Obesity induced infertility and hyperandrogenism are corrected by deletion of the insulin receptor in the ovarian theca cell.

Abbreviated Title: Cyp17IRKO Rescues DIO-Associated Infertility

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Abstract
Women with polycystic ovary syndrome (PCOS) exhibit elevated androgen levels, oligo-anovulation, infertility, and insulin resistance in metabolic tissues. The aims of these studies were to determine the role of insulin signaling in the development and function of ovarian theca cells, and the pathophysiologic effects of hyperinsulinism on ovarian function in obesity. We disrupted the insulin receptor (IR) gene specifically in the theca interstitial (TI) cells of the ovaries (Cyp17IRKO). No changes in reproductive development or function were observed in lean Cyp17IRKO female mice suggesting that insulin signaling in TI cell is not essential for reproduction. However, when females were fed a high fat diet (Diet Induced Obesity (DIO)), DIO-WT mice were infertile and experienced increased circulating testosterone levels, while DIO-Cyp17IRKO mice exhibited improved fertility and testosterone levels comparable to those found in lean mice. The levels of pIRS1 and CYP17 protein were higher in the ovary of DIO-WT compared to DIO-Cyp17IRKO or lean mice. Ex vivo studies using a whole ovary culture model demonstrated that insulin acts independently or additively with hCG to enhance androstenedione secretion. These studies reveal the causal pathway linking hyperinsulinism with ovarian hyperandrogenism and the infertility of obesity.
Introduction

Polycystic ovary syndrome (PCOS) is a heterogeneous endocrine disorder that affects 6-10% of reproductive aged women worldwide (1). About half of affected women have metabolic dysfunction, e.g. insulin resistance even in the absence of obesity (2). Since the pathologic features present in PCOS including hyperandrogenemia, hyperinsulinemia, hypersecretion of LH, and hyperlipidemia often co-exist, it is difficult to discern the relative contribution of each hormonal and metabolic abnormality to the dysfunction present in PCOS.

In an effort to unravel the pathophysiology of the multi-organ and multi-hormone dysfunction of PCOS, our laboratory has used tissue specific disruption of crucial pathway genes in affected organs. We previously reported that hyperinsulinemia in obese mice is associated with LH hypersecretion, female infertility and hypertestosteronemia much like some women with PCOS. Disruption of the insulin receptor specifically in gonadotrophs partially restored fertility, indicating that insulin signaling in the gonadotroph plays a role in the reproductive abnormalities seen in obesity induced infertility (3). However, the infertility phenotype was only partially rescued by the loss of insulin signaling in the gonadotroph, indicating that obesity is contributing to infertility via effects elsewhere in the reproductive axis.

DIO female mice exhibit high serum testosterone levels similar to some women with PCOS. The rate limiting step in androgen biosynthesis is mediated by the cytochrome P450 17a hydroxylase/17, 20 lyase enzyme encoded by the Cyp17 gene (4). This enzyme has two enzymatic functions; mediating the 17alpha hydroxylation of progesterone or pregnenolone, and subsequent conversion to dehydroepiandrosterenedione or androstenedione, respectively. In female rodents, P450Cyp17 activity is primarily present in the theca and interstitial cells (TI
cells) of the ovary, making them the primary source of androgen since the mouse adrenal gland does not produce androgen (5).

Cyp17 expression is not only responsive to LH from the pituitary but may also be regulated by other paracrine and endocrine signals such as IGF1 and insulin. For example, reducing serum insulin levels using metformin (6) decreased the secretion of serum 17 alpha-hydroxyprogesterone in response to GnRH agonists, suggesting that hyperinsulinemia may play a role in high androgen synthesis. Some of these effects could be mediated indirectly via increased pituitary LH secretion; however, insulin could serve as a co-gonadotropin on the ovary to contribute to increased androgen synthesis in obesity. In vitro, insulin stimulates ovarian androgen secretion in human and animal ovarian cells (7-11). Insulin receptors have been localized to the ovarian theca-interstitial (TI) cells (12, 13) and mediate insulin action on steroidogenesis in vitro (10, 14, 15) by stimulating androgen secretion alone or augmenting LH induced androgen secretion (7, 10, 16).

Ovarian steroidogenesis occurs in response to insulin in the ovaries of women with PCOS (10), even in the setting of peripheral insulin resistance, which suggests that ovarian insulin signaling is regulated differently than insulin signaling in other organs in hyperinsulinism. Tissue specific differences in insulin resistance were observed in studies from our laboratory demonstrating that obese female mouse with insulin resistance present in the liver, muscle and fat, retained sensitivity to insulin in the pituitary and the ovary (17). Consequently, basal insulin signaling in the pituitary and ovary was increased in the setting of obesity associated hyperinsulinemia. The anatomical and functional evidence thus warranted an analysis of the physiologic and pathologic role of insulin signaling in the theca cells in the development and function of the
ovary. Hence, we developed a mouse model in which the insulin receptor was specifically deleted in the TI cells of the ovary using CRE/LoxP technology.

