Published in final edited form as:

Reprod Toxicol. 2007 ; 23(3): 438–448. doi:10.1016/j.reprotox.2006.12.009.

Pioglitazone improves insulin action and normalizes menstrual cycles in a majority of prenatally androgenized female rhesus monkeys

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Abstract

PURPOSE OF THE STUDY—To determine whether pioglitazone will improve menstrual cyclicity in a fetal programming model for polycystic ovary syndrome.

BASIC PROCEDURES—Eight prenatally androgenized (PA) and 5 control female rhesus monkeys of similar age, body weight and body mass index received an oral placebo daily for 6–7 months followed, after at least 90 days, by daily oral dosing with pioglitazone (3mg/kg) for an additional 6–7 months. Blood was sampled thrice weekly to monitor ovulatory function, and a variety of endocrine challenges were performed to quantify changes in ovarian, gonadotropin and glucoregulatory function.

MOST IMPORTANT FINDINGS—Pioglitazone normalized menstrual cycles in 5 out of 8 (62%) PA females (pioglitazone responsive; Pio_{RESP}). Pioglitazone increased serum 17α -hydroxyprogesterone responses to an hCG injection in Pio_{RESP} PA females, while diminishing serum progesterone, and increasing DHEA and estradiol responses to hCG in Pio_{RESP} PA and all normal females.

PRINCIPAL CONCLUSIONS—Insulin resistance plays a mechanistic role in maintaining anovulation in a majority of PA female monkeys.

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Keywords

androgen excess; anovulation; insulin resistance; hyperglycemia; hyperlipidemia

Introduction

Consistent with a hypothesis for fetal programming of polycystic ovary syndrome (PCOS; 1, 2,3), prenatally androgenized (PA) female rhesus monkeys, exposed to androgen excess during fetal life, express multiple PCOS traits. By adulthood, PA female monkeys exhibit anovulation and intermittent menstrual cycles associated with adiposity-related hyperinsulinemia (4,5), as well as ovarian and adrenal hyperandrogenism (6,7), enlarged polyfollicular ovaries (8), and LH hypersecretion (5,9). A similar diversity of reproductive and metabolic PCOS-like traits is found in PA ewes (10), and lesser diversity of PCOS-like traits is found in PA female mice (11) and rats (12). Since hyperinsulinemia has been repeatedly implicated in the mechanism of anovulation in women with PCOS, compensatory hyperinsulinemia from adiposity-induced insulin resistance may play a key functional role in the mechanism impairing ovulation in PA females, particularly PA female rhesus monkeys, that most closely emulate the human PCOS phenotype (9).

In this regard, hyperinsulinemia from insulin resistance in PCOS women has been implicated in impaired glucoregulation (13) and reproductive dysfunction, including anovulation (14,15, 16) and miscarriage (17,18). It is, therefore, not surprising that weight loss programs (19,20) and insulin sensitizers (21,22,23,24) have been successful therapeutic interventions for PCOS women, in terms ovulation induction, conception and improved glucoregulation. Insulin-sensitizing agents, including metformin (24) and thiazolidinedione derivatives (25), are now commonly used in the management of infertility in both obese and non-obese women with PCOS. Pioglitazone, a relatively new thiazolidinedione derivative approved for treating type 2 diabetes (26), improves insulin sensitivity, decreases insulin secretion, reduces hyperglycemia, and induces ovulation in PCOS patients (27).

As a close mimic of PCOS, PA female rhesus monkeys provide an obvious opportunity to study pioglitazone's abilities to improve insulin regulation of glucose, as well as to improve ovulation. The outcome of this study will determine whether hyperinsulinemia is causally linked with the mechanism of anovulation in a fetal programming animal model for PCOS, and thus whether fetal programming of a PCOS mimic results in pathophysiological mechanisms similar to those underlying the human syndrome. To test this hypothesis, we administered daily placebo and pioglitazone to control and PA female monkeys for six to seven months to ascertain improvements in glucoregulation, menstrual cyclicity, and related ovarian and gonadotropin function. Since insulin sensitizers, such as pioglitazone, are not completely effective in enhancing menstrual cyclicity in women with PCOS, with improved menstrual cyclicity in 41–82% of PCOS patients (e.g., pioglitazone: 27, 28; troglitazone: 25, 29; metformin: 30, 31, 32), we anticipated a similarly dichotomous improvement in ovulatory function from pioglitazone treatment of PA female rhesus monkeys.

