ovaries. This study will make a useful addition to the literature.
concerning a potentially new and naturally derived compound that may prove effective in eradicating PCOS symptomatology in women.

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DISCLOSURES

No conflicts of interest are declared by the authors.

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