**Research Design and Methods**

**Mouse models.** Floxed-IR mice were obtained from Dr. C. Ron Kahn and have been previously described (18). Cyp17iCre mice were described in (19). Cyp17IRKO mice were generated by mating homozygous female (Cyp17iCre/-; fl/fl-IR) with heterozygous male (Cyp17iCre+/-; fl/wt-IR). Diet induced obese (DIO) mice were generated as previously described (17) in which 2 month old female mice were fed 60% high fat diet. Mice with genotyping (Cyp17iCre/-; fl/fl or fl/wt-IR) were used as control. Mice body mass and overnight fasted glucose were measured at age 6 months old. All procedures were performed with approval of the Johns Hopkins Animal Care and Use Committee.

**Genotyping and DNA extraction.** Primers for IR were as described in (20). These primers will detect WT band (280bp); fl/fl band (320bp) and KO band (220bp). Primers for Cyp17iCre were: cypcre-F: TCTGATGAAGTCAGGAAGAACC; cypcre-R: GAGATGTCCTTCACTCTGATTCC (19). DNA was extracted as in (21).

**Hormonal and glucose assays.** GnRH stimulation and glucose tolerance test. Basal morning levels of LH and FSH were measured by Luminex assay as previously described (21). Insulin and leptin were measured by Luminex assay (17) from overnight fasted mice. Androstenedione, testosterone and estradiol were measured by The University of Virginia, Center for Research in Reproduction, Ligand Assay and Analysis Core. LH was also measured after GnRH stimulation as previously described (3). Overnight fasted mice were injected with 2g/kg BW dextrose and glucose was recorded at 0, 15’, 30’, 60’, 90’ and 120’ as previously described (17).
Puberty and fertility examination. Puberty and estrous cyclicity were analyzed as in (21). Fertility was assessed as previously described (3, 21), briefly, female mice (5 month old) were mated with proven fertile male mice and fertility rates were evaluated as a percentage of the four mating trials that resulted in pregnancy as described in (3).

Quantitative Real-Time PCR. Ovary RNA was extracted by Trizol (Invitrogen, Grand Island, NY) according to the manufactory’s protocol. One µg of total RNA was reverse transcribed (iScript cDNA Synthesis Kit, BioRad, Hercules, CA) to cDNA. mRNA level of genes (Cyp17, Cyp19, StAR and LHR) related to androgen production in ovary were measured by iQSYBR green according to the manufactory protocol (Bio-Rad). Primers for Cyp17: sense 5’-GATCTAAGAAGCGCTCAGGCA3’ and antisense 5’-GGGCACTGCTCAGGATCAA-3’ (22); Cyp19: sense 5’-TTGGAATGCTGAAACCCC-3’ and antisense 5’-CAAGAATCTGCATGGA-3’ (23); StAR: sense 5’-CCCCAACAGCGATAGCAAG-3’ and antisense 5’-GCTGAATCCCCAAACCTTCT-3’; and LHR: sense 5’-GACCAAAAGCTGAGGCTGAGA and antisense 5’-CAATGTGGCCATCAGGGTAGA-3’ (24). Taqman quantitative PCR (BioResearch Technologies, Novato, CA) was performed for insulin receptor (IR) and GAPDH was used as the internal control. Primers for IR: sense 5’-ATGGGCTTCGGGAGAGGA-3’ and antisense: 5’-GGATGTCCATACGAGGCAC-3’ with the probe 5’-TGAGACAGCCTTGCTGTGACCATT-3’ labeled with FAM (5-carboxyfluorescein) and BHQ-1 (Black Hole Quencher-1). GAPDH: sense 5’-GAGCATCTTGGGCTACACT-3’ and antisense 5’-GGCATGGAAGGCTGAGG-3’ with the probe 5’-AGGACCAGGGTTTCTCAGGGA-3’ labeled with Cal fluoro red-610 and BHQ-2. Reactions were performed as described before (21).
**Western blot, insulin signaling assay and ovary culture.** Overnight fasted mice were injected with regular human insulin (1.5 unit/kg BW) or PBS, the ovary was collected 10 minutes after injection and used either for theca and granulosa cell separation (25) or for whole ovary incubation. Briefly, for the theca and granulosa cell separation, the ovary was taken from the bursa and immersed into McCoy’s 5A medium (Life Technologies, Grand Island, NY) supplied with 25mM Hepes, 0.1% BSA and antibiotics (26). Ovary was manual punctured with a 26-gauge needle and a fine tip tweezers. Granulosa cells were freed into the medium and centrifuged at 250g for 5 min at 4°C. The pellets were frozen in LN₂. The remaining cells of the ovary considered to be an enriched TI/stromal cells were centrifuged briefly and frozen in LN₂.