Materials and Methods

Animals

The 13 adult female rhesus monkeys (*Macaca mulatta*) used in this study (October 2000-June 2003) were captive-born and were housed at the National Primate Research Center, University of Wisconsin, Madison (WPRC) in accordance with routine care, management and assessment protocol (33). Each monkey was fed once daily with a meal of 16–30 biscuits (approximately 96–180g) of Purina Monkey Chow (Ralston Purina, Inc., St. Louis, MO; product # 5038), or

an equivalent amount of Harlan Monkey Chow (Harlan Teklad, Madison, WI, product # 2050; after January 2003). The meal was supplemented with either 1–2 pieces of fresh fruit or bread. The number of biscuits given was varied so that at least 1–3 biscuits were found when all remaining food in an animal's cage was removed between 1700h–1800h. The Institutional Animal Care and Use Committee of the University of Wisconsin-Madison approved all the procedures used in this study. Animal maintenance was in accordance with the recommendations of the Guide for the Care and Use of Laboratory Animals and Animal Welfare Act with its subsequent amendments.

PA females were selected for pioglitazone and placebo treatment based on the presence of lengthened (>30 days; n=1; 34), intermittent (>34 days; n=6; 35) or absent (n=1) menstrual cycles and not on gestational timing of androgen excess (Table 1 and Table 2). When initially selected for this study, the five control female monkeys were comparable in age and body weight to the eight PA female monkeys, but had a slightly higher BMI (Table 1). As anticipated, most (five out of eight; 62%), but not all, PA female rhesus monkeys used in this study demonstrated improvement in menstrual cyclicity on pioglitazone treatment. The eight PA female rhesus monkeys were thus subsequently divided into two separate groups according to whether menstrual cycle duration was diminished and ovulation rate was increased (pioglitazone responsive, PiO_{RESP} ; n=5) or not (pioglitazone unresponsive, PiO_{UNRESP} ; n=3) by pioglitazone treatment. The five control female monkeys were comparable in age, body weight, and BMI to the five PiO_{RESP} PA female monkeys (Table 1). The PiO_{RESP} PA female group included four female rhesus monkeys whose mothers received daily subcutaneous injections of testosterone propionate (TP), starting on gestational days 40–44 (early-treated), for 15–35 consecutive days (early-treated PA females, EPA), and one female monkey whose mother received daily subcutaneous injections of TP starting on gestational day 114 (late-treated, LPA) for 24 consecutive days. Control female monkeys were not exposed to TP *in utero*. The PiO_{UNRESP} PA female group included one early-treated PA female, one female monkey whose mother received daily subcutaneous injections of TP starting on gestational day 60 (mid-gestation) for 15 consecutive days (mid-treated PA female, MPA), and one late-treated PA female. The age and body weight of the three PiO_{UNRESP} PA female rhesus monkeys were similar to those of both control and PiO_{RESP} PA female monkeys; BMI was similar to that of PiO_{RESP} PA female monkeys, but lower than that found in control females ($p<0.02$).

