Effect of Glucocorticoid-Induced Insulin Resistance on Follicle Development and Ovulation

Katherine S. Hackbart, Pauline M. Cunha, Rudelle K. Meyer, and Milo C. Wiltbank

ABSTRACT

Polycystic ovarian syndrome (PCOS) is characterized by hyperandrogenemia, polycystic ovaries, and menstrual disturbance and a clear association with insulin resistance. This research evaluated whether induction of insulin resistance, using dexamethasone (DEX), in a monovular animal model, the cow, could produce an ovarian phenotype similar to PCOS. In all of these experiments, DEX induced insulin resistance in cows as shown by increased glucose, insulin, and HOMA-IR (homeostasis model assessment of insulin resistance). Experiment 1: DEX induced anovulation (zero of five DEX vs. four of four control cows ovulated) and decreased circulating estradiol (E2). Experiment 2: Gonadotropin-releasing hormone (GnRH) was administered to determine pituitary and follicular responses during insulin resistance. GnRH induced a luteinizing hormone (LH) surge and ovulation in both DEX (seven of seven) and control (seven of seven) cows. Experiment 3: E2 was administered to determine hypothalamic responsiveness after induction of an LH surge in DEX (eight of eight) and control (eight of eight) cows. An LH surge was induced in control (eight of eight) but not DEX (zero of eight) cows. All control (eight of eight) but only two of eight DEX cows ovulated within 60 h of E2 administration. Experiment 4: Short-term DEX was initiated 24 h after induced luteal regression to determine if DEX could acutely block ovulation before peak insulin resistance was induced, similar to progesterone (P4). All control (five of five), no P4-treated (zero of six), and 50% of DEX-treated (three of six) cows ovulated by 96 h after luteal regression. All anovular cows had reduced circulating E2. These data are consistent with DEX creating a lesion in hypothalamic positive feedback to E2 without altering pituitary responsiveness to GnRH or ovarulatory responsiveness of follicles to LH. It remains to be determined if the considerable insulin resistance and the reduced follicular E2 production induced by DEX had any physiological importance in the induction of anovulation.

dexamethasone, follicle, follicular development, glucocorticoid, hypothalamus, ovary, ovulation

INTRODUCTION

Polycystic ovarian syndrome (PCOS) is a heterogeneous reproductive disorder in women of reproductive age that has been estimated to affect up to 18% of the female population [1]. It is characterized by hyperandrogenemia, polycystic ovaries, and oligo-/amenorrhea. PCOS is one of the leading causes of anovulatory infertility in women as well as the most common cause of androgen excess disorders in women [2, 3]. Although insulin resistance is not included as a diagnostic feature, it has been reported that approximately 50–75% of women with PCOS have some degree of insulin resistance [4, 5]. Furthermore, it has been found that women with insulin resistance are over six times more likely to have PCOS than are non-diabetic women [6].

While it is widely accepted that hyperandrogenemia plays a role in the etiology of PCOS, there is also support for a central role of insulin resistance in the etiology of this syndrome. For example, women with PCOS who subsequently ovulated a dominant follicle had greater insulin sensitivity as determined by the homeostasis model assessment of insulin resistance (HOMA-IR) and decreased insulin concentrations after a glucose challenge [7]. Total testosterone and the free androgen index (FAI) were not different between the two groups of women [7]. Another study found that, in morbidly obese women, individuals who had menstrual dysfunction had greater insulin and HOMA-IR values [8]. Again, total testosterone and dehydroepiandrosterone sulfate did not differ between the two groups of women [8]. It has also been shown that treatment with an insulin sensitizer, rosiglitazone, can result in improved insulin sensitivity as well as the resumption of monthly menstruation in women who had been previously amenorrheic or oligomenorrheic without altering androgen concentrations [9]. Yet another study found that treatment with metformin, another insulin sensitizer, also increases the glucose-to-insulin ratio and decreases the number of small antral follicles, an ovarian characteristic associated with PCOS [10–12], without changing testosterone concentrations [13].

There is ample evidence, in numerous species, that glucocorticoids result in increased serum insulin and glucose [14–19], decreased insulin-mediated glucose uptake [14, 20], and insulin resistance [16–18, 21]. Dexamethasone (DEX) was utilized in this experiment since it is a potent glucocorticoid that is extensively utilized for treatment of cattle and humans with previous reports of a substantial increase in circulating insulin and glucose in cattle [19] and induction of an anovular condition in pigs [22]. Additionally, growth hormone has also been shown to induce hyperglycemia, hyperinsulinemia, and insulin resistance [23–26]. A previous study combined the long-acting, commercially available bovine growth hormone, recombinant bovine somatotropin (bST), with DEX to produce a particularly dramatic increase in insulin resistance in dairy calves [27]. Therefore, these two compounds were initially utilized to acutely induce insulin resistance, although most of
our studies were performed with only the DEX treatment because it was found to produce a similar degree of insulin resistance as the combined treatment.

For this research, the cow was chosen as the model for several reasons. Cows present a monovular phenotype similar to humans and therefore must also undergo a follicle selection process as opposed to polytocous mammalian species, such as rats, mice, and pigs, that naturally ovulate multiple follicles. The ultrasonically detected time of selection of a single dominant follicle in monovular species has generally been referred to as "follicular deviation" due to the deviation in growth rate of the dominant versus subordinate follicles [28].

