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Obesity-Induced Infertility and Hyperandrogenism Are Corrected by Deletion of the Insulin Receptor in the Ovarian Theca Cell

Women with polycystic ovary syndrome (PCOS) exhibit elevated androgen levels, oligoanovulation, 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) wild-type (DIO-WT) mice were infertile and experienced increased circulating testosterone levels, whereas DIO-Cyp17IRKO mice exhibited improved fertility and testosterone levels comparable to those found in lean mice. The levels of phosphorylated IRS1 and CYP17 protein were higher in the ovary of DIO-WT compared with DIO-Cyp17IRKO or lean mice. Ex vivo studies using a whole ovary culture model demonstrated that insulin acts independently or additively with human chorionic gonadotropin to enhance androstenedione secretion. These studies reveal the causal pathway linking hyperinsulinism with ovarian hyperandrogenism and the infertility of obesity.

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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). Because the pathologic features present in PCOS, including hyperandrogenemia, hyperinsulinemia, hypersecretion of luteinizing hormone (LH), and hyperlipidemia, often coexist, discerning the relative contribution of each hormonal and metabolic abnormality to the dysfunction present in PCOS is difficult.

In an effort to unravel the pathophysiology of the multiorgan and multihormone 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 (IR) specifically in

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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 by effects elsewhere in the reproductive axis.

Diet-induced obesity (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 17 α-hydroxylation of progesterone or pregnenolone and the subsequent conversion to dehydroepiandrostenedione or androstenedione, respectively. In female rodents, P450Cyp17 activity is primarily present in the theca-interstitial (TI) cells of the ovary, making them the primary source of androgen because the mouse adrenal gland does not produce androgen (5).

Cyp17 expression not only is 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 α-hydroxyprogesterone in response to gonadotropin-releasing hormone (GnRH) agonists, suggesting that hyperinsulinemia may play a role in high androgen synthesis. Some of these effects could be mediated indirectly by increased pituitary LH secretion; however, insulin could serve as a cogonadotropin 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). IRs have been localized to the ovarian 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 mice 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 IR 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). *Cyp17*iCre mice were described by Bridges et al. (19). Cyp17IR knockout (KO) mice (Cyp17IRKO) were generated by mating homozygous female (*Cyp17*iCre$^{-/-}$; fl/fl-IR) with heterozygous male (Cyp17iCre$^{+/-}$; fl/wt-IR) mice. DIO mice were generated as previously described (17), in which 2-month-old female mice were fed a 60% high-fat diet (HFD). Mice with genotyping (*Cyp17*iCre$^{-/-}$; fl/fl or fl/wt-IR) were used as the control. Mice body mass and overnight fasted glucose were measured at age 6 months. All procedures were performed with approval of the Johns Hopkins Animal Care and Use Committee.

Genotyping and DNA Extraction

Primers for IR have been described (20). These primers will detect the wild-type (WT) band (280 bp), fl/fl band (320 bp), and KO band (220 bp). Primers for *Cyp17*iCre were as follows: cypcre-F, TCTGATGAAGTCAGGAAGAACC; and cypcre-R, GAGATGTCCTTCACTCTGATTC (19). DNA was extracted as previously described (21).

Hormonal and Glucose Assays

GnRH Stimulation and Glucose Tolerance Test

Basal morning levels of LH and follicle-stimulating hormone (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 2 g/kg body weight (BW) dextrose, and glucose was recorded at 0, 15, 30, 60, 90, and 120 min, as previously described (17).

Puberty and Fertility Examination

Puberty and estrous cyclicity were analyzed as previously described (21). Fertility was assessed as previously described (3,21). Briefly, 5-month-old female mice 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 previously described (3).

Quantitative Real-Time PCR

Ovary RNA was extracted by Trizol (Invitrogen, Grand Island, NY) according to the manufacturer's protocol. Total RNA (1 μg) 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 the ovary were measured by iQ SYBR green according to the manufacturer's protocol (Bio-Rad). Primers for Cyp17 were sense 5′-GATCTAAGAAGCGCTCAGGCA3′ and antisense 5′-GGGCACTGCATCACGATAAA-3′ (22), for Cyp19 sense 5′-TTGGAAATGCTGAACCCCAT-3′ and

antisense 5′-CAAGAATCTGCCATGGGAAA-3′ (23), for StAR sense 5′-CCCAAAGAAGGCATAGCAAG-3′ and antisense 5′-GCTGAATCCCCCAAACTTCT-3′, and for LH receptor (LHR) sense 5′-GACCAAAAGCTGAGGCTGAGA and antisense 5′-CAATGTGGCCATCAGGGTAGA-3′ (24).

Taqman quantitative PCR (Bioresearch Technologies, Novato, CA) was performed for IR, and GAPDH was used as the internal control. Primers for IR were sense 5′-ATGGGCTTCGGGAGAGGA-3′ and antisense 5′-GGATGTCCATACCAGGGCAC-3′ with the probe 5′-TGAGACGACGGCTGTGCCATT-3′ labeled with 5-carboxyfluorescein and Black Hole Quencher-1 (BHQ-1); and for GAPDH were sense 5′-GGGCATCTTGGGCTACACT-3′ and antisense 5′-GGCATCGAAGGTGGAAGAGT-3′ with the probe 5′-AGGACCAGGTTGTCTCCTGCGA-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 min after injection and used for theca and granulosa cell (GC) separation (25) or for whole ovary incubation. Briefly, for the theca and GC separation, the ovary was taken from the bursa and immersed into McCoy's 5A medium (Life Technologies, Grand Island, NY) supplied with 25 mmol/L HEPES, 0.1% BSA, and antibiotics (26). The ovary was manually punctured with a 26-gauge needle and a fine-tip tweezers. GCs were freed into the medium and centrifuged at 250*g* for 5 min at 4°C. The pellets were frozen in liquid nitrogen. The remaining cells of the ovary considered to be an enriched TI/stromal cells were centrifuged briefly and frozen in liquid nitrogen.

