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

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ABSTRACT

This trial explores (i) prenatally androgenized (PNA) rats as a model of polycystic ovary syndrome (PCOS) and (ii) 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 weeks. Before treatment, compared with the 24 controls, the 24 PNA rats had (i) disrupted estrus cycles, (ii) higher 17-hydroxyprogesterone, \( P = 0.030 \), androstenedione \( P = 0.016 \), testosterone and insulin \( Ps = 0.000 \), and glucose \( P = 0.047 \) levels, and (iii) 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 (i) improved estrus cycles \( P = 0.045 \), (ii) 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 (iii) 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 human PCOS and that regulation of key molecules in insulin signaling and androgen synthesis within PNA ovaries may explain cryptotanshinone’s therapeutic effects.

Keywords: insulin resistance; insulin-signaling molecules; polycystic ovary syndrome
INTRODUCTION

Polycystic ovary syndrome (PCOS) is a common endocrine disorder that affects about 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 (1). The Androgen Excess & PCOS Society Position Statement, however, recently emphasized the androgenic component of PCOS, making hyperandrogenism fundamental to the syndrome (5). The effect was to exclude the phenotype of the non-hyperandrogenic woman with ovulatory dysfunction, which the Rotterdam criteria allow (1, 5).

Insulin resistance and compensatory hyperinsulinemia are prominent features of PCOS that occur in 60% to 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 colleagues (6) further demonstrated in vivo that insulin levels play a significant role in PCOS hyperandrogenemia, even in normo-insulinemic 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 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 non-classical 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
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 (2) 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 (9).

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 (26). More than 30 diterpene compounds—including tanshinone I, IIA, IIB, and cryptotanshinone—have been isolated from the herb and identified as major chemical constituents (33). 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 weight 250–300 g) were selected. On day 16, 17, and 18 of pregnancy, 10 of the females were injected s.c. 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
- **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 Co., LTD, 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 weight/day, 1mg cryptotanshinone was dissolved in 0.4ml vehicle) and the vehicle (same dosage volume) was administered orally for 14 d between 9:00 and 10:00 a.m.

**Estrus Cyclicity**

Estrus 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 (22). 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 twice: 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
OGTT was done with a glucose load of 3 g/kg weight (8). 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 h to 12 h and OGGT was done the following morning, independent of cycle day. At least 4 days after the OGTT, 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 hours 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 h to 12 h and OGGT was repeated on the morning of day 15. Between 4 day and 10 days after the last treatment (day 18-24 of the study)—when the rat was in diestrus or (in the case of the acyclic PNA rats treated with vehicle) 10 day following day 14 (day 24 of the study)—the rat was again fasted overnight for 10 h to 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 blood glucose test meter (Roche, Germany) (19). Fasting serum insulin concentrations, estradiol (E₂), LH, FSH, and androstenedione (A) were assessed with Chemiluminescent immunoassay kits (insulin kit, LKIN10310; estradiol kit, LKE210324; LH kit, LKLH10294; FSH kit, LKFS10298; androstenedione kit, LKAO10313;
Siemens Medical Solutions Diagnostics, Los Angeles, California, USA) and requires 50µL serum per assay (20, 25). Serum concentrations of testosterone (T) 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, Inc., Webster, TX USA) and requires 100µL serum per assay (12). The area under the curve (AUC) was calculated for glucose (AUC-Glu) using the trapezoidal rule (18). Homeostatic model assessment (HOMA) determined insulin resistance (HOMA-IR). The intraassay and interassay coefficients of variation and sensitivity were 7.4%, 6.8%, 0.6mmol/L (glucose); 5.8%, 4.7%, 2µIU/ml (insulin); 6.7%, 6.5%, 15pg/ml (E2); 6.0%, 3.3%, 0.1mIU/ml (LH); 1.9%, 2.2%, 0.1mIU/ml (FSH); 3.6%, 4.8%, 0.3ng/ml (A); 2.5%, 3.2%, 0.05ng/ml (T); and 2.3%, 1.9%, 0.03ng/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% (v/v) normal horse serum in PBS at 37°C for 1 h, the sections were incubated overnight in 10% (v/v) 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-4 (GLUT4) (Santa Cruz Biotechnology, Inc., 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, USA). 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 counter-stained 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 per 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 v/v; sodium pyrophosphate, β-glycerophosphate, EDTA, Na3VO4, leupeptin]), incubated for 30 min on ice, and centrifuged for 30 min at 16,000×g (4°C). The supernatant was saved as whole protein fraction. Total protein was assayed using the bicinchoninic acid method (Pierce Biotechnology Inc, Rockport, IL). After SDS-PAGE electrophoresis (200 V, 35 min), the protein (50 μg) was transferred to nitrocellulose filters (Pall-Gelman, Lawrence, KS) by electroblotting (25 mM Bicine, 25 mM Bis-tris, 1 mM ethylenediaminetetraacetic acid, 0.05 mM chlorobutanol, 20% methanol, pH 7.2).