The follicular dynamics associated with follicular deviation, as evaluated by ovarian ultrasonography, appears surprisingly similar in different monovular species (reviewed by Gintner et al. [29]). In particular, cows and women have a similar size of dominant follicle at the time of follicular deviation [29], accompanied by a decline in circulating follicle-stimulating hormone concentrations. Follicular growth in PCOS is marked by the growth of numerous follicles to a size of about 9 mm but lack of subsequent growth of a dominant follicle or, in other words, lack of follicular deviation. Due to the anatomical structure and large size of cattle, ovarian ultrasound can be performed frequently and repeated, using a minimally invasive transrectal approach. The meticulous observations permitted by this technique allow detailed analysis of the growth patterns of ovarian structures and dynamics of follicular growth during induction of insulin resistance, providing an opportunity to observe changes that occur in this process, particularly near the time of follicular deviation. The large blood volume present in cows is also advantageous, as multiple samples can be collected without the need to sacrifice the animal, allowing the systematic analysis of the dynamics in both ovarian morphology and corresponding circulating hormone concentrations during these experiments.

Thus, the purpose of this research was to induce insulin resistance and monitor the subsequent changes in follicular growth, deviation, and ovulation as well as circulating concentrations of reproductive hormones. This allowed determination of the effects that insulin resistance has on ovarian function and whether this aberrant condition is sufficient to induce anovulation and ovarian characteristics that are observed in patients with PCOS. It was our hypothesis that the acute induction of insulin resistance would prevent follicular deviation, resulting in multiple follicles of 8–9 mm in diameter (size at time of follicular deviation in cattle), and lack of ovulation in cows with insulin resistance, similar to what is hypothesized to occur in patients with PCOS [10].

**MATERIALS AND METHODS**

*Animals: Feeding, Housing, and Experimental Protocols*

Nonlactating, nonpregnant Holstein or crossbred (three-quarters Holstein, one-quarter Jersey) cows (n = 37; 59.1 ± 3.3 mo old at trial initiation) or heifers (n = 4; 26.8 ± 3.3 mo old at trial initiation; collectively referred to as "cows") were provided by the University of Wisconsin–Madison dairy herd for use in these studies and procedures were approved by the Animal Care and Use Committee for the College of Agriculture and Life Sciences of the University of Wisconsin–Madison. Cows were housed in individual stanchions with free access to a concrete lot during the day (experiment 1), individual tie stalls (experiments 2 and 3), or a free-stall barn (experiment 4). Animals were fed a lactating cow diet (experiment 1), an alfalfa silage-based diet (experiments 2 and 3), or a nonlactating cow diet (experiment 4) during the day. In all experiments, feed was removed at night to provide at least 12 h without feed prior to morning blood samples. All animals had ad libitum access to water at all times.

**Experiment 1: DEX and bST Treatment on Insulin Resistance, Follicle Growth, and Ovulation**

This experiment was performed in two replicates, with replicate 1 consisting of five cows and replicate 2 consisting of four different cows. The experimental protocol is detailed in Figure 1A. All cows (n = 9) were synchronized and monitored for a complete estrous cycle prior to treatment initiation to verify that all animals were cycling normally. Cows were synchronized using an intravaginal progesterone (P4)-releasing implant (CIDR; Eazi-Breed CIDR; Pfizer Animal Health, New York, NY) for 7 days, with i.m. treatments with progesterin-f2α (PGF2α; 25 mg; Latylase; Pfizer Animal Health) on the day of CIDR removal and the following day to ensure regression of the corpus luteum (CL) and aspiration of all follicles > 5 mm in diameter (Convex-array transducer with a 17-gauge, 60-cm aspiration needle attached to Aloka 900-V ultrasound machine; Corometrics Medical Systems, Wallingford, CT) on the day of CIDR removal and the following day (if a refilled aspiration site was observed). Ovarian structures were measured once daily using transrectal ultrasound in all animals for the remainder of the experiment. Two weeks after CIDR removal and aspiration, daily blood sampling was begun followed 1 day later by the initiation of daily treatments.

Cows were randomly assigned within replicate (replicate 1, n = 5; replicate 2, n = 4) to one of two treatment groups: control-daily treatment (n = 4) with 7.5 ml of 0.9% saline i.m. or DEX-daily treatment (n = 5) with 15 mg DEX Eazi-Breed CIDR (Pharmacia, Stockholm, Sweden) i.m. plus twice weekly treatments with bST (500 mg; Postilac; Elanco, Greenfield, IN; first on same day as first DEX injection and second wk later). One day before treatment initiation, all cows received a CIDR for 8 d with two treatments with PGF2α and follicle aspiration at CIDR removal as described during pretreatment period. Daily treatments with DEX/saline, ultrasound evaluation of ova ries, and blood sampling continued for 16 d after aspiration. Blood samples were collected each morning prior to feeding and treatment and were evaluated for glucose, insulin, P4, and estradiol (E2).