Measurement of protein concentrations and Western blot analysis were performed as described previously (17). Briefly, 5 μg protein of isolated theca cells from each individual mouse ovary was loaded onto the gel to perform the Western blot analysis. Primary antibodies used were rabbit polyclonal antibody to phosphorylated (p)AKT (Ser473) or to AKT, rabbit monoclonal antibody to IR-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), and 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 Luminex 200 (Austin, TX). pTyr-IRS1 protein expression was measured by pIRS1 Milliplex Map Phospho IRS1 Mapmates kits (EMD Millipore) in the Luminex 200. Alternatively, the ovary was incubated in a 24-well tissue culture plate with tissue culture well inserts (Millicell-CM, 0.4-μm pore size; EMD Millipore) (27,28) with McCoy's 5A medium. Medium was collected after 3 h of incubation (0 h), and the ovary was incubated with fresh medium with 1.6 IU/mL human chorionic gonadotropin (hCG). Medium was then collected 24 h later (24 h).

Histology and Immunostaining

The ovary was dissected from diestrous mice and fixed in 10% formalin phosphate buffer and sectioned to 5 μm 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 corpora lutea (CL), preantral follicle, and antral follicle were counted and examined with a Zeiss microscope. For the immunostaining, mice were overnight fasted and injected with 1.5 unit/kg BW insulin. Ovaries were collected after 10 min of injection and fixed in 4% paraformaldehyde. Each ovary was frozen in optimal cutting temperature medium and sectioned to 5 μm. Sections were incubated with primary (pAkt [Ser473]; Cell Signaling Technology) and secondary antibody goat anti-rabbit IgG Alexa Fluor 488 (Invitrogen, Eugene, OR) as described previously (21). Sections were photographed with an AxioCamMR camera and exported to AxioVision software.

Statistical Analysis

Data were analyzed by unpaired Student *t* test using GraphPad Prism (GraphPad Software, Inc., San Diego, CA), except where specifically addressed. Data are expressed as means ± SEM.

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 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 (GCs, hypothalamus, pituitary, and adrenal) have similar levels of IR expression (Fig. 1*A*) 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 compared with WT mice. As expected, there was no difference in IR expression in GCs isolated from control and Cyp17IRKO littermates (Fig. 1*B*). 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 with control mice (Fig. 1*C*). Histological analysis confirmed this finding. Expression of pAKT (green fluorescence) detected by immunostaining was similar in the ovarian GCs after insulin stimulation in control or Cyp17IRKO mice (Fig. 1*D*). However, pAKT expression in TI cells was much stronger in control than in Cyp17IRKO