The membrane was blocked for 2 h at room temperature in Tris-buffered saline (TBS; 100 mM
Tris, 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 p85α, 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 (1:1000 dilution in TBS) marked by one of the following: an HRP-linked rabbit anti-goat IgG, goat anti-rabbit IgG, or goat anti-mouse IgG (Dako Denmark A/S, 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, ver. 13.0; SPSS Inc., Chicago, IL). Values are expressed as means ± S.E. 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±24.51g vs. 248.33±17.85g, *P* = 0.078) at baseline (day 0). On day 7 and 14, mean body weight in the Control-cr was significantly lower than in the Control-v. On day 14, mean body weight in the PNA-cr group was significantly lower than in the PNA-v group (*P* = 0.043). During treatment, food intake was unchanged in all groups (Table 1).
Before treatment, compared with the 24 control females, the 24 PNA females had significantly higher (i) glucose levels at 30 min ($P = 0.047$) and 120 min ($P = 0.043$) during OGTT (ii) AUC-Glu ($P = 0.025$), (iii) insulin levels ($P = 0.000$), and (iv) HOMA-IR ($P = 0.008$, 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 -v groups (Table 2). In the PNA-cr treated group, serum glucose levels at 30 min 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 group (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 estrus cycles of $4.42 \pm 0.5$ d, while the PNA females (PNA-cr and -v) were completely acyclic or exhibited an extended estrus cycle of $10.25 \pm 0.97$ d. This difference between control and PNA rats was significant ($P = 0.036$). Following treatment with cryptotanshinone, estrus cycle determinations found that percentage of time spent in estrus and mean cycle length ($4.45 \pm 0.32$ d) 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 number
of theca and granulosa cell layer was respectively 2-3 and 6-9). Neither were significant differences between these groups observed in 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. But 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, 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 (i) differences in mean serum concentrations of LH, FSH, and E2 were nonsignificant and (ii) concentrations of 17-OH \( (P = 0.030) \), A \( (P = 0.016) \), and T \( (P = 0.000) \) 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, A, and T 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 (i) ERK-1 was expressed in theca and granulosa cells, (ii) PI3K p85\( \alpha \), IRS-1, and IRS-2 were primarily expressed within the ovarian stroma and theca, and (iii) GLUT4 was primarily expressed in
the ovarian theca, stroma, and CL (Fig. 1).

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 to in the PNA-v group, and in line with expression in control-cr and -v. No significant differences between control-cr, control-v, and PNA-cr were found (Fig. 2A, 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. 3C, D).

DISCUSSION

In this study, compared with control rats whose mothers had been injected with a sesame oil vehicle, the PNA female rats had abnormal estrus cycles, polycystic ovaries (characterized by cysts formed from atretic follicles, and diminished granulose layer) (21), and significantly higher (i) 17-OH, A, and T levels, (ii) 30- and 120-min OGTT glucose levels, (iii) AUC-Glu, and (iv) serum insulin concentrations and HOMA-IR. All these parameters in PNA rats were coincident with previous reports of primate, sheep, and rat PNA models for PCOS (3, 9). 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 over-express mRNA for key genes involved in androgen biosynthesis including LH receptor, steroidogenic acute regulatory (StAR) protein, CYP17, and CYP11A (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 PNA and control ovaries—both key insulin-signaling proteins and CYP17 protein are located in the theca and stroma of antral follicles. In recent study, a specific inhibitor of PI3-kinase, LY294002, inhibited insulin-induced 17α-hydroxylase activity in theca cells, indicating that the PI3-kinase pathway is a mediator of the insulin signal involved in the regulating of 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 (28). 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, -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 dexamethasone induced insulin resistance directly exaggerated the theca cells androgenic potential (24). In addition, it has been reported that insulin-sensitizing agent metformin directly inhibits androgen synthesis in ovary theca cells cultured in intro (4).