**Experiment 2: Effect of DEX Treatment on a Gonadotropin-Releasing Hormone Challenge**

A preliminary experiment was performed to determine the effect of DEX administered at a lower dose (10 vs. 15 mg/day), in an attempt to reduce the immune-suppressing effects of elevated glucocorticoid treatment. Additionally, DEX was administered alone, without additional growth hormone treatment, to ascertain if DEX was sufficient to induce insulin resistance and anovulation. DEX alone and at a lower dose dramatically increased concentrations of glucose and insulin and the HOMA-IR values (data not shown) and prevented ovulation in all treated cows. Therefore, all subsequent experiments used DEX treatment at 10 mg/day and without additional bST treatment.

In experiment 2, cows (n = 7) were randomly assigned to one of two treatments: DEX (10 mg/day) or control (5 ml of 0.9% saline). Cows were treated for 13 days, and then the gonadotropin-releasing hormone (GnRH) challenge was administered, as described below. This experiment used a crossover experimental design, and therefore all cows subsequently were assigned to the other experimental treatment group. The experimental protocol is shown in Figure 1B.

To synchronize cows for this experiment, a CIDR treatment for 7 days was again utilized with treatment with 25 mg PGF2α at the time of CIDR removal. To prevent an early luteinizing hormone (LH) surge or ovulation, all cows then received a new CIDR, and 3 days later, all follicles ≥5 mm in diameter were removed by follicular aspiration, and the CIDR was replaced with another new CIDR. Daily ovarian ultrasonography was initiated to allow tracking of follicle growth. Seven days after follicular aspiration, the CIDR was removed, and 6.5 h later, 200 μg of GnRH (OvaCyst; Butler Animal Health Supply, Dublin, OH) were administered i.m. Blood samples were collected at 0, 1, 2, 3, and 4 h in relation to GnRH administration to determine LH concentrations in response to the GnRH challenge. Animals underwent ovarian ultrasound for 3 more days to determine ovulation in response to the exogenous GnRH. Ovulation was defined as the disappearance of an ovulatory-sized (>10 mm in diameter) follicle. Beginning the day of the last ovarian ultrasound, DEX-treated cows received one-half dose of DEX for 3 days, one-quarter dose for 3 more days, 1 day with no treatment, followed by 1 more day of one-quarter dose. The day after this last DEX injection, or 11 days after the GNRIH injection, a new CIDR was inserted, and cows went through this protocol a second time. During this second period, animals were again resynchronized and assigned to the opposite treatment as in the first period. Blood samples were evaluated for glucose and insulin during the treatment period and for circulating LH concentrations during the GnRH challenge.
Experiment 3: Effect of DEX Treatment on Response to an E2 Challenge

Cows in this experiment (n = 8) were randomly assigned to one of two treatments: DEX (10 mg/day) or control (5 ml of saline). A crossover experimental design was utilized so that half of the cows went through the DEX treatment during the first period followed by control and the other half received the control treatment followed by DEX treatment. The protocol for this experiment was similar to experiment 2, except that cows were challenged with 0.5 mg of E2-17β administered i.m. at 12 h after CIDR removal. Blood samples were collected at 0, 1, 2, 4, 6, 8, 10, 12, 14, 16, 18, and 20 h in relation to the E2 injection to determine both E2 and LH concentrations. It was determined that cows underwent an E2 surge if posttreatment concentrations of E2 reached at least 8 pg/ml, a value found by Sartori et al. [30] to be the concentrations attained by cows undergoing a natural estrous cycle. Likewise, an LH surge was defined by LH concentrations reaching at least 8.8 ng/ml, a concentration found by Haughian et al. [31] to be obtained by naturally ovulating dairy heifers. Ovaries were evaluated for 3 days following the E2 challenge to determine ovulation (dominant follicle disappearance). Cows were again weaned off of the DEX as described in experiment 2, and a CIDR was inserted 11 days after the E2 challenge as the start of the second period. Cows were again assigned to the opposite treatment from the one they had received in the first period. The experimental protocol is presented in Figure 1C.

Experiment 4: Effect of Acute DEX or P4 Treatment on Ovulation

This experiment was designed to determine whether the anovulation that was observed in experiment 1 could be induced by acute DEX treatment, similar to acute effects of P4 treatment. Cows (n = 17) were randomly assigned to one of three treatment groups: control (5 ml/day of 0.9% saline i.m.; n = 5), DEX (10 mg/day i.m. for 5 days; n = 6), or P4 (CIDR intravaginally for 5 days; n = 6). The CIDRs had previously been used in lactating dairy cows in an unrelated experiment for 10 days to generate a lower level of P4 release than from a new CIDR. Used CIDRs were cleaned and autoclaved before use in this experiment.

In this experiment, blood samples were taken every 12 h to provide a more accurate determination of the E2 peak. Samples taken in the morning were taken prior to feeding and treatment administration, thus providing a measure of basal glucose and insulin. Cows on Day 7 of the estrous cycle received two treatments with PGF2α to regress the CL (first AM, second PM); twice-daily blood sampling and ovarian...
ultrasoundography (to determine ovulation) were initiated, with treatments being started 1 day after the first PGF2\alpha treatment. Cows were evaluated for a total of 12 days. Daily treatment with DEX/saline began on Day 1 with the last treatment on Day 5. The CIDR was inserted on Day 1 and removed on Day 6 (to mimic timing of DEX treatment). A total of 15 of the 17 cows had ovulated by this time. The two DEX cows that remained anovular continued to have blood samples drawn and ovarian ultrasound performed once daily for 8 additional days and then received 100 µg GnRH to induce ovulation (both cows ovulated 2 days later).