Measurement of protein concentrations and western blot analysis were performed as described in (17). Briefly, 5µg protein of isolated theca cells from each individual mouse ovary was loaded onto the gel to perform the western blot analysis. Primary antibodies used included: rabbit polyclonal antibody to pAKT (Ser 473) or to AKT; rabbit monoclonal antibody to insulin receptor β (4B8) (Cell Signaling Technology, Danvers, MA); rabbit monoclonal antibody to cytochrome P45017A1 (Cyp17) (Abcam, Cambridge, MA); rabbit polyclonal antibody to LHR (Santa Cruz Biotechnology, Santa Cruz, CA); mouse monoclonal antibody to actin Clone C4 (EMD Millipore, Billerica, MA). pAKT and total AKT was also measured by western blot or by Bio-rad Bio-Plex Pro Assays in the Luminex200 (Austin, Texas). pTyr-IRS1 protein expression was measured by pIRS1 Milliplex Map Phospho IRS1 Mapmates kits (EMD Millipore) in the Luminex200. Alternatively, the ovary was incubated in a 24-well tissue culture plate with tissue culture well inserts (Millicell-CM, 0.4µm pore size; EMD Millipore) (27, 28) with McCoy’s 5A medium. Medium was collected after 3hrs of incubation (0 hour) and ovary was incubated with fresh medium with 1.6IU/ml hCG. Medium was then collected 24 hours later (24hrs).
Histology and immunostaining. The ovary was dissected from diestrus mice and fixed in 10% formalin phosphate buffer and sectioned to 5 microns thickness in its entirety by Johns Hopkins Medical Laboratories (Histology group). Every 10th section was collected and ovarian sections were stained with hematoxylin and eosin. The copora lutea, preantral follicle, antral follicle were counted and examined with a Zeiss microscope. For the immunostaining, mice were overnight fasted and injected with 1.5unit/kg BW insulin. Ovaries were collected after 10 minutes of injection and fixed in 4% paraformaldehyde. Each ovary was frozen in OCT and sectioned to 5 microns. Sections were incubated with primary (Phospho-Akt (Ser473), Cell Signaling Technology) and secondary antibody goat anti-rabbit IgG Alexa fluro-488 (Invitrogen, Eugene, Oregon) as described in (21). Sections were photographed with an AxioCamMR camera and exported to AxioVision Software.

Statistical analysis. Data was analyzed by unpaired student’s t test using GraphPad Prism (GraphPad Software, Inc. San Diego, California) except where specifically addressed. Data are expressed as means ± S.E.M. and different letters represent statistically significant. P<0.05 was defined as statistically significant.

Results

Generation of Cyp17IRKO mice. Mice with insulin signaling absent in the ovarian theca cells (Cyp17IRKO mice) were generated by mating homozygous floxed IR mice (3, 18) with Cyp17-iCre mice that express the iCre recombinase gene in the theca-interstitial (TI) cells of the ovary (19). Quantitative real-time PCR determined that Cyp17IRKO has significantly lower IR mRNA levels in the TI cells of the ovary than control littermates. Other tissues (GC, hypothalamus, pituitary and adrenal) have similar levels of IR expression (Fig. 1A) between control and KO. In addition, western blot showed that fasted basal levels of IR and pAKT protein were also
dramatically decreased in TI cells from Cyp17IRKO mice when compared to WT mice. As expected, there was no difference in IR expression in granulosa cells isolated from control and Cyp17IRKO littermates (Fig. 1B). As a confirmation of disrupted insulin signaling, the insulin induced increase in pAKT, as assessed by Luminex assay, was significantly attenuated in the TI residues of Cyp17IRKO mice compared to control mice (Fig. 1C). Histological analysis confirmed this finding. pAKT expression (green fluorescence) detected by immunostaining was similar in the ovarian GC cells after insulin stimulation in either control or Cyp17IR-KO mice (Fig. 1D). However, pAKT expression in TI cells was much stronger in control than Cyp17IR-KO mice. Further probing the insulin signaling pathway, we examined activation of the IRS1 scaffolding protein. We had previously demonstrated that insulin signaling was mediated by IRS1 in lean and obese mice (17). Basal p-Tyr-IRS1 levels were increased significantly in TI cells, but not GC cells, between control and Cyp17IR-KO mice (Fig. 1E).

**Metabolic status of DIO Cyp17IRKO and DIO control mice.** To determine the role of obesity on IR signaling in the ovary, mice were fed a HFD as previously described (3, 17). Body mass was recorded from postnatal day 20 to day 180. In either the lean or DIO state, there is no difference in weight between control and cyp17IRKO mice (Fig. 2A). Fasting glucose, insulin and leptin levels were measured at 6 months of age with no significant difference between Cyp17IRKO or control mice fed either a normal chow or high fat diet (Fig. 2B-D). Liver pAKT was significantly reduced in DIO mice when compared to lean mice after insulin stimulation (Fig. 2E) indicating that insulin resistance was present in both control and Cyp17IRKO obese mice. As further confirmation of the metabolic derangements of the DIO state, DIO mice (control and KO) demonstrated glucose intolerance compared to lean mice (Fig. 2F). Thus,
metabolic status was determined by the dietary intervention, with no difference in metabolic status between control and Cyp17IRKO mice.

**Puberty and cyclicity.** As an assessment of reproductive status, pubertal development and estrous cyclicity were evaluated. Vaginal opening and first estrus are two indicators of female puberty onset (Safranski et al., 1993), and there was no difference between control and cyp17IRKO mice in either measure of puberty (Fig. 3A). Folliculogenesis in the ovary was also examined by H&E histology. There are no differences in the number of corpora lutea (CL), preantral follicles or antral follicles between control and Cyp17IRKO lean female mice (Suppl.1). To assess the function of the ongoing reproductive cyclicity of the female mice, vaginal cytology was collected for 16 consecutive days from 5 to 6 month old mice. There was no difference between control and Cyp17IRKO lean mice; however, DIO mice exhibited irregular estrous cycles, consistent with our earlier findings (Brothers et al 2010). The duration spent in the different phases of the cycle was similar in lean control and Cyp17IRKO mice (Fig. 3B). Control-DIO mice had fewer days in proestrus (PE) and estrus than lean mice, remaining predominately in persistent diestrus and metestrus, however, the Cyp17IRKO-DIO mice cycled comparably to the lean mice with significantly more days in PE and E than Control-DIO litter mates (Fig. 3C and D) indicating estrous cycling was partially restored in DIO Cyp17IRKO mice.

