Supraphysiological leptin levels shift the profile of steroidogenesis in porcine ovarian follicles toward progesterone and testosterone secretion through increased expressions of CYP11A1 and 17β-HSD: a tissue culture approach

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Abstract

Evidence from both clinical and animal studies suggests that exposure to excess androgens results in cyst formation. The present in vitro study assessed the effects of supraphysiological concentrations of leptin (20 and 40 ng/ml) on progesterone (P₄), androstenedione (A₄), testosterone and estradiol (E₂) secretion by ELISA and the expression of CYP11A1, CYP17, 17β-hydroxysteroid dehydrogenase (17β-HSD) and CYP19 by western blot to answer the question of whether leptin could be an independent risk factor for cyst formation in pigs. Small- and medium-sized ovarian follicles were collected from prepubertal and cycling pigs. Increased P₄ and testosterone secretions were observed in both small- and medium-sized follicles in prepubertal and cycling animals whereas there was no change in E₂ secretion. Leptin treatment resulted in an increase in CYP11A1 and 17β-HSD protein expression but had no effect on CYP17 and CYP19 expression in follicles of either size from prepubertal and cycling pigs. Results of presented data suggest that leptin in elevated doses, by stimulatory effect on CYP11A1 and 17β-HSD protein expression resulting in elevated P₄ and testosterone secretions could be an independent risk factor for cyst formation in both prepubertal and cycling pigs.

Introduction

Cystic ovarian disease is a common reproductive disorder in women and female domestic animals, which can lead to temporary or permanent infertility. Evidence from both clinical and animal studies suggests that polycystic ovary syndrome (PCOS) is a developmental syndrome that manifests in adolescence and adulthood, in which exposure to excess androgens during the fetal or prepubertal phases of growth essentially ‘reprograms’ multiple tissues (Abbott et al. 2005, Xita & Tsatsoulis 2009).

Although the etiology and pathogenesis of this disorder remains unknown it has been proposed that hyperleptinemia and leptin resistance may play a role in the pathogenesis of the disease. Obese swine express ~306% higher levels of porcine leptin in sera than do contemporary, crossbred swine (Ramsay et al. 1998). Moreover obese swine express increased levels of leptin mRNA and protein compared with control swine of a similar body weight. Gonzalez-Anover et al. (2012) compared leptin levels in prepubertal gilt pigs of obese and lean genotypes and identified a profound increase in plasma leptin levels in the obese genotypes.

Leptin receptor gene expression in the porcine ovary has been characterized and porcine leptin receptor cDNA has been cloned and sequenced (Ruiz-Cortes et al. 2000). Leptin receptor levels are detectable in ovarian follicles collected from prepubertal animals, with a sixfold increase in expression in early antral and antral follicles of cycling animals (Gregoraszczuk et al. 2007). Serum leptin levels in pigs have been reported to be <10 ng/ml (Qian et al. 1999). We previously showed leptin levels to be almost consistent (2 ng/ml) in the ovarian follicular fluid of small-, medium-, and large-sized follicles collected from cycling pigs (Gregoraszczuk et al. 2004).

Using a wide range of leptin doses studies have reported both stimulatory and inhibitory effects on ovarian cell steroid secretion. Ruiz-Cortes et al. (2003) demonstrated that leptin at a dose of 10 ng/ml increased progesterone (P₄) accumulation in luteinized porcine granulosa cells in vitro whereas a dose of 1000 ng/ml
decreased P₄ accumulation. These authors further suggested that these results are physiologically significant because the 10 ng/ml dose is in the same order of magnitude as that found in normal circulation, which ranges from 1.5 to 5 ng/ml (Barb et al. 2001). Later Gregoraszczuk et al. (2004) demonstrated that leptin at a dose matching that in follicular fluid had no effect on basal testosterone and estradiol (E₂) secretions in small and medium ovarian follicles, whereas in large pre-ovulatory follicles decreased E₂ secretions were observed concomitant with an increase in P₄ secretion. On the other hand, leptin at all concentrations (2, 20, and 200 ng/ml) increased E₂ secretion and significantly diminished caspase-3 activity in ovarian follicles collected from prepubertal pigs (Gregoraszczuk et al. 2006). Sirotkin & Meszarosová (2010) noted an increase in P₄ secretion by porcine granulosa cells after the addition of 1 and 10 ng/ml leptin but observed no effect at a dose of 100 ng/ml.