Blood Sampling, Ultrasonography, and Hormonal Assays

Blood samples were collected from the coccygeal vein or artery and, when necessary due to difficulty in sampling from the tail (experiment 1), from the jugular vein. Samples to be analyzed for insulin, LH, P4, and E2 were collected into evacuated serum tubes (Vacutainer; Becton, Dickinson and Company, Franklin Lakes, NJ). Samples for the analysis of glucose levels were collected into evacuated plasma tubes containing potassium oxalate and sodium fluoride (Vacutainer; Becton, Dickinson) as a glycolytic inhibitor. After collection, samples were placed immediately on ice until processed in the laboratory. Blood samples were centrifuged at 1600 \( g \) for 20 min at 4°C, aliquoted into multiple tubes, and stored at \(-20^\circ C\) until analyzed.

Ovarian structures were determined by transrectal ultrasonography using an Aloka 500-V with a 7.5-MHz linear-array transducer (Corometrics Medical Systems, Wallingford, CT). Images were frozen when the ovarian structures were judged visually to be at their maximal size. Structure size was determined by taking the average of two perpendicular measurements. All structures were mapped and recorded for subsequent analysis.

For P4 determination, nonextracted serum samples were analyzed using an antibody-coated-tube RIA kit (Coat-A-Count; Diagnostic Products Corporation, Los Angeles, CA) with an intra-assay coefficient of variation (CV) of 9.7%. Insulin values were determined using a porcine insulin RIA kit (Millipore Corporation, Billerica, MA) that has 90% specificity for bovine insulin that had an average intra-assay CV of 6.3%. Estradiol concentrations were determined using an RIA-based double-antibody E2 kit (Estradiol Double Antibody; Siemens Healthcare Diagnostics, Los Angeles, CA) as described previously [32], except that standards were made using steroid-free serum rather than plasma to reflect the serum samples collected. The sensitivity of the assay was 0.2 pg/ml with an average intra-assay CV of 2.5%. Samples for LH were determined as described [33]. The sensitivity of the assay was 0.1 ng/ml with an intra-assay CV of 13%. Glucose samples were analyzed using the glucose oxidase/peroxidase method described previously [34]. The sensitivity of the assay was 0.3 mg/dl with an average intra-assay CV of 4.1%.

The HOMA-IR, a measure of the insulin sensitivity of an individual, was calculated according to the formula presented in Matthews et al. [35]:

\[
\text{Inulin(units/ml)}/(22.5e^{-\frac{\ln(glucose(mmol/l))}{10}})
\]

or, more simply,

\[
(\text{Inulin(units/ml)} \times \text{Glucose(mmol/l)})/22.5
\]

Statistical Analysis

All hormonal values, follicle diameter, and E2:dominant follicle volume were analyzed using the MIXED procedure (SAS Institute Inc., Cary, NC) with repeated measures. Period (in experiments 1–3), treatment, and day were used as fixed variables, whereas cow was used as a random variable. For the intensive samples of E2 (experiment 3) and LH (experiments 2 and 3), period, treatment, and hour were used as fixed effects with cow as a random variable. In experiment 3 only, LH values were log (base 10) transformed prior to analysis. When comparing ovulatory versus anovulatory DEX cows in experiment 4, hour and ovulatory status were fixed effects; cow was a random effect. Ovulation rates and proportions of cows ovulating single versus multiple follicles were analyzed using Fisher exact test.

RESULTS

Experiment 1: DEX and bST Treatment on Insulin Resistance, Follicle Growth, and Ovulation

One DEX-treated cow was removed from the experiment at 14 days after the second set of follicular aspirations (performed once treatments had been initiated) due to treatment-related illness, and another DEX cow was removed 12 days after this same follicular aspiration due to rectal injury. The data collected from these cows up until removal from the experiment are included in the following results.

Treatment with DEX and bST increased \((P < 0.001)\) circulating concentrations of glucose (Fig. 2A) with glucose increasing from \(\leq 60\) to \(\geq 100\) mg/dl on the first day after treatment with continued elevation during the subsequent 23 days of experimental treatments. Circulating insulin concentrations also increased \((P = 0.026)\) 2.8-fold by 1 day after treatment with further increases \((P < 0.01)\) to \(\geq 6\)-fold increase compared to control after 3 days of treatment (Fig. 2B). There was also an increase \((P < 0.001)\) in the homeostasis model assessment for insulin resistance (HOMA-IR [35]) of \(\approx 10\)-fold by 3 days after treatment initiation (Fig. 2C). All three parameters remained significantly elevated in treated cows for the remainder of the experiment (Fig. 2).

Treatment with DEX/bST prevented ovulation in all cows. No DEX-treated cow ovulated by 16 days after follicle aspiration, whereas all control cows ovulated by 7 days after follicle aspiration. This was further confirmed by an increase in P4 concentrations in control cows at 10 days after aspiration, which reached significance \((P = 0.011)\) by Day 12 and remained significantly \((P < 0.001)\) elevated for the remainder of the trial. In addition, there was a CL observed in all control cows. In contrast, P4 concentrations in DEX-treated cows remained consistently low \((<0.1\, \text{mg/ml})\) Fig. 3) with no observable CL. Treatment also affected \((P = 0.048)\) the proportion of cows that developed a single versus multiple dominant follicles with 100% of control cows (four of four) developing a single dominant follicle after follicle aspiration but only 20% of DEX-treated cows (one of five) developing a single dominant follicle and the other 80% having two to three large dominant follicles.