Experimental design

Six to 7 months of daily treatment started in October or later and ended at the beginning of June to avoid potential seasonal oligomenorrhea during the summer months (35,36). Pioglitazone treatment followed the placebo phase by at least 90 days to avoid the summer month period of potential seasonal oligomenorrhea. Crushed pioglitazone (3mg/kg/day, Actos, Eli Lilly and Company, Indianapolis, IN) or equivalent amounts of placebo (Takeda Pharmaceuticals America, Inc. Lincolnshire, IL) tablets were wrapped in peanut butter sandwiches or marshmallows. This daily dose of pioglitazone improved insulin sensitivity in rhesus monkeys within two weeks in a previous study at WPRC (37), was similar to daily doses of troglitazone employed to improve insulin sensitivity and menstrual cyclicity in PCOS women (21,22,25,29), but was ~6–10 times greater than daily doses of pioglitazone given to PCOS women (30–45mg/day, ~0.3–0.5mg/kg/day; 27, 28). The sandwiches or marshmallows were fed to each female monkey at 0700 h - 0800 h every morning before they received their daily food allotment. Immediately before and just before cessation of each placebo and pioglitazone treatment, body weights were measured, and blood chemistry panels and overnight-fasted serum levels of glycosylated hemoglobin were determined from blood samples collected between 0600h–0900h. A cross-over experimental design was not employed so as to avoid the potential long-term consequences of pioglitazone encountered in women,

such as weight gain and fat accumulation (38), and increased insulin sensitivity (39), that might compromise subsequent placebo treatment.

During each treatment phase, endocrine tests were performed during the early follicular phase of separate menstrual cycles or anovulatory 30-day periods. A frequently sampled, intravenous glucose tolerance test (FSIGTT) to assess insulin sensitivity was performed during the 3rd or 4th menstrual cycle or anovulatory 30-day period from the onset of treatment, an hCG test was carried out in the subsequent menstrual cycle or anovulatory 30-day period (4th–5th from treatment onset) to assess ovarian steroidogenic responses, and a GnRH stimulation test was performed during one of the next two menstrual cycles or anovulatory 30-day periods (5th–7th from treatment onset).

Menstrual cycle assessment

Each monkey underwent saphenous venipuncture three times per week between 0600h and 0900h, and the resultant serum was assayed for progesterone. Due to the fact that menstrual discharge was not usually observed in approximately one-third of ovulatory PA female rhesus monkeys (40), both decline in serum progesterone values and the first day of menstruation were used to determine menstrual cycle phase onset and duration. The day that serum progesterone levels exceeded 0.4 ng/ml was designated as the first day of a luteal phase, while the day that serum progesterone levels declined below 0.4 ng/ml was defined as the onset of the follicular phase (40). Ovulatory menstrual cycles were identified by two serum progesterone levels above 1 ng/ml within 15 days prior to menses or serum progesterone falling below 0.4 ng/ml (33,40). Area under curve (AUC [nmol/L*day]) serum progesterone for each luteal phase was determined using the trapezoidal rule (41).

Ovulation rate

Based on the ovulatory menstrual cycles determined above, the ovulation rate was calculated for each female monkey as the number of ovulatory events identified divided by the number of ovulations that could have occurred during the treatment phases (one per 28 days, as per control female data in this study and typical for this nonhuman primate, 42). Ovulation rates were averaged across monkeys within each female group and within treatments.

Glucoregulation assessment by frequently sampled, intravenous glucose tolerance test (FSIGTT)

FSIGTTs were performed according to the tolbutamide-modified, minimal model protocol validated in rhesus monkeys (37,43). Briefly, after an overnight fast, each animal was anesthetized with ketamine hydrochloride (15 mg/kg, im) and diazepam (1.25 mg/kg, im). Supplemental ketamine was administered, as appropriate, to maintain anesthesia (5–10 mg/kg, im). A catheter (Polyethylene tubing, Intramedic TM, PE60, Becton Dickinson and Company, Sparks, MD) was placed into the vena cava through the saphenous vein for blood sampling (33 samples over 195 min) and for administration of glucose (300 mg/kg at 0 min) and tolbutamide (5 mg/kg at 20 min). Serum glucose and insulin levels were determined from these blood samples. Insulin sensitivity (S_I ; the measure of the fraction of glucose cleared from the circulation per unit increase in insulin) and glucose effectiveness (S_G ; the measure of the ability of glucose to increase its own uptake and to suppress hepatic glucose production at basal insulin levels) were determined using the modified minimal model method (37,43). Further measures derived from the FSIGTT were basal insulin (I_b ; mean of the four prechallenge plasma insulin values, –15, –10, –5, and –1 min), basal glucose (G_b ; average of the four prechallenge plasma glucose values), acute insulin responses to glucose (AIR_g; average elevation of posthepatic plasma insulin concentration above the baseline for the 2, 3, and 4 min samples), acute insulin response to tolbutamide (AIR_{tol}; average elevation of posthepatic plasma insulin concentration above the baseline for the 22, 23, and 24 min samples), glucose disappearance rate (K_G ; slope