In a previous study in human subject, Yen 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 (32). Although there are discrepancies between our model rats and Yen’s PCOS subjects, both studies indicate 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, 27). Tanshinone’s therapeutic effect in acne treatment is based on reduction of T levels (11). We found that treatment with cryptotanshinone restored normal estrus cyclicity in PNA females and decreased T
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. showed that tanshinone reduces adipose mass and body weight, improves glucose tolerance without affecting food intake in a high fat diet–induced obese animal model (11). The molecular mechanisms behind the potent antidiabetic 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 (PPARγ) (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 higher than in control group (control-v, -cr) or PNA-cr ovaries; CYP17 expression in these three groups (control-v and -cr, 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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REFERENCES


8. de Paula Martins W, Santana LF, Nastri CO, Ferriani FA, de Sa MF, and Dos Reis RM. Agreement among insulin sensitivity indexes on the diagnosis of insulin resistance in polycystic


FIGURE LEGENDS

Figure 1. Immunohistochemical staining of rat ovaries for IRS-1, IRS-2, PI3K p85α, GLUT4, ERK-1, and CYP 17 in control vehicle ovaries. 1: IRS-1 was primarily localized within the ovarian stroma and theca (arrows); 2: IRS-2 was primarily localized within the ovarian stroma and theca (arrows); 3: PI3K p85α was primarily localized within the ovarian stroma and theca (arrows); 4: GLUT4 was primarily expressed in the ovarian stroma (short thick arrows) and corpus luteum (long arrow in upper left corner); 5: ERK-1 was expressed in theca cells (short thick arrows) and granulosa cells (long arrows); 6: CYP17 was primarily expressed in theca cells (short thick arrow) and granulosa cells (long arrows).

Figure 2. Protein expressions of IRS-1, IRS-2, PI3K p85α, GLUT4, ERK-1, and CYP17 in rat ovaries. A and B: IRS-1, IRS-2, PI3K p85α, and GLUT4 protein levels were significantly lower in the PNA group than in the control group. After cryptotanshinone treatment, protein levels were significantly higher in PNA-cr rats than PNA-v rats. The data represent means ± S.E. C and D: ERK-1 and CYP17 protein levels were significantly higher in the PNA group (testosterone propionate–injected mothers) than in the control group (oil-injected mothers) before treatment. After treatment, protein levels in the PNA-cr group (cryptotanshinone treatment) were significantly lower than the PNA-v group (vehicle treatment). Data represent means ± S.E. Note: #P < 0.05 vs. control-v; *P < 0.05 vs. PNA-v.
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>Body weight (g)</th>
<th>Food intake</th>
<th>Ovarian</th>
<th></th>
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</thead>
<tbody>
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<td></td>
<td>Day 0</td>
<td>Day 7</td>
<td>Day 14</td>
<td>(g/day/rat)</td>
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<tr>
<td>Control-cr</td>
<td>244.0±1.77</td>
<td>234.0±1.12</td>
<td>234.0±1.90</td>
<td>16.5±0.11</td>
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<tr>
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<td>255.0±1.79</td>
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<td>270.0±2.21</td>
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</tr>
<tr>
<td>P value</td>
<td>0.475</td>
<td>0.039</td>
<td>0.043</td>
<td>0.513</td>
</tr>
</tbody>
</table>

PNA, prenatally androgenized; Control-cr = oil-injected mothers, cryptotanshinone treatment (n = 12); Control-v = oil-injected mothers, saline vehicle treatment (n = 12); PNA-cr = testosterone propionate–injected mothers, cryptotanshinone treatment (n = 12); PNA-v = testosterone propionate–injected mothers, saline vehicle treatment, (n = 12).

Values are means ± SEM.