**Fertility is partially rescued in Cyp17IRKO DIO mice.** Lean or DIO female mice (5 month old) were mated with 4 different proven fertile male mice, in which the male mouse was rotated into each female cage for 7 days. Female mice remained in the cage alone for another 23 days to assess whether pregnancy had occurred. Lean female mice had a similar fertility rate (90%) in both groups (WT and CYP17IRKO). Although female DIO mice had an impaired ability to
produce offspring, Cyp17IRKO DIO mice had a significantly improved fertility rate compared to their control DIO litter mates (60% versus 30%, respectively; Fig. 4). WT DIO mice have similar number of vaginal plugs as lean mice, indicating a similar number of mating events (3). Fertility rate was correlated with numbers of CL which were decreased in DIO-Control compared to lean-Control, lean-KO and DIO-KO (Suppl. 2 and 3) indicating reduced numbers of ovulatory cycles in the WT-DIO mice. While the control DIO mice had fewer pregnancies than KO DIO mice, the number of pups in each litter was not significantly different (10-13 pups/litter).

**Testosterone is reduced in Cyp17IRKO mice.** Morning blood samples were collected during metestrous and diestrous for measurement of hormone levels. Basal LH levels were higher in DIO mice than lean mice (Fig. 5A) as previously reported (3). To evaluate the role of pituitary function in the altered LH levels, a GnRH stimulation test was performed. DIO mice had a significantly increased response to GnRH (Fig. 5A), while no difference was found in pituitary response between control and Cyp17IRKO mice, either lean or DIO.

Control DIO mice had significantly increased testosterone levels when compared to lean mice while the increase was attenuated in Cyp17IRKO DIO mice (Fig. 5B). We investigated whether the increased testosterone was secondary to increased Cyp17 or LH receptor expression at the mRNA or protein level in Control-DIO mice. Cyp17IRKO DIO mice, whether lean or DIO, had reduced IR expression when compared to lean or DIO control mice, respectively (Fig. 6A).

While there were no significant changes in the level of mRNA for LH receptor, Cyp19 and StAR (Fig. 6B-D), there was a significantly increased Cyp17 mRNA expression in DIO control (Fig. 6E) which was abrogated in KO-DIO mice. Protein level was measured by western blot analysis from TI cells. The basal level of CYP17 and LH receptor was similar between lean control and
KO (suppl. 4). CYP17 protein levels were increased in Control-DIO mice compared to Control-lean and KO-DIO; LH receptor levels were not different between any groups (Fig. 6F). Intensity of protein level by western blot was quantified by densitometry and the observed differences between control lean and DIO mice in Cyp17 and LH receptor protein expression are quantified in Fig. 6F1 and 6F2. Additionally, both control lean and DIO mice expressed higher CYP17 protein while LH receptor expression did not change after insulin injection (Fig. 6G, G1 and G2). Estradiol levels were also measured and were not significantly different among the groups (Suppl. 5)

**Cyp17IRKO ovary has reduced secretion of androgens in ex vivo culture.** Overnight fasted mice were injected with insulin or PBS and one ovary was used for **ex vivo** culture (Fig. 7A). Androstenedione was measured in the cultured medium (Fig. 7B). Ovaries from all groups secreted similar levels of androstenedione after hCG. However, only ovaries from WT lean mice injected with insulin had increased secretion of androstenedione and ovaries from Cyp17IRKO mice had no response to insulin. Androstenedione was increased significantly after insulin plus hCG treatment in both WT-lean and WT-DIO mice compared to hCG or insulin alone. There was no change of androstenedione concentration between hCG alone and insulin plus hCG in either Cyp17IRKO-lean or -DIO groups.

**Discussion**

PCOS is a complex and multi-factorial disease that is the leading cause of infertility in women. Its development and progression is difficult to understand due to the interconnected nature of the hormonal disturbances present in the syndrome. The diagnosis of PCOS includes amenorrhea, hyperandrogenism and polycystic ovaries, and women with PCOS frequently exhibit insulin resistance, with or without obesity, and increased LH secretion. Additionally, the common
features of PCOS may be due to malfunction of the reproductive system at the level of the hypothalamus, pituitary, or gonad, independently or together.