Treatment also affected E2 concentrations, with overall concentrations being higher in control than DEX cows \((1.3 \pm 0.1\, \text{vs. } 0.80 \pm 0.1\, \text{pg/ml}; P = 0.004)\). Concentrations of E2 increased after follicle aspiration with greater E2 in control than DEX cows \((2.1 \pm 0.3\, \text{vs. } 0.7 \pm 0.1\, \text{pg/ml}; P < 0.001)\) from Day 3 to Day 6 after aspiration (Fig. 4A). Surprisingly, there was no difference in circulating E2 in the DEX-treated cows during this same time period. This was not due to absence of a dominant follicle since the average diameters of dominant follicles did not differ between treatments (Fig. 4B). This is clearly illustrated by evaluation of the ratio of circulating E2 to total dominant follicle volume. Control cows had approximately a 4.5-fold greater \((0.0053 \pm 0.0012\, \text{vs. } 0.0012 \pm 0.0003\, \text{pg/ml mm}^2); P = 0.008)\) ratio than DEX cows from 3 to 6 days after follicle aspiration, though this ratio was only different between treatments on Day 3 when comparing each day individually (Fig. 4C). At 7 days after aspiration, circulating E2 decreased in the control cows coincident with the expected LH surge and subsequent ovulation.

Experiment 2: Effect of DEX Treatment on Response to a GnRH Challenge

Glucose \((57.4 \pm 0.4\, \text{vs. } 83.5 \pm 1.1\, \text{mg/dl})\), insulin \((20.7 \pm 1.2\, \text{vs. } 79.4 \pm 5.9\, \text{µU/ml})\), and HOMA-IR values \((3.0 \pm 0.18\, \text{vs. } 16.8 \pm 1.33)\) were again increased \((P < 0.001)\) by treatment with DEX. Significant increases were observed 1 day after initiation of treatments; these parameters remained elevated in DEX cows for the remainder of the experiment (Fig. 5). Glucose was maximally elevated by 1 day after treatment initiation, whereas insulin and HOMA-IR had a further increase between 1 and 3 days after treatment initiation.
Circulating E2 concentrations were again altered by DEX treatment with greater \((P < 0.05)\) E2 on Days 6 and 7 in control cows compared to DEX cows (Supplemental Figure S1; all Supplemental Data are available online at www.biolreprod.org). Treatment with GnRH caused an LH surge in 100% of control (seven of seven) and DEX (seven of seven) cows with no treatment difference in LH concentrations at any time point (Fig. 6). Ovulation occurred in 100% of control (seven of seven) and DEX (seven of seven) cows. There was no difference between treatment groups for the proportion of cows that ovulated a single follicle (control = 71%, five of seven; DEX = 43%, three of seven) or multiple follicles (control = 29%, two of seven; DEX = 57%, four of seven). Average size of the ovulatory follicle(s) was not different between treatment groups.

Experiment 3: Effect of DEX Treatment on Response to an E2 Challenge

In this experiment, one DEX cow was considered an outlier by SAS (more than two standard deviations above the mean) for serum insulin after treatment. The data from this cow were not used in the analysis for insulin and HOMA-IR; however, all other hormonal values were utilized because they were not found to be outliers. Similar to experiments 1 and 2, treatment with DEX increased \((P < 0.001)\) circulating insulin \((17.9 \pm 1.0\) vs. \(66.9 \pm 4.3\) μU/ml) and glucose \((54.8 \pm 0.3\) vs. \(81.1 \pm 1.1\) mg/dl) and HOMA-IR values \((2.4 \pm 0.1\) vs. \(13.5 \pm 1.0\)). Glucose was maximally elevated by 1 day after treatment and remained elevated for the remainder of the experiment (Supplemental Figure S2A). In contrast, both insulin and HOMA-IR values were significantly increased by 1 day after treatment but did not reach maximal values until 3 days after treatment initiation (Supplemental Figure S2, B and C).

Concentrations of E2 were decreased \((P < 0.001)\) in DEX compared to control cows \((1.7 \pm 0.12\) vs. \(0.9 \pm 0.07\) pg/ml) prior to the E2 challenge as well as during the E2 challenge \((20.2 \pm 2.1\) vs. \(13.0 \pm 1.5\) pg/ml). As in previous experiments, control cows had greater E2 from 3 to 7 days after follicle aspiration (Supplemental Figure S3). During the E2 challenge, all control and DEX-treated cows had elevated E2 concentrations with each cow attaining concentration above 8 pg/ml, the value previously described. Nevertheless, control cows had greater \((P < 0.05)\) E2 concentrations than DEX cows at 1, 2, 4, and 6 h after E2 treatment than DEX cows (Fig. 7A). Individual E2 profiles are shown in Supplemental Figure S4, A and B.