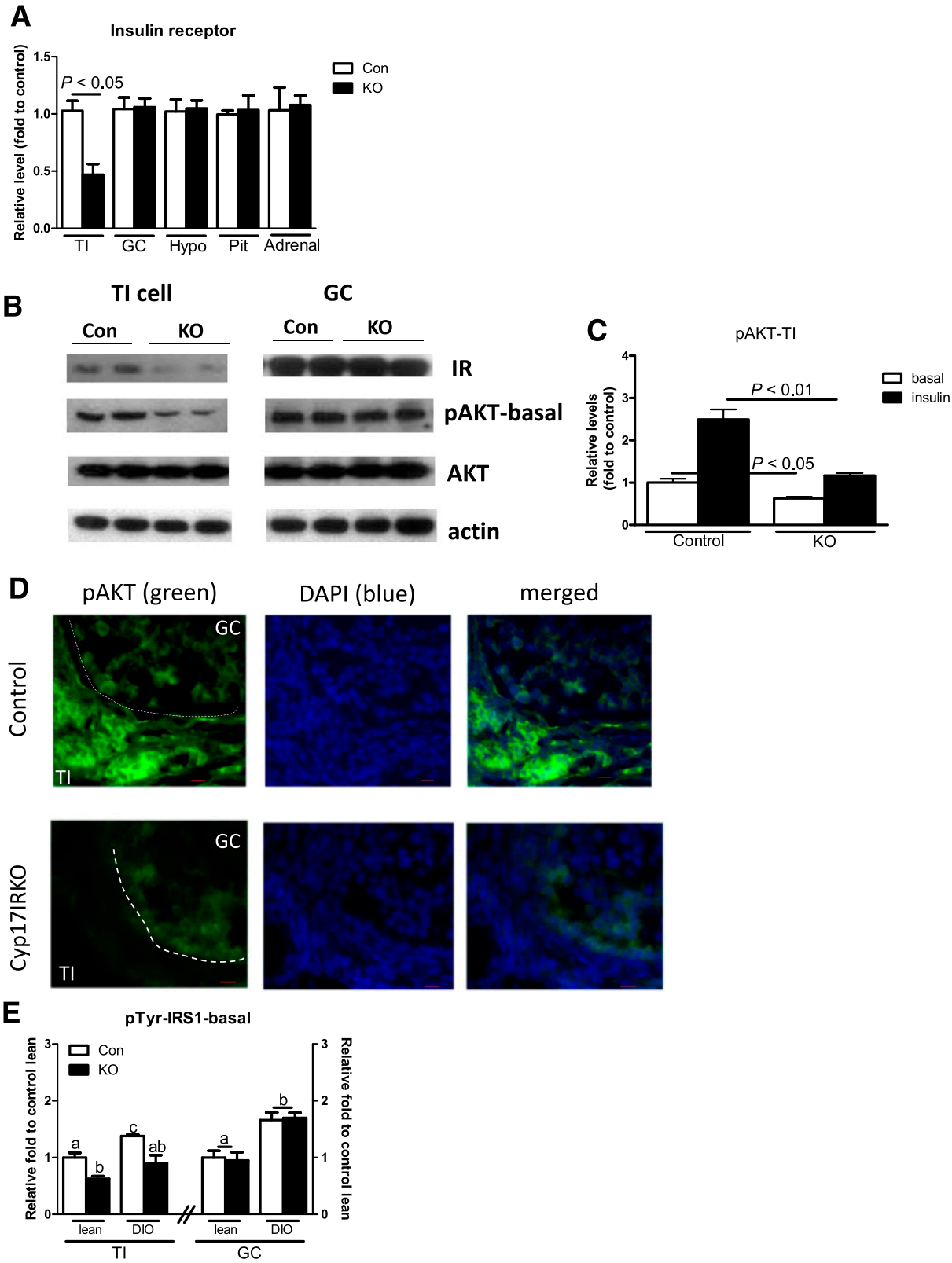

Figure 1—IR signaling is disrupted in the TI cells of the Cyp17IRKO mice ovary. *A*: IR mRNA level was measured by quantitative real-time PCR and was significantly reduced in the TI cells of the Cyp17IRKO ovary compared with control (Con) littermates, but no difference in IR expression was observed in GC, hypothalamus (Hypo), pituitary (Pit), or the adrenal gland. *B*: TI cells and GCs 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 the Cyp17IRKO ovary, but no change was observed in GCs. *C*: pAKT protein expression of TI cells was measured by Luminex after insulin stimulation. pAKT was significantly increased in the control TI cells compared with 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)

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 pTyr-IRS1 levels were increased significantly in TI cells, but not in GCs, between control and Cyp17IRKO mice (Fig. 1*E*).

Metabolic Status of DIO Cyp17IRKO and DIO Control Mice

To determine the role of obesity on IR signaling in the ovary, mice were fed an 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 was no difference in weight between control and Cyp17IRKO mice (Fig. 2*A*). Fasting glucose, insulin, and leptin levels were measured at 6 months of age, with no significant difference between Cyp17IRKO or control mice fed a normal chow diet or an HFD (Fig. 2*B–D*). Liver pAKT was significantly reduced in DIO mice compared with lean mice after insulin stimulation (Fig. 2*E*), indicating that insulin resistance was present in 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 with lean mice (Fig. 2*F*). 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, and there was no difference between control and Cyp17IRKO mice in either measure of puberty (Fig. 3*A*). Folliculogenesis in the ovary was also examined by hematoxylin and eosin histology. There were no differences in the number of CL, preantral follicles, or antral follicles between control and Cyp17IRKO lean female mice (Supplementary Fig. 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 (3). The duration spent in the different phases of the cycle was similar in lean control and Cyp17IRKO mice (Fig. 3*B*). 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 littermates (Fig. 3*C* 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 months old) were mated with four 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 of 60% compared with 30% for their control DIO littermates (Fig. 4). The number of vaginal plugs in WT-DIO mice was similar to that in 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 with lean-control, lean-KO, and DIO-KO (Supplementary Figs. 2 and 3), indicating reduced numbers of ovulatory cycles in the WT-DIO mice. Although 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 metestrus and diestrus for measurement of hormone levels. Basal LH levels were higher in DIO mice than in lean mice (Fig. 5*A*), 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. 5*A*), whereas no difference was found in pituitary response between control and Cyp17IRKO mice, either lean or DIO.

Control-DIO mice had significantly increased testosterone levels compared with lean mice, whereas the increase was attenuated in Cyp17IRKO-DIO mice (Fig. 5*B*). We investigated whether the increased testosterone was secondary to increased *Cyp17* or LHR expression at the mRNA or protein level in control-DIO mice. Cyp17IRKO-DIO mice, whether lean or DIO, had reduced IR expression compared with lean or DIO control mice, respectively (Fig. 6*A*). Although no significant changes were noted in the level of mRNA for LHR, *Cyp19*, and *StAR* (Fig. 6*B–D*), there was a significant increase in *Cyp17* mRNA expression in DIO control (Fig. 6*E*) that was

expressed in the GCs in the ovary. Strong expression of pAKT was observed in the TI cells of the control ovary; however, there is no staining in the TI cells of pAKT in the Cyp17IRKO. The dashed line separates the GC and TI cells. *E*: pTyr-IRS1 levels were measured at basal level in TI cells and in GCs in lean and DIO mice. pTyr-IRS1 was significantly reduced in the KO TI cells compared with the control TI cells in the lean and DIO state. However, there was no change in GC between KO and control. Data are mean ± SEM and $n = 4–8$. Bars with different letters represent values that are significantly different. $P < 0.05$.