Obese women with PCOS have higher testosterone levels than lean women with PCOS, suggesting an additional compounding effect of insulin in PCOS (29). With hyperinsulinemia, energy storage tissues such as muscle, fat, and liver exhibit diminished activation of downstream insulin signaling components, indicating insulin resistance. In contrast, in obese female mice with hyperinsulinemia, the pituitary and ovary exhibit heightened activation of downstream insulin receptor pathways (17) indicating retained insulin sensitivity of the reproductive tissues in the face of peripheral insulin resistance. Previous work from our laboratory demonstrated that heightened insulin signaling in the pituitary contributed to high LH levels and infertility in obese females (3). Using tissue specific deletion of the insulin receptor in the gonadotroph, this model demonstrated that rescue of the neuroendocrine dysfunction (high LH levels) did not completely correct the infertility, suggesting that insulin signaling elsewhere in the reproductive axis also plays a role in the development of the infertility. Since the ovary also retains insulin sensitivity in obese female mice (17), we hypothesized that insulin may directly stimulate androgen synthesis at the level of the ovary and by this mechanism contribute to hyperandrogenemia and infertility. Previous studies indicating that insulin signaling in ovarian cells stimulates androgen synthesis have used in vitro culture techniques, leaving open the question of the true functional significance of the observations. The studies presented here are the first to investigate the significance of in vivo insulin signaling in theca cells and subsequent reproductive function. The use of tissue specific knockout animals allowed us to unravel the relative contribution of individual reproductive tissues to one feature of PCOS, hyperandrogenism. We demonstrate that
insulin signaling in the theca interstitial cells contributes to obesity associated infertility and hyperandrogenemia by augmenting CYP17 expression and activity.

Insulin receptor signaling in the TI cells is not required for normal development and reproductive function of the ovary as indicated by the normal puberty, estrous cycling, fertility (Fig. 3 and 4) and similar basal serum LH and pituitary response to GnRH (Fig. 5A) of the Cyp17IRKO mice compared to control mice. Since obesity is associated with high circulating insulin levels and infertility (3, 30, 31), we explore the role of enhanced insulin signaling on TI cell function. In tandem, we used a conditional KO strategy to isolate the direct effects of enhanced insulin signaling on ovarian function from the indirect effects mediated at the level of the pituitary and conveyed to the ovary by LH. These studies, therefore, provide a unique opportunity to disassociate the effects of high LH and insulin on the function of the ovary. After 3 to 4 months of a high fat diet, Cyp17IRKO mice have identical metabolic characteristics to WT mice including a significant weight gain, fasting hyperglycemia, hyperinsulinemia and hyperleptinemia relative to chow fed mice (Fig. 2) indicating similar metabolic regulation. These findings recapitulate the metabolic patterns of obese rodent models (3, 17, 30, 31).

Although Control-DIO mice had irregular estrous cycles, and diminished fertility, Cyp17IRKO-DIO mice exhibited improved estrous cyclicity (Fig. 3 C-E) and a higher fertility rate compared to Control-DIO mice (Fig. 4). The Control-DIO mice had a lower number of corpora lutea compared to Cyp17IRKO-DIO mice (suppl. 2 and 3), indicating that ovulatory events are more frequent in the Cyp17IRKO mice, and likely account for the difference in fertility between the groups. However, the fertility rate of the DIO Cyp17IRKO mice remains lower than in lean control or lean Cyp17IRKO mice, which may be due to the pathogenic effects of obesity on multiple target tissues including the pituitary (3), uterine endometrium at implantation (32) or
egg quality (33). As the ovary is regulated by the pituitary, we next evaluated pituitary function. Cyp17IRKO-DIO and Control-DIO mice had similar relatively high basal LH levels and enhanced GnRH stimulated pituitary response when compared to lean mice (Fig. 5A). These data confirm our previous data that the elevated baseline LH levels present in the obese state are due to increased GnRH responsiveness of the pituitary (Fig. 5A), through enhanced insulin signaling in the pituitary (3). Elevated LH independent of obesity may contribute to altered estrous cycling, ovulation and infertility as has been shown in the pituitary ER alpha receptor deficient mouse (34). The rescue of fertility in the current study cannot be explained by changes in LH secretion as both DIO-Control and KO have higher LH levels than mice fed normal chow. LH receptor protein levels and hCG responses were not different between control and Cyp17IRKO mice in the DIO state, indicating that LH sensitivity was preserved in the ovaries from Cyp17IRKO mice (Fig 6F, F2 and Fig. 7B). Therefore, these studies isolate a direct role for insulin at the level of the TI cells in mediating the infertility of obesity. Increased basal LH is not observed in all models of DIO induced infertility C57BL/6 mice (35), which may due to mice strain differences (30) or experimental conditions.

Insulin has been well documented to synergize with LH to stimulate androgen synthesis in vitro. Whether insulin can stimulate theca cells to secrete androgen independently of LH is controversial (8, 10, 36-41). In some women with PCOS, LH levels are normal but serum androgen levels are still high, thus implicating insulin as a co-gonadotroph contributing to the hyperandrogenism (42). Given the potential dual roles of insulin and LH on androgen synthesis, we investigated whether the restored cyclicity and fertility may due to the reduced androgen levels in the Cyp17IRKO-DIO mice (Fig. 5B). Testosterone levels were lower in lean Cyp17IRKO compared to lean control mice, but this difference did not reach statistical
significance. This may be due to the sensitivity of the testosterone assay, or it could be that in lean mice with normal insulin levels, LH is the sole regulator of androgen synthesis and production while in hyperinsulinemic conditions, insulin action contributes to augment androgen production (Fig. 7). The enhanced testosterone levels seen in the DIO mice when compared to the other experimental groups support this assertion. Since the mouse liver produces very low levels of sex hormone-binding globulin (SHBG), the modest differences in the total serum testosterone levels likely reflect greater changes in free testosterone levels (43).