The LH concentrations during the E2 challenge were elevated \((P < 0.005)\) in control compared to DEX cows at all times from 8 to 20 h after E2 treatment (Fig. 7B). In this experiment, none of the DEX-treated cows reached concentrations >2.8 ng/ml during the 20-h sampling period, much less than the 8.8 ng/ml described previously. Thus, it was concluded that no DEX-treated cows had an LH surge during the course of sampling. On the other hand, all control cows were determined to have experienced an LH surge with peak
concentrations >13.4 ng/ml in each cow. Individual LH profiles are shown in Supplemental Figure S4, C and D.

The occurrence of ovulation in this experiment was based on ultrasound evaluations of the ovaries at 36 h, 60 h, and 7 days after E2 treatment. All control cows had “normal ovulation” (100%, eight of eight) defined as an ovulatory follicle present at 36 h that had ovulated by 60 h with a CL present 7 days after E2 treatment. In contrast, only 25% (two of eight) DEX cows had ovulation observed between 36 and 60 h after E2 treatment. Surprisingly, another 25% (two of eight) of DEX cows had not ovulated by 60 h but had a CL present on Day 7. These animals were considered “late ovulators.” The other four of eight DEX cows remained anovular at Day 7. When considering “normal” ovulations that occurred within the time frame expected (eight control cows and two DEX cows) versus late and anovular cows (zero control and six DEX), DEX treatment reduced (P = 0.007) the percentage of
cows ovulating to the E2 challenge. When considering all ovulatory cows (eight control and four DEX) versus anovular cows (zero control and four DEX), DEX treatment tended ($P = 0.077$) to decrease the percentage of cows ovulating. Size of the largest dominant follicle (control = 13.7 ± 1.0 mm; DEX = 14.2 ± 0.5 mm) and development of multiple dominant follicles (control = two of eight; DEX = two of eight) were not different between treatments.

**Experiment 4: Effect of Acute DEX or P4 Treatment on Ovulation**

Once again, DEX treatment significantly increased circulating glucose and insulin concentrations and HOMA-IR values by Day 1 after treatment initiation (Fig. 8). Insulin concentrations and HOMA-IR values remained significantly elevated in DEX cows for the remainder of the sampling period, 3 days after the last administration of DEX. Glucose concentrations were still elevated in DEX cows 2 days after the last DEX injection but were no longer significantly elevated at 3 days after treatment cessation.

The positive control in this experiment was the P4 treatment group. At 24 h after PGF2α administration (prior to treatments), there was no difference between any of the groups. The P4-treated group had an elevation in circulating P4 concentrations by 12 h after treatment initiation compared to control and DEX cows; there was no difference between control and DEX cows. This elevation in P4-treated cows continued until the end of the sampling period. At no time point were there differences in circulating P4 between DEX and control cows (Fig. 9).

Ovulation was observed in all control cows (negative control), while all P4-treated cows remained anovular while the CIDR was in place (positive control). In DEX-treated cows, however, 50% (three of six) ovulated in a similar time frame as control cows (Fig. 10). One DEX cow (17%) remained anovular until 12 days after PGF2α administration when spontaneous ovulation occurred. Because this was past the time of the DEX treatment and similar to P4-treated cows, this cow was considered to be anovular. The remaining 33% (two of six) remained anovular for 20 days, after which they were successfully induced to ovulate with GnRH (Fig. 10).

One ovulatory DEX cow had increased E2 concentrations (4.0 pg/ml) at 24 h prior to ovulation, unlike other ovulatory cows in which E2 concentrations had dropped to 0 or near 0 pg/ml (0.48 ± 0.14 pg/ml) at this time. This value was deemed an outlier by SAS, and this cow was not used in E2 analyses. The E2 concentrations in P4-treated cows decreased to 0 pg/ml by 24 h after CIDR insertion and remained at an average concentration of <0.5 pg/ml for the remainder of the sampling period (Fig. 11A). Average E2 concentrations in control cows increased to 5.3 pg/ml by 24 h after treatment initiation, after which concentrations began to decrease until reaching ~0 pg/ml at 48 h (Fig. 11A). DEX-treated cows did not reach their greatest concentration of 3.4 pg/ml until 36 h after treatment initiation, after which E2 concentrations decreased, but not lower than 1.4 pg/ml. However, when comparing ovulatory DEX to ovulatory control cows with samples normalized to the time of ovulation, E2 profiles were very similar with no differences between treatments at any time prior to ovulation (Fig. 11B). When comparing ovulatory DEX to anovulatory DEX cows, there was no difference at either 12 or 24 h after treatment initiation. However, at 36 h, E2 concentrations in ovulatory cows were significantly greater, suggestive of an E2 surge. In contrast, at 48 h ovulatory cows had significantly decreased E2 concentrations, indicative of the LH surge-induced drop in E2 production. Anovulatory cows, on the other hand, had E2 near 2 pg/ml throughout this time, consistent with lack of an E2 surge or an LH surge (Fig. 11C).
DISCUSSION