Kozłowska et al. (2009) published a study demonstrating the expressions and localization patterns of CYP11A1, 3ß-hydroxysteroid dehydrogenase (3ß-HSD) and CYP19 in porcine ovaries with a polycystic status induced by dexamethasone (DXM) injections on days 7–21 of the estrous cycle. They found higher expressions of these markers in the follicles from polycystic pigs than in the medium-sized follicles from control pigs. Based on this publication, the aim of the presented data was to answer the question of whether leptin could be an independent risk factor for cyst formation. We plan to use supraphysiological leptin levels (20 and 40 ng/ml) and as a marker P₄, androstenedione (A₄), testosterone and E₂ secretions and expressions of steroidogenic enzymes CYP11A1, CYP17, 17β-hydroxysteroid dehydrogenase (17β-HSD) and CYP19 as a marker of leptin action on small- and medium-sized ovarian follicles. Additionally because data on the prepubertal period are scarce we compared the action of leptin on ovarian follicles collected from pubertal and cycling pigs.

Results

Effects of leptin on steroid secretions in small- and medium-sized follicles

In ovarian follicles collected from prepubertal animals, basal steroid secretions (unstimulated) were 5.9 ng/ml for P₄, 1.6 ng/ml for A₄, 2.08 ng/ml for testosterone and 0.22 ng/ml for E₂ in the small follicles (SFs) and 12.5 ng/ml for P₄, 1.85 ng/ml for A₄, 6.2 ng/ml for testosterone and 0.66 ng/ml for E₂ in the medium follicles (MFs). The effects of 20 and 40 ng/ml leptin on the concentrations of steroid secretions were similar in the SFs (P₄, 6.9 and 10.1 ng/ml; A₄, 1.8 and 1.6 ng/ml; testosterone 5.9 and 8.7 ng/ml; and E₂, 0.35 and 0.47 ng/ml respectively) and MFs (P₄, 14.5 and 14.6 ng/ml; A₄, 1.6 and 1.5 ng/ml; testosterone 8.0 and 8.8 ng/ml; and E₂, 0.4 and 0.5 ng/ml respectively) (Fig. 1).

In ovarian follicles collected from mature animals, the control steroid secretions were 8 ng/ml for P₄, 2.6 ng/ml for A₄, 5.9 ng/ml for testosterone and 0.7 ng/ml for E₂ in the SFs and 7.8 ng/ml for P₄, 4.2 ng/ml for A₄, 10 ng/ml for testosterone and 1 ng/ml for E₂ in the MFs. For both sizes of follicles, both concentrations of leptin (20 and 40 ng/ml) increased P₄ (SFs, 10.8 and 10 ng/ml; MFs, 10.2 and 11 ng/ml respectively) and testosterone (SFs,
12.8 and 12.8 ng/ml; MFs, 13.7 and 13.1 ng/ml respectively) secretions and had no effect on E2 secretion. Leptin at both doses significantly inhibited A4 secretion in both the SFs (1.5 ng/ml) and MFs (2.5 ng/ml) (\(P!0.05\)) compared with that from the pigs under control conditions (Fig. 2).

**Effects of leptin on CYP11A1, CYP17, 17\(\beta\)-HSD and CYP19 protein expressions**

In ovarian follicles collected from prepubertal animals, both concentrations of leptin significantly increased CYP11A1 protein expression in both sizes of follicles and had no effect on CYP19 expression. Furthermore leptin had no effect on CYP17 expression in the SFs but had an inhibitory effect on the expression of CYP17 in the MFs. Only the 20 ng/ml concentration of leptin increased 17\(\beta\)-HSD protein expression in the SFs (\(P!0.05\); Fig. 3).