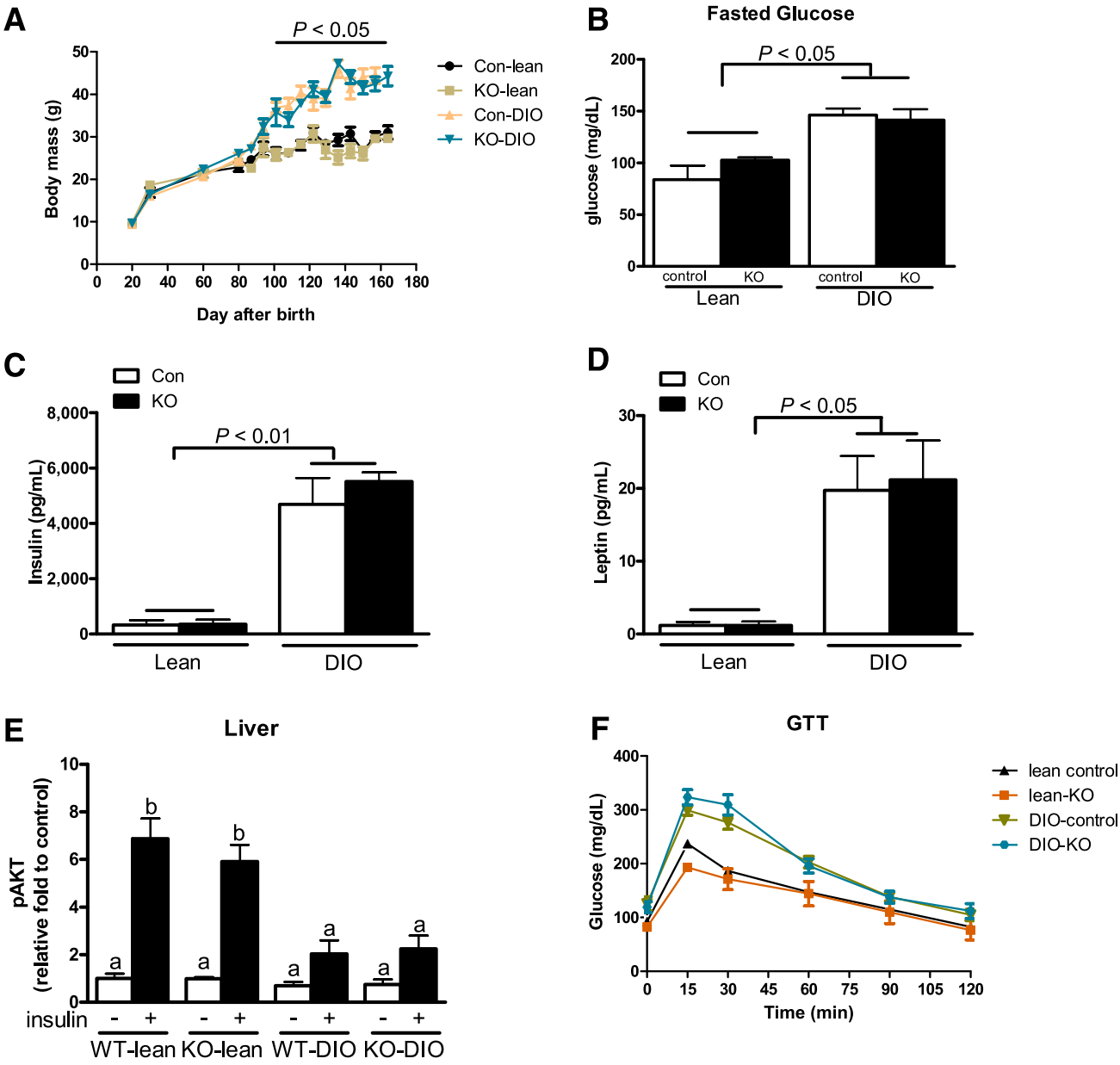

Figure 2—Metabolic function is not altered in Cyp17IRKO mice. Body mass (*A*) and levels of fasted glucose (*B*), fasted insulin (*C*), fasted leptin (*D*), and pAKT (*E*) were measured by Luminex assay. *E*: DIO mice (control and KO) showed insulin resistance in liver, characterized by attenuated increase in insulin induction of pAKT (measured by Luminex) compared with lean mice. *F*: Glucose tolerance test (GTT) was conducted in lean and DIO mice. There was no significant difference between control (Con) and Cyp17IRKO-lean mice or between control-DIO and Cyp17IRKO-DIO mice for these parameters. One-way ANOVA with Tukey post hoc test was used for statistics in *A*–*E*. Data are mean ± SEM and n = 5–8. Bars with different letters represent values that are significantly different. $P < 0.05$.

abrogated in KO-DIO mice. Protein level was measured by Western blot analysis from TI cells. The basal level of CYP17 and LHR was similar between lean control and KO (Supplementary Fig. 4). CYP17 protein levels were increased in control-DIO mice compared with control-lean and KO-DIO mice; LHR levels were not different among any groups (Fig. 6*F*). Intensity of protein level by Western blot was quantified by densitometry, and the observed differences between control lean and DIO mice in Cyp17 and LHR protein expression are quantified in Fig. 6*F1* and F2. In addition, control lean and DIO mice both expressed higher CYP17 protein, whereas LHR expression did not change after insulin injection (Fig. 6*G*, *G1*, and *G2*). Estradiol levels were also measured and were not significantly different among the groups (Supplementary Fig. 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. 7*A*).