The experiments in this study were designed to evaluate a novel animal model for PCOS by analyzing the role of high insulin and insulin resistance on follicle growth and anovulation in a monovular species. Treatment of cows with DEX produced two key physiological responses that are typical of PCOS: insulin resistance and anovulation. The positive effects of glucocorticoids, such as DEX, on hepatic gluconeogenesis have been well characterized. Glucocorticoids stimulate transcription of the two rate-controlling enzymes of the gluconeogenic pathway, PEPCK and G6Pase, in the liver, thus inducing considerable quantities of hepatic glucose secretion [36–38]. The resultant increase in circulating glucose concentrations produces a corresponding rise in insulin release from the pancreas. Persistent elevation of circulating insulin leads to down-regulation of insulin action due to either decreased insulin receptor [39] or desensitization of key insulin signaling pathways [40, 41], ultimately leading to insulin resistance. In addition, glucocorticoids may directly inhibit insulin signaling pathways in tissues such as hypothalamus [42], skeletal muscle [43, 44], pancreas [45], and T-lymphocytes [46]. Along with the profound insulin resistance produced by DEX treatment, there was also a severe anovulation induced by DEX treatment. The use of targeted hormonal challenges and daily ovarian ultrasound allowed resolution of the precise physiological lesion and the follicular dynamics that were associated with the DEX-induced anovulation.

From a whole-animal perspective, induction of anovulation by DEX treatment may be mediated by direct or indirect actions of DEX on the ovary, particularly the follicle, the hypothalamus, and/or the pituitary. It is clear that there was a direct or indirect action of DEX on the follicle because, despite the development of similarly sized follicles in both DEX-treated and control animals, circulating E2 was suppressed and follicles did not ovulate in the DEX-treated group. The former may be a result of a reduction in both aromatase activity [47, 48] as well as a decrease in aromatase mRNA expression [47, 49], characteristics found in granulosa cells from PCOS patients. Interestingly, DEX has also been shown to decrease aromatase activity in human granulosa cells [50]. Further, DEX has been found to decrease LH pulse frequency [51] and amplitude [52], and therefore decreased gonadotropins may underlie depressed follicular E2 production, although it is clearly not profound enough to decrease follicular growth rate. Insulin, on the other hand, might be expected to increase E2 production as it stimulates production of androgen precursors [53–55] and increases the activity of aromatase [56]. If insulin is indeed increasing E2 in this model, it is apparent that the negative effects of DEX far outweigh any stimulatory effects of the increased insulin concentrations. It is important to note,
however, that while some have indeed found circulating E2 to be decreased in PCOS [57, 58], as would be expected with decreased conversion of androgen to E2 with reduced aromatase, others have reported increased E2 in PCOS women [59, 60]. Thus, it is uncertain how relevant the reduced E2 concentrations observed in this study are to the physiology of PCOS.

Conversely, DEX may also alter metabolism of E2 since DEX has been found to induce CYP3A mRNA expression and/or increase CYP3A protein concentrations, a key enzyme in steroid metabolism, in the liver of mice [61], sheep [62], and humans [63, 64]. This is supported by the results from experiment 3, in which all cows received the same dose of exogenous E2 but concentrations were significantly decreased in DEX-treated cows. On the other hand, high insulin might be expected to reduce E2 metabolism since it has been shown to reduce CYP3A expression or CYP3A activity in the cow [65, 66] and the rat [67]. It seems likely that follicular E2 production is the primary driver of the DEX-induced decrease in circulating E2; however, further research is needed to determine the role, if any, of altered E2 metabolism in circulating E2 and to mechanistically define changes in follicular E2 production.

In spite of the reduced follicular E2 production, these follicles had ovulatory capacity, as demonstrated by 100% ovulation in experiment 2 when DEX-treated cows were challenged with GnRH. This experiment also demonstrated that the pituitary had normal amounts of LH and was normally responsive to a GnRH surge. Thus, the physiological lesion induced by DEX did not involve pituitary LH secretion or follicular LH responsiveness, at least from a gross perspective when challenged with GnRH.

Although circulating E2 concentrations were clearly reduced after DEX, the lack of E2 positive feedback at the hypothalamus was also clearly evident in the presence of DEX and is probably the rate-limiting lesion in DEX-induced anovulation. Challenge of the DEX-treated cows with an E2 dose that produced circulating E2 more than 4-fold greater than the physiological E2 surge did not produce a normal LH surge in any of the cows. Although 25% of the cows had ovulation within a normal time period after the E2 challenge, even these cows did not exhibit a normal LH surge. In addition, half of the cows remained anovular for 2 wk after the E2 challenge, highlighting the profound unresponsiveness of the hypothalamus to E2, at least in this subset of cows. Additionally, two out of three of the anovular DEX-treated cows in experiment 4 remained anovular until administration of GnRH. This anovulatory condition seems similar to a common anovulatory abnormality in cattle, termed follicular cysts. Cows with follicular cysts develop large follicles that do not ovulate naturally or in response to an induced E2 surge, apparently due to E2 unresponsiveness of the hypothalamus [68]. Similar to the DEX-treated cows, cows with follicular cysts are capable of ovulating after GnRH administration [68]. Hypothalamic unresponsiveness to E2 and growth of large anovular follicles can be induced in cattle that are exposed to an E2 surge without subsequent ovulation and/or P4 exposure [68, 69], the latter of which appears necessary to restore hypothalamic sensitivity to E2 [reviewed by Wiltbank et al. [70]). This is the physiological situation that is likely to be induced in DEX-treated anovular cows that received E2 since they experienced an E2 surge without subsequent ovulation or P4 exposure. Thus, it seems likely that the most important physiological lesion producing anovulation during DEX treatment is the inhibition of E2 positive feedback in the hypothalamus.