In ovarian follicles collected from cycling animals, 40 ng/ml leptin increased CYP11A1, 17\(\beta\)-HSD and CYP19 protein expression but had no effect on CYP17 expression in the SFs. In the case of MFs both concentrations of leptin increased CYP11A1 expression and decreased CYP17 expression with no effect on 17\(\beta\)-HSD and CYP19 expression observed with either leptin concentration (Fig. 4).

**Discussion**

Several studies show that prenatal, perinatal, or postnatal androgen exposure in monkeys (Abbott et al. 2002), sheep (West et al. 2001), rats (Foecking & Levine 2005, Foecking et al. 2005, Mannerås et al. 2007), and mice (Sullivan & Moenter 2004) can induce cyst formation. Exposure of female monkey and sheep fetuses to excess androgen concentrations resulted in an excess of LH and enlarged ovaries that were polyfollicular, anovulatory and hyperandrogenic (Birch et al. 2003, Abbott et al. 2005). Evidence from both clinical and animal studies suggests that PCOS is a developmental syndrome that manifests in adolescence and adulthood, in which exposure to excess androgens during the fetal or prepubertal phases of growth essentially ‘reprograms’ multiple tissues (Abbott et al. 2005, Xita & Tsatsoulis 2009).

Numerous studies have focused on the impact of obesity on the hyperandrogenic state in women with PCOS and a possible link with leptin (Panidis et al. 2003). To our knowledge, there are no data concerning leptin and cyst formation in pig. However, there are data showing that obese swine express \(\approx 306\%\) higher levels of leptin (Ramsay et al. 1998). Moreover obese swine express increased levels of leptin mRNA and protein compared with control swine of a similar body weight. The presented data have been whether leptin could be an independent risk factor for cyst formation in both prepubertal and cycling pig. Increased P4 and testosterone secretions, with no changes in E2 secretions, were observed in response to leptin in both SFs and MFs from prepubertal animals and regularly cycling animals, which correlated with an increase in CYP11A1 expression and 17\(\beta\)-HSD expression. These results suggest that leptin acts mainly to increase P4 and testosterone secretion resulting in follicular atresia of SFs and MFs, and a failure to produce ovulatory follicles. Additionally the data support the idea that follicular cells
begin to luteinize at a premature stage of follicle development in response to leptin.

Faes et al. (2007) showed that P₄ concentration increased with follicle size only in atretic follicles and was greater in 5–8 mm atretic follicles than in healthy follicles of the same size. Others have described elevated androgen levels during follicular atresia in rodents and during granulosa cell apoptosis (Billing et al. 1993, McGee & Hsueh 2000).

The conversion of 17-ketosteroids to 17β-hydroxysteroids by 17β-HSD is essential for the formation of testosterone, dihydrotestosterone and E₂. There is evidence that the granulosa cells in arrested follicles in polycystic ovaries prematurely express CYP11A1 (Jakimiuk et al. 1998) and produce an excessive amount of P₄, compared with cells in control ovaries at a similar developmental stage (Willis et al. 1998). In anovulatory cycles, which are typical for cystic ovaries, follicle

![Figure 3](image1.png)

**Figure 3** Effects of leptin on CYP11A1, CYP17, 17β-HSD and CYP19 protein expression in small (SF) and medium (MF) follicles of prepubertal pigs. (A) immunoblot analysis and (B) densitometry results. The ratio of band intensity to β-actin for three independent experiments is shown. Intensity is expressed in arbitrary units. Statistically significant differences (P<0.05) between points in each graph are indicated with different letters, same letters indicating no significant difference, with a < b < c.<d < e.