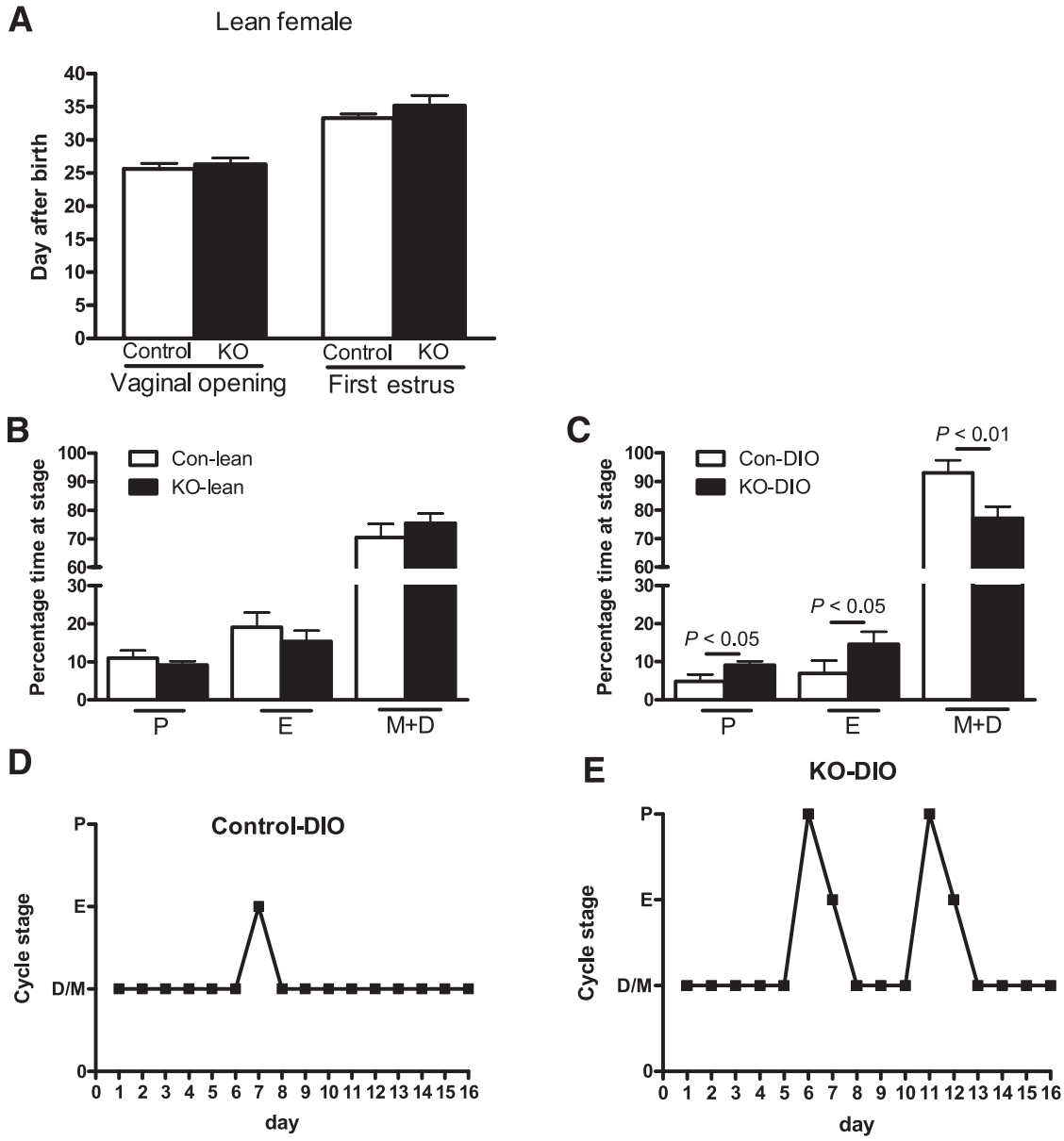

Figure 3—Insulin signaling plays role in obesity-induced fertility. *A*: Lean Cyp17IRKO mice exhibited similar age of puberty onset by examination of vaginal opening and first estrus. *B*: Percentage of time spent in each stage (D, diestrus; E, estrus; M, metestrus; P, proestrus) was not significantly different between control (Con) and Cyp17IRKO-lean mice. *C*: Percentage time spent in each stage was significantly different between control and Cyp17IRKO-DIO mice. Percentage of time in proestrus and estrus was shortened, and met-estrus/diestrus was increased in control-DIO mice compared with Cyp17IRKO-DIO mice. Representative data of vaginal cytology from control-DIO (*D*) and Cyp17IRKO-DIO (*E*) mice. Data are mean ± SEM and n = 6–10.

Androstenedione was measured in the cultured medium (Fig. 7*B*). 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 with hCG or insulin alone. There was no change of androstenedione concentration between hCG alone and insulin and hCG in Cyp17IRKO-lean or -DIO groups.