Women with PCOS who are insulin resistant can exhibit hyperresponsiveness to insulin stimulated ovarian steroidogenesis (10, 44), likely mediated by the PI3K-AKT pathway (45, 46). Insulin increases pAKT in the ovary via the pIRS1 and pIRS2 scaffolding proteins (17) in the basal state and in the presence of systemic hyperinsulinemia and peripheral insulin resistance. TI cell AKT phosphorylation is lower in the CYP17IRKO mice compared to control mice in basal and insulin stimulated conditions (Fig. 1 B-D), and this decreased phosphorylation AKT of TI cells may associated with decreased pTyr-IRS1 (Fig. 1E), confirming altered downstream insulin receptor signaling in the KO ovary.

LH is the major factor leading to androgen secretion as it increases transcription of genes necessary for steroidogenesis, such as Star, Cyp11a, and Cyp17. Insulin also increases Cyp17 expression and enzymatic activity in human and animal theca cells (45, 47). Indeed, in the DIO state, theca cell CYP17 enzyme levels were higher in the control DIO mice than in the control lean or Cyp17IRKO DIO mice (Fig. 6F and F1), indicating that increased signaling via the insulin receptor in theca cells increases Cyp17 levels. CYP17 expression is not completely abolished in the KO animals, as LH signaling is intact (Fig. 6F and suppl. 4). As confirmation of the effect of insulin on CYP17 expression, we observed that exogenous insulin administration
increases CYP17 protein levels in the lean state, but in the hyperinsulinemic state associated with DIO, basal CYP17 expression was elevated but was not appreciably increased by exogenous insulin (Fig. 6G and G1). The effect of insulin on CYP17 expression was not due to an alteration in LH receptor levels, as indicated in figure 6G and G2. It has been reported that theca cells expressed higher mRNA levels for LH receptor, StAR and Cyp17 in polycystic ovaries (48). In our study, we do not observe increased mRNA expression of LH receptor and StAR, and do observe an increased Cyp17 mRNA level in DIO versus lean mice (Fig. 6 A-E). We document increased fasting basal levels of CYP17 protein (Fig. 6 F and G) in Control-DIO and insulin stimulated CYP17 protein expression in both lean and DIO control mice. Thus, insulin may increase serum testosterone in the DIO state in part by increasing CYP17 mRNA and protein expression independent of insulin’s effects on serum LH.

To investigate whether the insulin-induced increase in CYP17 expression was accompanied by an increase in CYP17 enzymatic activity, we measured theca cell secretion of androstenedione in response to insulin and LH. We injected 1.5 unit/kg BW insulin to overnight fasted mice, as this dose is the lowest dose that can stimulate the IRS-PI3K-pAKT signaling pathway in the ovary (17). In addition, we measured androstenedione levels in cultured medium rather than testosterone since this hormone is the major secreted androgen in the ovary (Suppl. 6 and (49, 50)) and differences in testosterone secretion between groups may not be detectable due to its low level of secretion at the time point we measured. Insulin alone stimulated androstenedione secretion in the control lean and DIO mice (Fig. 7B), but not in the Cyp17IRKO animals, indicating that insulin receptor signaling in the TI cells is necessary for this effect. After hCG administration alone, androstenedione secretion increased in all groups, which was expected since all the mice have a functional LH receptor. Preinjection in vivo with insulin prior to hCG
administration resulted in a secretion of a greater amount of androstenedione from control mice than from the Cyp17IRKO mice, indicating that insulin augments LH stimulated androgen secretion. Thus, insulin alone or in combination with LH increases TI cell androgen secretion, likely via an increase in CYP17 expression rather than enhanced LH receptor signaling.

The experiments herein indicate retained insulin sensitivity in the ovarian theca cells of hyperinsulinemic DIO mice has functional consequences, as control DIO mice exhibit higher CYP17 protein levels, androstenedione secretion, and serum testosterone compared to lean control mice or Cyp17IRKO mice with DIO. We suggest that the enhanced androgen secretion in the DIO state contributes to the ovarian dysfunction resulting in the reproductive abnormalities as the primary role of theca cell in ovarian steroidogenesis is androgen synthesis. However, we cannot exclude the possibility that altered insulin signaling affects other functions of the theca cell resulting in reproductive dysfunction.

In summary, our study indicates a direct role of insulin signaling in the theca-interstitial cells of the ovary to produce androgen in an obesity model of infertility (Fig. 8). Along with previous studies from our laboratory (3, 17), it demonstrates the multi-organ effect of hyperinsulinemia to induce abnormal production of ovarian androgens and subsequent ovarian dysfunction. We suggest that hyperinsulinemia is a trigger for the hyperandrogenemia and multi-hormonal dysfunction in women with PCOS. Our findings may contribute to the development of new therapies for the treatment of obesity related infertility by focusing efforts to reduce insulin action in reproductive tissues.

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S.W. developed the project, the researched data and wrote the manuscript; S.D. contributed to write the manuscript; S.R. and F.W. reviewed and edit the manuscript. C.K reviewed the manuscript; A.N. helped some experiments; A.W. helped develop the project, performed some experiments and wrote the manuscript; A.W. takes full responsibility for the manuscript and its originality. Authors have no conflict of interest.

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**Figure legends**

Fig. 1 Insulin receptor (IR) signaling is disrupted in the theca interstitial (TI) cells of the Cyp17IRKO mice ovary.