DEX was utilized in this work because of the profound effects that it has on hepatic glucose production and induction of insulin resistance. Nevertheless, it is appropriate to consider whether the effects of DEX are due to the insulin resistance induced by DEX or some other effect of DEX. The classical glucocorticoid and P4 receptors are members of the family of steroid hormone receptors and share remarkable similarity. Indeed, P4 is able to bind to and activate the glucocorticoid receptor [71, 72] and vice versa [73, 74]. Although DEX has a 476-fold greater affinity for the glucocorticoid receptor than the P4 receptor [74], the large, repeated doses of DEX administered in these experiments may have provided enough glucocorticoid to activate P4 receptor pathways. Many of our results are consistent with DEX acting as a progestin in the hypothalamus. For example, successful ovulation was induced by exogenous GnRH, but anovulation continued in most cows when exogenous E2 was administered. It has been shown in sheep that cortisol, a natural glucocorticoid, is capable of delaying and attenuating the LH surge in response to an E2 surge [75]. Experiment 4 was conducted to test the acute effect of DEX close to the time of ovulation to determine if it would prevent ovulation similar to P4 administration at this same time, before insulin levels rose dramatically. While P4 blocked ovulation in all cows, acute DEX blocked ovulation in 50% of cows, suggesting that glucocorticoid may be acting in a similar albeit
weaker manner as P4 treatment. Nevertheless, DEX cows that
had a normal E2 surge had normal times of ovulation as
compared to DEX-treated cows that lacked an E2 surge
during the sampling period and did not ovulate. Thus, DEX may
be acting as a progestin sufficient to block E2 action at the
hypothalamus but not enough to reset hypothalamic sensitivity
to E2. Both the reduced follicular E2 production effects and
the progestin-like attributes of DEX could participate in induction
of anovulation in different circumstances. Obviously, further
research is needed to clearly differentiate the physiological role
of insulin resistance as well as P4-like actions in DEX-induced
anovulation.

The results of this research may also contribute to the
extensive scientific research on the reproductive effects of
stress [76–78] since stress-induced release of cortisol, an
endogenous glucocorticoid, could have actions similar to DEX.
Cortisol can decrease LH pulse amplitude [52] and frequency
[51, 79] as well as attenuate the estradiol-induced LH surge
[75, 80]. In research with sheep, a glucocorticoid-induced
reduction in pituitary responsiveness to GnRH has been
indicated [81]; however, we observed no change in the
magnitude of the LH surge following a large (200 µg) dose of
GnRH. It seems clear that reduced reproductive efficiency
during stress could be due to reduced LH pulse amplitude and
frequency, reduced follicular E2 production, and particularly
reduced hypothalamic E2 positive feedback [76–78].

Finally, discussion of this animal model and its relevance to
human PCOS is warranted. It is apparent that the ovarian
phenotype that was induced by DEX treatment is not analogous
to that observed in PCOS. Treated cows developed one to three
large, ovulatory-sized follicles that were fully capable of
ovulating when an LH surge was induced rather than the
multiple small follicles that fail to progress past deviation as is
observed in women with PCOS [10–12]. Although the large
dominant follicles can be observed in PCOS women [7],
women with the typical PCOS phenotype generally lack
ovulatory-sized follicles. This phenotype was not observed in
any cow in any of our experiments, indicating that deviation
and attainment of dominance are not hindered in this model.
Thus, this model appears more consistent with the large
follicle, anovular condition previously described in cattle [68,
69] than with the PCOS condition. While many animals models
are capable of producing anovulation, it is critical in future
research to consider whether these models of PCOS produce a
physiology that truly mimics PCOS or, for instance, more
closely resembles this large anovular follicle condition.

In conclusion, it seems likely that DEX treatment, although
producing a profound insulin resistance, is blocking ovulation
through a mechanism independent of the induced insulin
resistance, perhaps by blocking the E2-induced GnRH surge
and subsequently the ovulatory LH surge. This is different than
the proposed mechanism of anovulation in PCOS in that
follicles fail to go through deviation and thus fail to obtain
ovulatory capacity. Thus, it is concluded that this model is
likely to not represent a physiological model of human PCOS
but may be more consistent with a large anovular follicle
phenotype, generally termed follicular cysts, that has been
observed in many species, including cattle and humans.
Although the present work failed to definitively demonstrate
direct effects of hyperinsulinemia and/or insulin resistance on
follicle development and function, a role for hyperinsulinemia
and insulin resistance in the ovarian characteristics of human
PCOS cannot yet be conclusively dismissed. Further, this
research provides important information on physiological
reproductive pathways that can be altered by glucocorticoids
in mammals, including women. This may be important in
understanding the mechanisms by which stress and/or cortisol may affect reproduction as well as the possible reproductive effects of long-term clinical treatment with glucocorticoids.

ACKNOWLEDGMENT

Many thanks to Jerry N. Guenther, Robb W. Bender, Paulo D. Carvalho, Murillo M. Meschiatti, Hermanta K. Shrestha, and Patrick G. Kusilek for assistance with animal-related data collection and Melba O. Gatal for assistance with the LH assays. Thank you also to Peter M. Crump for aid with the statistical analyses.

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