of the log linear regression of plasma glucose concentration between 10 and 19 min), and disposition index (DI or β -cell compensation index; product of S_I and AIRg) (36). AUC for glucose and insulin were determined using the trapezoidal rule (41) as AUC luteal phase progesterone described before. Total free (non-esterified) fatty acid plasma levels were determined from the first (–15 min) pre-glucose infusion and the last (180 min) post-glucose infusion samples.

hCG stimulation test

Briefly, each monkey was injected intramuscularly with 200 IU recombinant hCG (Serono Laboratories, Norwell, MA), and blood samples (5ml) were withdrawn at 0h (0800 h day 1), 24h (0800 h day 2), 48h (0800 h day 3) and 72h (0800 h day4). Serum was assayed for hCG-mediated ovarian theca cell steroid hormone release, including progesterone, and steroid hormones indicative of androgen biosynthetic enzyme activity (17 α -hydroxyprogesterone (17 α OHP), dehydroepiandrosterone (DHEA), androstenedione, and testosterone (44). Serum was also assayed for estradiol, at least partially reflecting ovarian granulosa cell aromatization of hCG-stimulated theca cell androgen release. The dose of hCG, timing of blood samples and steroid hormones assessed are comparable to those employed in determining ovarian hyperandrogenism in young PCOS women (45) and PA female monkeys (6,9). Direct gonadotropic (hCG) stimulation of ovarian theca cells (e.g., 46) was employed rather than indirect, GnRH analogue mediated, endogenous gonadotropic (LH and FSH) stimulation of both ovarian theca and granulosa cells (e.g., 47) in order to avoid endogenous LH hyperresponsiveness to exogenous GnRH stimulation in PA monkeys (9) that could potentially confound interpretation of ovarian responses (46).

GnRH stimulation test

Monkeys were anesthetized with an intramuscular injection of 15 mg/kg ketamine hydrochloride at ~0745h, and a venous catheter (the same catheter as described for the FSIGTT) was inserted through the saphenous vein and positioned in the inferior vena cava for the whole procedure. At ~0800h, each monkey was infused with 20 μ g GnRH (47; 0.5ml, Sigma L-7134; Sigma Chemical Company, St. Louis, MO), and blood samples (3ml) were withdrawn at 0, 2, 5, 10, 20, 30, 40, and 60 minutes after GnRH infusion for immunoactive LH and FSH determinations. The GnRH test was designed to assess pituitary gonadotropin responses to exogenous GnRH (9), but not ovarian responses to GnRH-stimulated gonadotropin release.

Assay procedures

All hormones were assayed in the WPRC Hormone Assay Services Laboratory (40,49,50). Circulating levels of total free fatty acids (51), and glycosylated hemoglobin (52) were assayed, as previously described. Glucose was measured by the glucose oxidase method (37). Insulin, LH, and FSH were determined by RIA (37,40). RIA determinations for DHEA, androstenedione, and estradiol were performed following diethyl ether extraction of serum and solvent fraction separation by celite chromatography (6,7). Similar determinations for progesterone and testosterone, and 17 α OHP were made by EIA (6). RIA determinations of cortisol (50) were made following diethyl ether extraction without prior chromatography. Intra- and inter-assay CVs for quality control preparation (QC) values were, respectively, glucose: 2.9% and 4.0%, insulin: 4.6% and 7.9%, progesterone: 3.7% and 19.4%, 17 α OHP: 5.6% and 9.8%, DHEA: 8.2% and 13.4%, androstenedione: 3.7% and 7.9%, testosterone 3.5% and 17.7%, estradiol: 9.3% and 20.0%, FSH: 3.2% and 4.0%, and LH: 4.2% and 4.3%.