![Figure 4](image2.png)

**Figure 4** Effects of leptin on CYP11A1, CYP17, 17β-HSD and CYP19 protein expression in small (SF) and medium (MF) follicles of cycling pigs. (A) immunoblot analysis and (B) densitometry results. The ratio of band intensity to β-actin for three independent experiments is shown. Intensity is expressed in arbitrary units. Statistically significant differences (P<0.05) between points in each graph are indicated by different letters, identical letters indicating no significant difference, with a < b < c.
development is arrested at the small antral stage. Consequently the granulosa cells fail to express aromatase, and estrogen production is minimal. By acting on the above-mentioned enzymes to influence ovarian steroid synthesis, leptin may play an essential role in the creation and/or course of cyst formation both in prepubertal and in regularly cycling animals. It has previously been reported that alterations to the concentrations of steroid hormones in the cystic ovaries of swine (Kozłowska et al. 2009) are accompanied by changes in the expressions and cellular distributions of CYP11A1, 3β-HSD, 17α,20-lyase, 20α-HSD, and/or CYP19.

Kozłowska et al. (2009) showed that the expression of CYP11A1, -CYP17, -17β-HSD in cysts was higher than that in MFs of control gilt pigs. Moreover, an injection of DXM resulted in increased levels of P450scC in the walls of SFs relative to control gilt pigs. Elevated testosterone secretion induced by leptin could result in a failure to activate or inhibition of aromatase. In follicles from both prepubertal and mature animals in vitro exposure to high doses of leptin had no effect on E2 secretion and CYP19 protein expression. Granulosa cells in antral follicles normally begin to express aromatase (at a size of 7 mm; Jonard & Dewailly 2004) and follicular arrest in cystic ovaries occurs when an excess of intra-ovarian 5α-reduced androgen inhibits granulosa cell aromatase activity in vitro and impairs follicle growth (Goodarzi et al. 2011).

Results of presented data suggest that leptin in supraphysiological doses by stimulatory effect on CYP11A1 and 17β-HSD protein expression resulting in elevated P4 and testosterone secretion could be an independent risk factor for cyst formation in both prepubertal and cycling pig.

**Materials and Methods**

**Reagents**

M199, fetal bovine serum (FBS; heat-inactivated), antibiotic/antimycotic solution (100×), Tris, Na-deoxycholate, Nonidet NP-40, SDS, protease inhibitors (EDTA-free), dithiothreitol (DTT), Tween 20, bromophenol blue and leptin were obtained from Sigma Chemical Co.

**Tissue culture**

Porcine ovaries were obtained from a local slaughterhouse. Ovaries were collected in a bottle filled with sterile saline and transported to the laboratory. SFs (3–4 mm; n=6) and MFs (4–5 mm; n=6) were obtained from prepubertal or regularly estrous cycling animals on days 4–6 (for SFs) and days 10–12 (for MFs) of the estrus cycle, as described previously (Rak-Mardyla & Gregoraszczuk 2012). Estrus was designated as day 0. In each experiment six ovaries from three animals were selected. As each ovary yielded four to six follicles the total number of follicles for each preparation varied between 24 and 36. This procedure was chosen to minimize potential variation between follicles and animals. After isolation, follicles were slit using small scissors to facilitate penetration of the compounds into the tissue and the removal of oocytes and follicular fluids. Whole follicular walls including theca and granulosa cells were individually placed in 24-well plates in M199 medium without phenol red supplemented with antibiotic/antimycotic solution and 5% FBS. The treatment consisted of two doses of leptin, 20 and 40 ng/ml. Serum leptin levels in the pig have been reported to be <10 ng/ml (Qian et al. 1999). The doses of leptin were chosen based on the data of Ruiz-Cortes et al. (2003), which suggested that a 10 ng/ml dose employed in vitro is of the same order of magnitude as that found in circulation. The follicles were maintained at 37 °C in a humidified atmosphere containing 5% CO2. After 24 h of incubation, the medium was separated from the follicles and stored at −20 °C for steroid hormone determination. Whole follicles were frozen at −70 °C for CYP11A1, CYP17, 17β-HSD and CYP19 protein expression analysis.