A. IR mRNA level was measured by q-RT-PCR and was significantly reduced in the TI cells of Cyp17IRKO ovary compared to control littermates, but no difference in IR expression was observed in granulosa cells (GC), hypothalamus (Hypo), pituitary (Pit) and the adrenal gland. 
B. TI cells and granulosa cells were separated from ovaries of overnight fasted mice. Western blot showed that the basal protein levels of IR and pAKT were reduced in TI cells of Cyp17IRKO ovary while no change was observed in granulosa cells.
C. pAKT protein expression of TI cells was measured after insulin stimulation by Luminex. pAKT was significantly increased in the control TI cells compared to the Cyp17IRKO TI cells. Data are mean±SEM and N= 5-6.
D. Immunostaining of pAKT in the ovaries of control and Cyp17IRKO mice after insulin stimulation. Green fluorescence (pAKT) expressed in the granulosa cells (GC) in the ovary. Strong expression of pAKT was observed in the TI cells of control ovary. However, there is no staining in the theca-interstitial (TI) cells of pAKT in the Cyp17IRKO. Dashed line separated the GC and TI cells.
E. pTyr-IRS1 levels were measured at basal level in TI cells and GC in lean and DIO mice. pTyr-IRS1 was significantly reduced in the KO TI cells compared to the control TI cells in either lean and DIO state. However, there was no change in GC between KO and Control. Data are mean±SEM and N= 4-8.

Fig. 2 Metabolic function is not altered in Cyp17IRKO mice.

A. Body mass; B, fasted glucose; C, fasted insulin; D, fasted leptin; E, pAKT were measured by Luminex assay. F, glucose tolerance test was conducted in both lean and DIO mice. There was no significant difference either between control and Cyp17IRKO lean mice or control-DIO and Cyp17IRKO-DIO mice in these parameters. E, DIO mice (control and KO) showed insulin resistance in liver characterized by attenuated increase in insulin induction of pAKT (measured by Luminex) compared to lean mice. F, Glucose tolerance test was compared between either lean control and lean KO group or DIO control and DIO KO groups. There was no significantly
different between control and KO in the same state. One way-ANOVA with Tukey post hoc test was used for (A-E) statistics. Data are mean ±SEM and N=5-8.

Fig. 3 Insulin signaling plays role in obesity induced fertility.

A. Lean Cyp17IRKO mice exhibited similar age of puberty onset by examination of vaginal opening and first estrous.
B. Percentage time spent in each stage was not significantly different between control and Cyp17IRKO lean mice.
C. Percentage time spent in each stage was significantly different between control and Cyp17IRKO DIO mice. Percentage time in proestrus and estrus was shortened, and metesturs/diestrus increased in control-DIO mice compared to Cyp17IRKO-DIO mice.
D and E are representative of vaginal cytology data from Control-DIO and Cyp17IRKO-DIO mice. Data are mean ±SEM and N=6-10

Fig. 4 Insulin signaling in the TI cells contributes to infertility in diet induced obesity.

Lean and DIO, WT and Cyp17IRKO female mice were randomly mated with 4 different proven fertile WT males. Cyp17IRKO-DIO mice have a significantly improved fertility rate compared with WT-DIO mice although the fertility is still impaired compared to the lean female mice. Data are represented as mean ±SEM and N=5-6.

Fig. 5 High fat induced hyperandrogenism is attenuated in the Cyp17IRKO mice.

A. Basal LH levels and LH levels following GnRH stimulation were compared between control and Cyp17IRKO mice under lean or DIO state. Both control and Cyp17IRKO DIO mice had significantly higher response to GnRH stimulation compared to lean mice.
B. Testosterone is significantly increased in Control-DIO mice compared to Control-Lean mice. However, the levels in Cyp17IRKO-DIO mice are not significantly changed compared to lean mice. Data are represented as mean ±SEM and N=5-13.

Fig. 6 Quantitative real-time PCR was performed and protein expression was measured by western blot.

A-E, mRNA levels of IR, LH receptor, Cyp19, Cyp17 and StAR were measured in mouse ovary. As expected IR was decreased in KO, and Cyp17 gene expressions was significantly changed among groups. Data are represented as mean ±SEM and N=6-18.
F. Increased basal level of Cyp17 in TI cells of Control-DIO mice was observed by western blot. Intensity of Cyp17 level (F1) and LHR level (F2) in western blot was quantified by densitometry.
G. Western blot showing protein levels of Cyp17, LHR and beta-actin before and after insulin treatment. Protein levels were quantified by densitometry and are graphed in (G1, Cyp17) and (G2, LHR) and expressed relative to levels of beta-actin. Levels of Cyp17 protein increase significantly in response to insulin in lean-control mice, but due to higher basal Cyp17 levels in control-DIO mice, the small increase in Cyp17 levels in response to insulin was not significant.
LH receptor is not altered among the groups. One-Way ANOVA with Tukey post hoc test was used. Data are represented as mean ±SEM and N=5-6.