Statistical analysis

Hormonal data and parameters from the FSIGTT/minimal-model analyses were log transformed to achieve homogeneity of variance and to increase linearity when appropriate

(53). Durations of follicular and luteal phases, as well as menstrual cycle length were analyzed using Fully factorial (M) ANOVA with one between subjects factor (types of female monkeys) and two within subject factors (treatment and time) (Systat, version 7.0, Evanston, IL). AUC of luteal phase serum progesterone related to $P_{io_{resp}}$ PA females, serum hormone levels and ratios from the hCG tests, and gonadotropin levels from GnRH tests were all submitted to statistical analysis using SAS Proc GLM with repeated measures (SAS, version 8, SAS Institute Inc. Cary NC). When significant ($P<0.05$) statistical interactions were identified by ANOVA, appropriate posthoc univariate or least significant difference (LSD) analyses were performed on the variables S_g , G_b and S_I , and from FSIGTT/minimal-model were analyzed by nonparametric analyses (Wilcoxon signed rank test or Mann-Whitney U test); other parameters from the FSIGTT/minimal-model analyses were subjected to SAS Proc GLM with repeated measures with one between subjects factor (types of female monkeys) and one within subject factors (treatment) because of unbalanced data. All log transformed parameters are expressed as the antilog of the transformed mean (95% confidence limit); nonparametric analyzed data are expressed as median $\pm$ interquartile interval; while all other data are shown as either mean $\pm$ SEM.

As anticipated, the PA female group comprised two distinct sub-groups according to whether their menstrual cycles normalized ($P_{io_{RESP}}$; $n=5$) or did not normalize ($P_{io_{UNRESP}}$; $n=3$) on pioglitazone treatment. We, therefore, also performed additional statistical comparison of only $P_{io_{RESP}}$ PA females against controls, as described above.

Results

Body weights and blood chemistry panels

Body weights did not differ by treatment or by type of female (Table 1). Body weights, however, decreased ($p<0.005$) by approximately 0.8kg (~8.2%) at the end of either treatment phase, as may be typical for this seasonally breeding primate (54). Body weight was regained in both female groups between the end of placebo treatment and the onset of pioglitazone treatment (Table 1). Blood chemistry panels showed no liver enzyme abnormalities during 6–7 months of pioglitazone treatment (data not shown).

FSIGTT test

As expected, pioglitazone diminished (p -values <0.04 – 0.004) basal plasma insulin levels (Table 3). Measures of S_I , K_g , S_G and DI , however, were not different between placebo and pioglitazone treatment for either control or PA female monkeys. Pioglitazone-mediated changes in acute insulin responses in both control and PA female rhesus monkeys were varied. In both female groups, AIR_g was unaltered by pioglitazone; AIR_{TOL} , however, was decreased ($p<0.01$), suggesting insulin sensitizer-induced changes in activity at the pancreatic beta cell sulfonylurea-activated, ATP-regulated potassium (KATP) channel.

Pioglitazone did not alter basal serum glucose levels (G_b) in PA females (Table 3). Conversely, fasting basal plasma total free fatty acid levels remained higher ($p<0.04$) in PA versus control female monkeys during both placebo and pioglitazone treatments (Table 3), while pioglitazone had no effect on total free fatty acid levels in either female group. Fasted total free fatty acid levels were positively correlated with fasting basal glucose levels in PA females, alone, during placebo treatment ($p<0.05$, $r^2=0.92$).

Glycosylated hemoglobin

Serum glycosylated hemoglobin values decreased ($p<0.007$) during pioglitazone treatment in both control and PA female monkeys (Table 4). No such improvement in serum glycosylated

hemoglobin values occurred during a similar period of time on placebo treatment. There were no differences in glycosylated hemoglobin values between female groups.

Menstrual cycle characteristics

Menstrual cycle irregularities were prevalent among PA, but not control females, at the onset of this study (Table 1). While receiving pioglitazone treatment, menstrual cycle duration in PA females became similar to that in controls (Table 2 and Table 5). Pioglitazone treatment also increased AUC serum luteal phase progesterone values in ovulatory females from both animal groups.