DISCUSSION

PCOS is a complex and multifactorial disease that is the leading cause of infertility in women. Its development and progression are 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. In addition, the common features of PCOS may be due to malfunction of the reproductive system at the

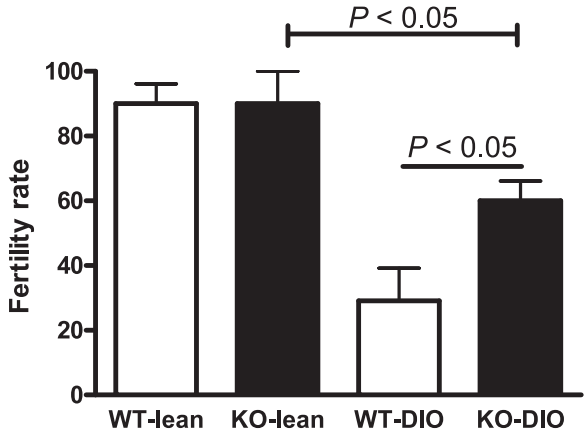

Figure 4—Insulin signaling in the TI cells contributes to infertility in DIO. Lean and DIO, WT, and Cyp17IRKO female mice were randomly mated with four different proven fertile WT males. Cyp17IRKO-DIO mice have a significantly improved fertility rate compared with WT-DIO mice, although the fertility is still impaired compared with the lean female mice. Data are represented as mean ± SEM and n = 5–6.

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 IR 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). Tissue-specific deletion of the IR in the gonadotroph in 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. Because 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 KO 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 TI cells contributes to obesity-associated infertility and hyperandrogenemia by augmenting CYP17 expression and activity.

IR 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 (Figs. 3 and 4), and similar basal serum LH and pituitary response to GnRH (Fig. 5*A*) of the Cyp17IRKO mice compared with control mice. Because obesity is associated with high circulating insulin levels and infertility (3,30,31), we explored 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

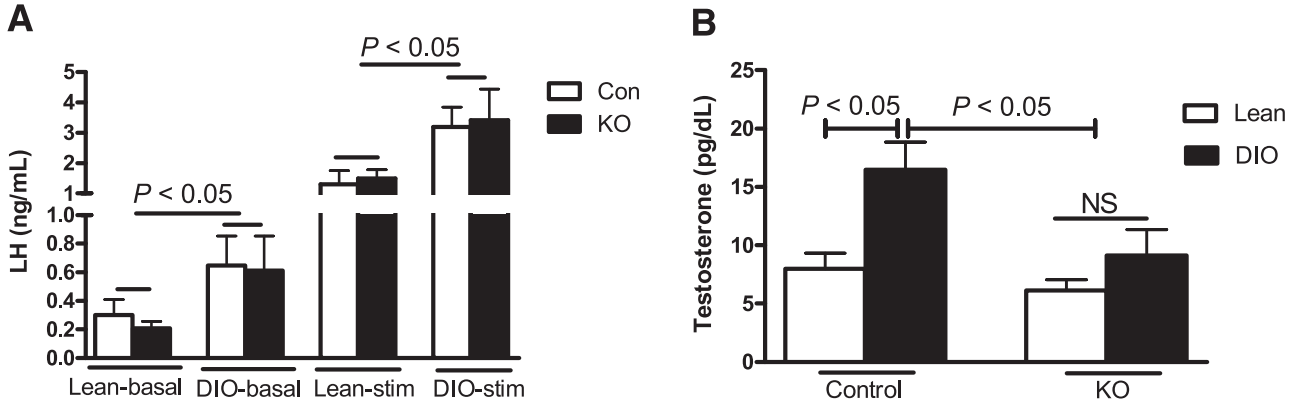

Figure 5—High-fat induced hyperandrogenism is attenuated in the Cyp17IRKO mice. *A*: Basal LH levels and LH levels after GnRH stimulation (stim) were compared between control (Con) and Cyp17IRKO mice in the lean or DIO state. Control and Cyp17IRKO-DIO mice both had a significantly higher response to GnRH stimulation compared with lean mice. *B*: Testosterone is significantly increased in control-DIO mice compared with control-lean mice. However, the levels in Cyp17IRKO-DIO mice are not significantly changed compared with lean mice. Data are represented as mean ± SEM and n = 5–13. NS, not significant.