**Analysis of steroid levels**

The concentrations of P4, A4, testosterone and E2 in culture media were determined by enzyme immunoassay (EIA) using commercial ELISA kits (DRG Diagnostic, Marburg, Germany). All samples were run in duplicate in the same assay. The sensitivity limits for P4, A4, testosterone and E2 were 0.045, 0.019 and 0.083 ng/ml, and 9.714 pg/ml respectively. The coefficients of variation for inter- and intra-run precision were 4.34 and 6.99% respectively for P4; 12.1 and 5.6% for A4; 6.71 and 3.28% for testosterone; and 6.72 and 2.71% for E2. The range of the assay was 0–40 ng/ml for P4; 0.019–10 ng/ml for A4; 0–16 ng/ml for testosterone; and 0–2000 pg/ml for E2. Cross-reactivity of testosterone to A4 was 0.9% and of testosterone to E2 was <0.1%. Cross-reactivity of E2 to A4 and testosterone was 0% and to estrone (E1) was 0.2%, based on the assays DRG EIA-1559 (for testosterone) and EIA-2693 (for E2).

**Immunoblot**

Whole follicles were homogenized twice in ice-cold lysis buffer (50 mM Tris–HCl, pH 7.5, 100 mM NaCl, 0.5% Na-deoxycholate, 0.5% NP-40, 0.5% SDS and protease inhibitory EDTA free). For each experiment three follicles per treatment group were used. The homogenate was centrifuged at 15 000 g at 4 °C for 30 min and the protein concentration in the lysates determined using the Bradford reagent (Bio-Rad Laboratories) with BSA as a standard. Samples containing 40 μg total protein were reconstituted in the appropriate amount of sample buffer (125 mM Tris, pH 6.8, 4% SDS, 25% glycerol, 4 mM EDTA, 20 mM DTT and 0.01% bromophenol blue). The samples were separated by 10% SDS–PAGE in a Bio-Rad Mini-Protean II Electrophoresis Cell and then electrotransferred to a PVDF membrane. The membrane was washed and the nonspecific binding sites were blocked with 5% milk/0.2% Tween 20 in 0.02 M TBS for 2 h. The membranes were incubated overnight at 4 °C with anti-CYP11A, -CYP17, -17β-HSD and -CYP19 antibodies, diluted 1:200 (sc-18040, sc-46084, sc-26963 and sc-14244 respectively; Santa Cruz Biotechnology). Anti-β-actin antibody
(A5316, Sigma Chemical Co.), diluted 1:3000, was used as a loading control. After incubation with the primary antibody, the membrane was washed with TBS/0.02% Tween 20 and then incubated for 1 h with HRP-conjugated donkey anti-goat IgG (sc-2020 for CYP11A, CYP17, 17β-HSD and CYP19; Santa Cruz Biotechnology), diluted 1:2000, or an HRP-conjugated secondary antibody (P0447, DakoCytonmation, Glostrup, Denmark), diluted 1:5000 for β-actin. Signals were detected by ECL using Western Blotting Luminol Reagent (sc-2048; Santa Cruz Biotechnology) and visualized using the Chemidoc XR5+ System (Bio-Rad Laboratories). All bands visualized by ECL were quantified by densitometry using Image Lab 2.0 Software (Bio-Rad Laboratories).

Statistical analysis
Each experiment was repeated three times (n=3). For ELISA each sample was run in quadruplicate. Statistical analysis was performed using GraphPad Prism 5, La Jolla, CA, USA. The data were analyzed by one-way ANOVA with Tukey honestly significant differences multiple range test to assess differences between small- and medium-sized follicles and differences between control and leptin-treated groups.

Declaration of interest
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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