As anticipated by examining raw menstrual cycle data, two distinct sub-groups of PA female monkeys existed: those that had menstrual cycle duration normalized by pioglitazone (PiORESP PA) and those that did not (PiOUNRESP PA; Table 2 and Table 5). Therefore, data were re-analyzed omitting the PiOUNRESP PA females. In 62% (5 out of 8) PA females (PiORESP), menstrual cycle duration was reduced to normal by pioglitazone due to a reduction in follicular phase length (Table 5). Follicular phase duration in control females remained unchanged on pioglitazone treatment compared to placebo (Table 5). Luteal phase duration in PiORESP PA females was increased by pioglitazone, resulting in normalization of luteal phase length in these females. AUC luteal phase serum progesterone values remained increased ($p < 0.02$) by pioglitazone treatment in both control and PiORESP PA females. The combined effect of pioglitazone on reducing the follicular phase and prolonging the luteal phase served to normalize the ovulation rate in PiORESP PA females (Table 5).

Pioglitazone-induced associations between insulin sensitivity and menstrual cycle parameters involving PiORESP PA females

there were three significant changes in insulin and glucose parameters derived from the FSIGTT when PiORESP PA females alone were compared to controls (Table 3). Pioglitazone treatment increased S_I in both female groups (~15% in control and ~157% in PiORESP PA female monkeys) and normalized basal serum glucose levels (G_b) in PiORESP PA females, alone (Table 3). Total free fatty acids only tended ($p < 0.07$) to be elevated in PiORESP PA compared to control females. In addition, two menstrual cycle parameters were significantly correlated with insulin sensitivity when PiORESP PA and control monkeys received pioglitazone, but not placebo. PiORESP PA and control females, combined, demonstrated a negative relationship ($r^2 = 0.50$, $p < 0.03$) between insulin sensitivity and menstrual cycle duration, while AUC serum luteal phase progesterone values were positively associated ($r^2 = 0.53$, $p < 0.03$) with insulin sensitivity. There were no associations found between insulin sensitivity and either follicular or luteal phase duration.

hCG stimulation test

Prior to the administration of hCG in the placebo phase, basal serum testosterone levels tended ($p < 0.07$) to be elevated in PA compared to control females (Table 1). Following hCG injection, serum testosterone levels showed similar increases 24h after hCG injection in both female monkey groups on both treatments. Serum androstenedione levels tended ($p < 0.07$) to be higher in PiORESP PA versus control female monkeys in both treatments combined (Figure 1). Serum estradiol levels, in contrast, were higher ($p < 0.001$) in both pioglitazone-treated monkey groups, and were increased ($p < 0.05$) by 24h after hCG injection (Figure 1). Pioglitazone decreased serum androstenedione/DHEA ($p < 0.01$) and progesterone/estradiol ($p < 0.0009$) ratios, while it increased the estradiol/testosterone ratio ($p < 0.001$) in both control and PiORESP PA females (Figure 2), creating a more estrogenic than androgenic milieu in all pioglitazone-treated females.

Pioglitazone decreased ($p<0.05$) serum progesterone responses to hCG injection in both female groups compared to levels achieved on placebo treatment (Figure 1). Pioglitazone, however, increased serum 17α OHP levels in PiO_{RESP} PA female monkeys ($p<0.01$) before and after hCG injection without altering serum 17α OHP levels in controls. Overall, there were no differences in magnitude of steroid hormone or steroid hormone ratio responses to hCG that differed between the female groups.

GnRH stimulation test

Prior to the administration of GnRH in the placebo phase, basal serum LH and FSH levels, and the LH:FSH ratio, were similar in PA and control females (Table 1). Pioglitazone did not alter serum LH and FSH levels, or their responses ($p<0.05$) to exogenous GnRH in either female group (data not shown). The serum ratio of LH/FSH, however, was higher ($p<0.03$) in PiO_{RESP} PA female monkeys, regardless of treatment (Figure 3). The serum LH/FSH ratio was also increased by pioglitazone treatment in both control and PiO_{RESP} PA female monkeys ($p<0.05$).