Fig. 7 The Cyp17IRKO mice blocked insulin stimulated androgen secretion. A. Mice were overnight fasted and 1.5unit/KgBW insulin was injected. Ovary was collected 10 minutes after injection (called -3hr). Collected ovary was cultured in McCoy 5A medium for 3 hrs. Medium was collected (called 0 hr) and new medium was added with hCG. At 24hr medium was collected again and androstenedione was measured. B. Androstenedione secretion from cultured ovary was analyzed by one-way ANOVA with Tukey post hoc test in each group. Different letters represent significant differences among groups. Insulin increased ovary secretion of androstenedione in WT-lean group. Ovary from mice pre-injected with insulin has significantly increased androstenedione secretion in hCG treated medium in both WT-lean and WT-DIO groups. However, insulin is not able to increase androstenedione secretion in CYP17IRKO groups of either lean or DIO status. There are no significant differences among any groups treated with hCG alone. Data are represented as mean ±SEM and N=5-11.

Fig. 8 A model summarizing the insulin and LH receptor pathways in producing androgen in theca cells of the ovary in the lean and obese state. In lean mice, LH is a major resource to trigger androgen secretion in TI cells of ovary. In DIO mice, both insulin and LH induce androgen production by increasing Cyp17 protein level. Without insulin receptor, the KO-DIO mice have attenuated androgen production and improved fertility compared to the Control-DIO mice.
Fig. 1

A. Insulin receptor

B. TI cell

Granulosa cell

C. pAKT-TI

D. pAKT (green) DAPI (blue) merged

E. pTyr-IRS1-basal
Fig. 2

A

Body mass (g)

Day after birth

P<0.05

B

Fasted Glucose

P<0.05

C

Insulin (pg/ml)

P<0.01

D

Leptin (pg/ml)

P<0.05

E

Liver

P<AKT (relative fold to control)

F

GTT

Glucose (mg/dl)
Fig. 3

A  Lean female

Day after birth

Control KO

Vaginal opening  Control KO

First Estrus

B

Percentage time at stage

Con-lean  KO-lean

P  E  M+D

C

Percentage time at stage

Con-DIO  KO-DIO

P  E  M+D

P<0.05  P<0.05  P<0.01

D

Cycle stage

Control-DIO

P  E  D/M

day

0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16

E

KO-DIO

P  E  D/M

day

0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
Fig. 5

A

B

LH (ng/ml)

Testosterone (pg/dl)

Con
KO
DIO-stim
Lean-stim

P<0.05

P<0.05

P<0.05

P<0.05

DIO-basal
Lean-basal

NS

Control
KO

Lean
DIO

Page 32 of 39
Fig. 6

A. Ovary
Fold difference IR
(versus lean con)

Lean Con Lean KO DIO Con DIO KO
0.0 0.5 1.0 1.5

P<0.001 P<0.05

B. Fold difference LHR
(versus lean con)

Lean Con Lean KO DIO Con DIO KO
0.0 0.5 1.0 1.5

P<0.001

C. Fold difference cyp19
(versus lean control)

Lean Con Lean KO DIO Con DIO KO
0 1 2 3

NS

D. Fold difference star
(versus lean control)

Lean Con Lean KO DIO Con DIO KO
0 1 2 3

P<0.05

E. Fold difference cyp17
(versus lean control)

Lean Con Lean KO DIO Con DIO KO
0 1 2 3

P<0.05

F. TI cell

Con-lean Con-DIO KO-DIO

Cyp17

actin

LHR

G. TI-control

Lean Con DIO

Cyp17

Actin

LHR

insulin
Fig. 7

A

Fasting mice | Insulin injection | hCG | Medium collection

Hours -18 | -3 | 0 | 24

Insulin injection

Fasting mice

B

Androstenedione (ng/ml)

WT-lean | KO-lean | WT-DIO | KO-DIO

Basal-0' | Preins-0' | hCG-24h | PreIns-hcg-24h

Comparison of Androstenedione levels in different conditions.
Fig. 8

Theca cell

Insulin

LH

Cholesterol ↓
StAR →
↓
↓

CYP17
Androgens

lean

Insulin

LH

Cholesterol ↓
StAR →
↓
↓

CYP17
Androgens

Infertility

Con-DIO

Insulin

LH

Cholesterol ↓
StAR →
↓
↓

CYP17
Androgens

KO-DIO

Insulin

LH

Cholesterol ↓
StAR →
↓
↓

CYP17
Androgens

lean

Insulin

LH

Cholesterol ↓
StAR →
↓
↓

CYP17
Androgens

Infertility
Supplemental legends:

Suppl. 1 Ovary of lean mice was sectioned through. A. Corpora Lutea (CL), preantral follicles, antral follicles were counted from the every 10th section. There are no significant difference between control and KO lean mice. Data are represented as mean ±SEM and N=4.

Suppl. 2 CL is significantly reduced in Control DIO mice compared to other three groups. Data are represented as mean ±SEM and N=6-11.

Suppl. 3 A representative section of ovary by H&E staining in Control-DIO and KO-DIO mice. CL was labeled in each section.

Suppl. 4 Western blot shows similar expression protein level of Cyp17 and LH receptor between lean control and lean KO mice.

Suppl. 5 Basal serum estradiol level was not altered between control and KO in any state. Data are represented as mean ±SEM and N=9-14.

Suppl. 6 Androstenedione and Testosterone were measured from the same sample of cultured medium. Androstenedione was much higher than testosterone when expressed as the same unit (ng/ml). Data are represented as mean ±SEM and N=6.