*P*<0.05 vs. PNA-v (one-way ANOVA followed by Bonferroni t test)

*P*<0.05 vs. control-v (one-way ANOVA followed by Bonferroni t test)
Table 2. Results of oral glucose tolerance test (OGTT), homeostatic model assessment of insulin resistance (HOMA-IR), insulin, and area under the curve (AUC) for glucose (Glu) 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>OGTT-Glu (mM/L)</th>
<th>AUC-Glu</th>
<th>Insulin (mIU/ml)</th>
<th>HOMA-IR</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
<td>30</td>
<td>60</td>
<td>120</td>
</tr>
<tr>
<td><strong>Before treatment</strong> (baseline)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PNA (n = 24)</td>
<td>6.42±0.06</td>
<td>9.85±0.05&lt;sup&gt;a&lt;/sup&gt;</td>
<td>10.03±0.09</td>
<td>8.16±0.07&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Control (n = 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>
</tr>
<tr>
<td><strong>After treatment</strong> (day 14)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PNA-cr (n = 12)</td>
<td>5.64±0.05</td>
<td>8.30±0.03&lt;sup&gt;b&lt;/sup&gt;</td>
<td>10.18±0.21</td>
<td>8.60±0.04&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>PNA-v (n = 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>
</tr>
<tr>
<td>Control-cr (n = 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>
</tr>
<tr>
<td>Control-v (n = 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>
</tr>
</tbody>
</table>

Control-cr = oil-injected mothers, cryptotanshinone treatment; Control-v = oil-injected mothers, saline vehicle treatment; PNA-cr = testosterone propionate–injected mothers, cryptotanshinone treatment; PNA-v = testosterone propionate–injected mothers, saline vehicle treatment; PNA, prenatally androgenized. Values are means ± SEM. <sup>a</sup> P < 0.05 vs. control, and <sup>aa</sup> P < 0.01 vs. control (independent t-test). <sup>b</sup> P < 0.05 vs. PNA-v (one-way ANOVA followed by Bonferroni t test).
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>LH (mIU/ml)</th>
<th>FSH (mIU/ml)</th>
<th>E₂ (pg/ml)</th>
<th>17-OH (ng/ml)</th>
<th>A (ng/ml)</th>
<th>T (nM/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Baseline (diestrus)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PNA (n = 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 (n = 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><strong>After treatment (diestrus)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PNA-cr (n = 12)</td>
<td>4.75±0.01</td>
<td>7.71±0.03</td>
<td>34.26±0.72</td>
<td>31.29±0.78</td>
<td>0.49±0.01</td>
<td>47.91±2.39</td>
</tr>
<tr>
<td>PNA-v (n = 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 (n = 12)</td>
<td>5.09±0.12</td>
<td>7.99±0.09</td>
<td>30.30±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 (n = 12)</td>
<td>4.70±0.01</td>
<td>8.02±0.09</td>
<td>33.77±0.72</td>
<td>24.70±0.80</td>
<td>0.56±0.01</td>
<td>73.09±1.55</td>
</tr>
</tbody>
</table>

Control-cr = oil-injected mothers, cryptotanshinone treatment; Control-v = oil-injected mothers, saline vehicle treatment; PNA-cr = testosterone propionate–injected mothers, cryptotanshinone treatment; PNA-v = testosterone propionate–injected mothers, saline vehicle treatment; PNA, prenatally androgenized; 17-OH, 17-hydroxyprogesterone; A, androstenedione; T, testosterone. Values are means ± SEM. *P < 0.05 vs. control, and **P < 0.01 vs. control (independent t-test). bP < 0.05 vs. PNA-v and b**P < 0.05 vs. PNA-v (one-way ANOVA followed by Bonferroni t test).
Figure 2

Panel A: Western blot analysis showing the expression levels of β-actin, IRS-1, IRS-2, PI-3K85α, and GLUT4 in Control-cr, Control-v, PNA-cr, and PNA-v groups. The molecular weights of these proteins are indicated.

Panel B: Bar graph showing the protein expression levels (OD mm²) of IRS-1, IRS-2, PI 3-kinase, and GLUT4 in the different groups. The graph includes error bars indicating standard deviation.

Panel C: Western blot analysis showing the expression levels of β-actin, ERK-1, and CYP17 in Control-cr, Control-v, PNA-cr, and PNA-v groups. The molecular weights of these proteins are indicated.

Panel D: Bar graph showing the protein expression levels (OD mm²) of ERK and CYP17 in the different groups. The graph includes error bars indicating standard deviation.