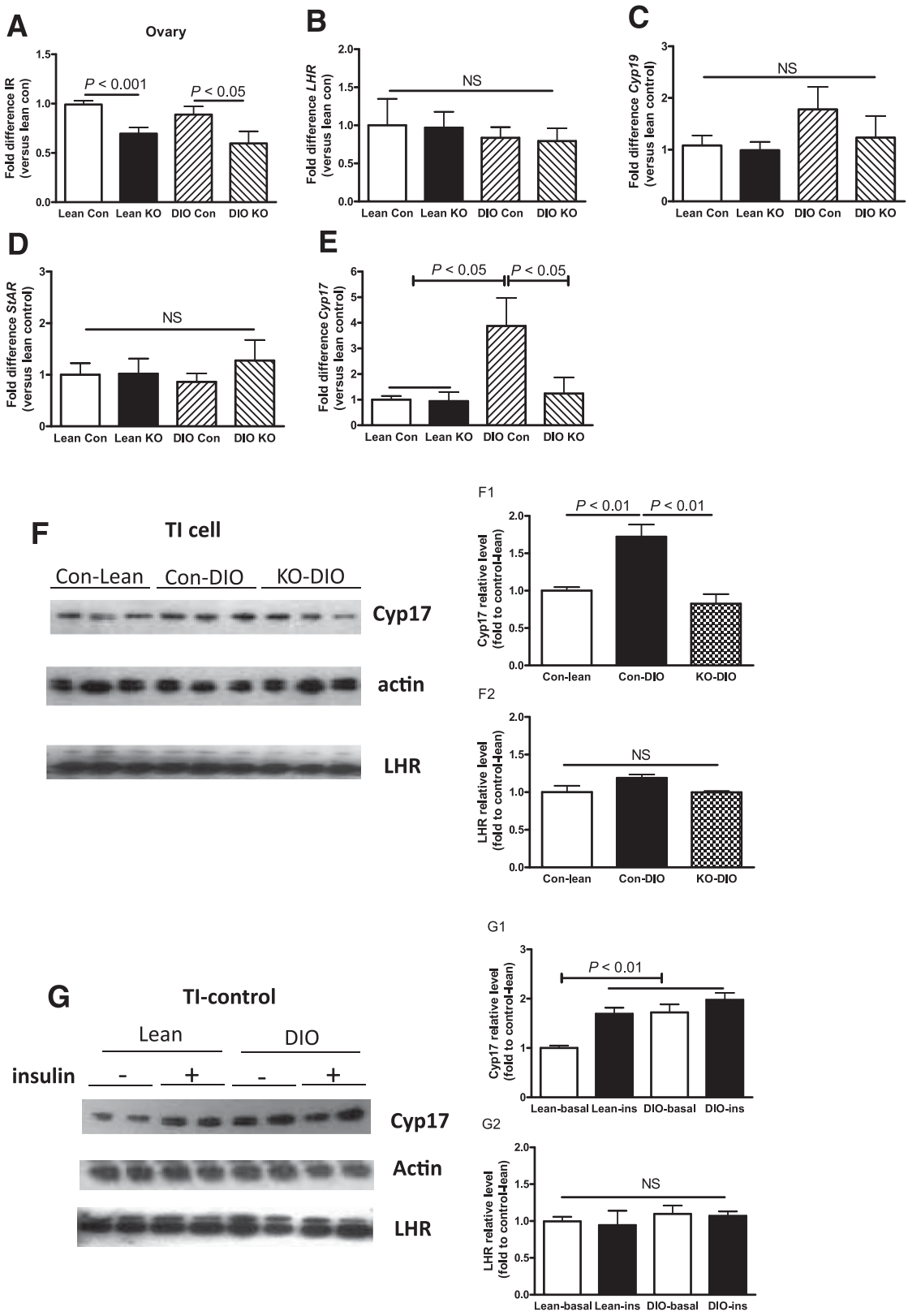

Figure 6—Quantitative real-time PCR was performed, and protein expression was measured by Western blot. mRNA levels of IR (*A*), *LHR* (*B*), *Cyp19* (*C*), *StAR* (*D*), and *Cyp17* (*E*) were measured in mouse ovary. As expected, IR was decreased in KO, and *Cyp17* gene expression was significantly changed among groups. Data are represented as mean ± SEM and n = 6–18. *F*: Increased basal level of Cyp17 in TI cells of control (Con)-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 β-actin before and after insulin (ins) treatment. Protein levels were quantified by densitometry and are graphed for Cyp17 (*G1*) and LHR (*G2*) and expressed relative to levels of β-actin. Levels of Cyp17 protein increased significantly in response to insulin in lean control mice, but due to higher basal Cyp17 levels in

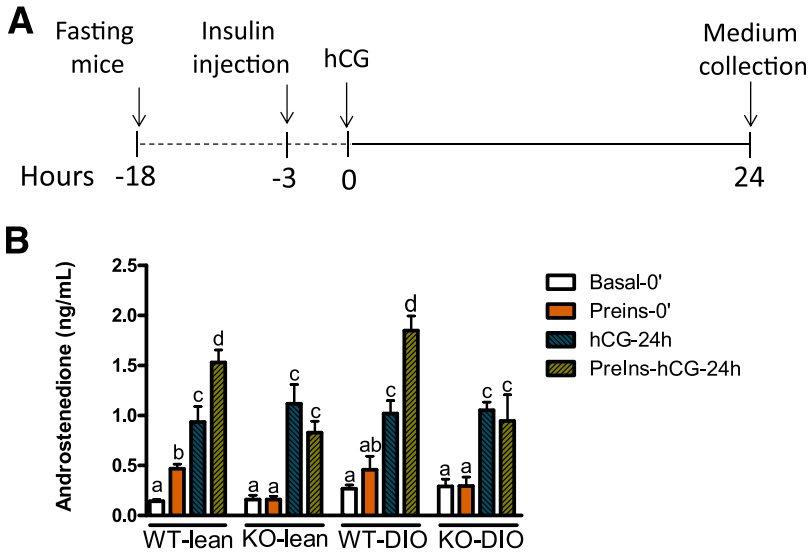

Figure 7—The Cyp17IRKO mice blocked insulin-stimulated androgen secretion. *A*: Mice were overnight fasted, and 1.5 unit/kg BW insulin was injected. The ovary was collected 10 min after injection (called −3 h) and was cultured in McCoy 5A medium for 3 h. Medium was collected (called 0 h), and new medium was added with hCG. At 24 h, 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. Insulin increased ovary secretion of androstenedione in the WT-lean group. The ovaries from mice preinjected with insulin (Preins) have significantly increased androstenedione secretion in hCG-treated medium in WT-lean and WT-DIO groups. However, insulin is not able to increase androstenedione secretion in Cyp17IRKO groups of 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. Bars with different letters represent values that are significantly different. $P < 0.05$.

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 an HFD, 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 than control-DIO mice (Fig. 4). The control-DIO mice had a lower number of CL compared with Cyp17IRKO-DIO mice (Supplementary Figs. 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).

Because 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 compared with lean mice (Fig. 5*A*). These results 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. 5*A*), 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 α-receptor–deficient mouse (34). The rescue of fertility in the current study cannot be explained by changes in LH secretion because DIO-control and KO mice both have higher LH levels than mice fed normal chow. LHR 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. 6*F* and *F2* and Fig. 7*B*). 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.

control-DIO mice, the small increase in Cyp17 levels in response to insulin was not significant. LHR 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. NS, not significant.

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 cogonadotroph 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 be due to the reduced androgen levels in the Cyp17IRKO-DIO mice (Fig. 5*B*). Testosterone levels were lower in lean Cyp17IRKO compared with 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, whereas in hyperinsulinemic conditions, insulin action contributes to augment androgen production (Fig. 7). The enhanced testosterone levels seen in the DIO mice when compared with the other experimental groups support this assertion. Because the mouse liver produces very low levels of sex hormone–binding globulin, 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 phosphatidyl inositol 3-kinase (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 with control mice in basal and insulin-stimulated conditions (Fig. 1*B–D*), and this decreased pAKT of TI cells may be associated with decreased pTyr-IRS1 (Fig. 1*E*), confirming altered downstream IR signaling in the KO ovary.

LH is the major factor leading to androgen secretion because 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. 6*F* and *F1*), indicating that increased signaling by the IR in theca cells increases Cyp17 levels. CYP17 expression is not completely abolished in the KO animals, because LH signaling is intact (Fig. 6*F* and Supplementary Fig. 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; however, in the hyperinsulinemic state associated with DIO, basal CYP17 expression was elevated but was not appreciably increased by exogenous insulin (Fig. 6*G* and *G1*). The effect of insulin on CYP17 expression was not due to an alteration in LHR levels, as indicated in Fig. 6*G* and *G2*. Jakimiuk et al. (48) reported that theca cells expressed higher mRNA levels for LHR, *StAR*, and *Cyp17* in polycystic ovaries. In our study, we did not observe increased mRNA expression of LHR and *StAR*, and did observe an increased *Cyp17* mRNA level in DIO versus lean mice (Fig. 6*A–E*). We documented increased fasting basal levels of CYP17 protein (Fig. 6*F* and *G*) in control-DIO and insulin-stimulated CYP17 protein expression in 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, because 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 because this hormone is the major secreted androgen in the ovary (Supplementary Fig. 6 [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. 7*B*), but not in the Cyp17IRKO animals, indicating that IR signaling in the TI cells is necessary for this effect. After hCG administration alone, androstenedione secretion increased in all groups, which was expected because all of the mice have a functional LHR. Preinjection in vivo with insulin before 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 combined with LH increases TI cell androgen secretion, likely by an increase in CYP17 expression rather than enhanced LHR signaling.

The experiments in this study indicate that retained insulin sensitivity in the ovarian theca cells of hyperinsulinemic DIO mice has functional consequences, because control-DIO mice exhibit higher CYP17 protein levels, androstenedione secretion, and serum testosterone than 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 because 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 TI 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

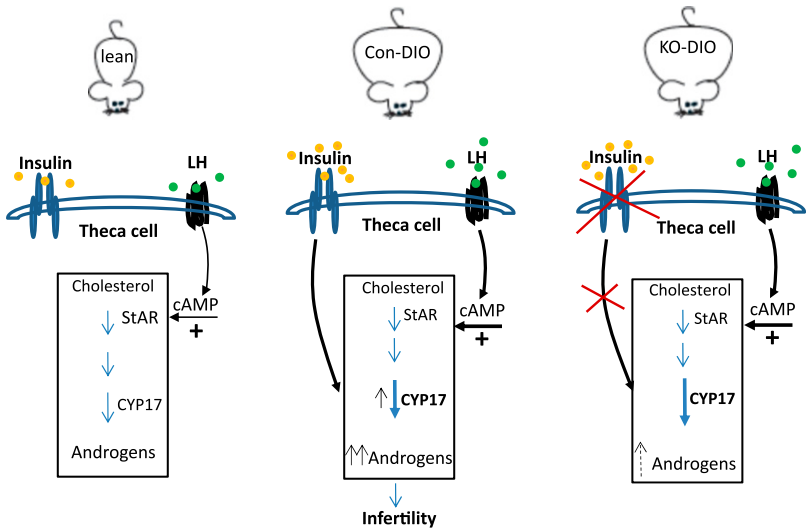

Figure 8—A model summarizing the insulin and LHR pathways in producing androgen in theca cells of the ovary in the lean and obese state. In lean mice, LH is a major resource to trigger androgen secretion in TI cells of the ovary. In DIO mice, insulin and LH both induce androgen production by increasing the Cyp17 protein level. Without IR, the KO-DIO mice have attenuated androgen production and improved fertility compared with the control (Con)-DIO mice.

demonstrates the multiorgan 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 multihormonal 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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