Pioglitazone unresponsive PA female monkeys

There were no improvements in menstrual cycle frequency and duration in response to insulin sensitizer treatment in a minority (38%) of PA females ($\text{PiO}_{\text{UNRESP}}$; Table 2 and Table 5). One $\text{PiO}_{\text{UNRESP}}$ PA female monkey (EPA) remained anovulatory during placebo and pioglitazone treatments (and had the lowest BMI of all study females), while an additional two $\text{PiO}_{\text{UNRESP}}$ PA females (one MPA and one LPA) showed irregular inter-menstrual intervals during both treatment phases, indicating episodes of anovulation. The latter two $\text{PiO}_{\text{UNRESP}}$ PA females exhibited the longest and third longest intervals between menstrual cycles during the placebo phase before pioglitazone treatment (Table 2). There were no obvious differences in insulin and glucose parameters derived from the FSIGTT or serum levels of glycosylated hemoglobin between PiO_{RESP} PA and $\text{PiO}_{\text{UNRESP}}$ PA females (Table 3 and Table 4). hCG stimulation testing also yielded different results from those found in PiO_{RESP} PA females. While receiving pioglitazone, hCG-stimulated serum androstenedione levels were lower [overall mean (95%CI): 213 (173, 264) pg/ml, $p<0.006$] in $\text{PiO}_{\text{UNRESP}}$ PA females than in PiO_{RESP} PA females [356 (296, 427) pg/ml]. No differences were found in the serum levels of the other steroid hormones measured. Since one $\text{PiO}_{\text{UNRESP}}$ PA female did not receive an hCG test on placebo treatment, no placebo-pioglitazone comparisons could be made for the $\text{PiO}_{\text{UNRESP}}$ PA female group. LH and FSH responses to GnRH were not different to those observed in PiO_{RESP} PA females (data not shown).

Discussion

Pioglitazone exerts its biological effects through binding to peroxisome proliferator-activated receptor γ (PPAR γ), an intra-cellular receptor belonging to the nuclear hormone receptor superfamily of ligand-dependent transcription factors (55,56). While PPAR γ receptors are predominantly expressed in adipose tissue, they have also been located in the reproductive system (hypothalamus, pituitary, ovarian granulosa and theca cells), cardiovascular system (endothelial cells, vascular smooth muscle cells), immune system (macrophages), liver, kidney, and in malignant tissue (55,56,57,58). Pioglitazone has also been recently implicated in diminishing extra-cellular matrix production (59) and affecting a diversity of organ systems through mechanisms apart from its anti-diabetic properties (55,58). Thus, while this thiazolidinedione derivative is therapeutically employed to diminish insulin resistance, anovulation and hirsutism in PCOS women (27,28), it has multiple and complex effects in unrelated organ systems and should not be expected to have straightforward physiological outcomes (56,60).

Employing pioglitazone in this study has functionally reinforced our previous observations showing an association between anovulation and hyperinsulinemia from adiposity-dependent insulin resistance in PA female rhesus monkeys (4,5). The present study provides the first evidence for a mechanistic link between hyperinsulinemia, insulin resistance and anovulation in this fetal programming model for PCOS. Pioglitazone improved menstrual cyclicity in 62% of PA female rhesus monkeys (PiORESP PA) compared to previous placebo treatment. This normalization of menstrual cyclicity, however, did not persist following cessation of pioglitazone treatment. In the subsequent 12–18 months of a separate study (61), menstrual cycles in the same PiORESP PA female monkeys reverted to a prolonged duration of ~39–40 days.

The proportion of PiORESP PA that experienced ovulatory improvement in response to pioglitazone is surprisingly similar to that achieved in PCOS women receiving various insulin sensitizers. For example, six months treatment of PCOS women with 45mg/day of pioglitazone improved ovulatory function in 67% of patients (28), while a similar duration of treatment with 400mg/day of troglitazone, a thiazolidinedione chemically similar to pioglitazone, improved ovulation in 61% of patients (29). Pioglitazone-induced ovulatory improvement, however, depends upon its dose and duration as well as the patient population treated. For example, ~41% PCOS patients receiving 30mg pioglitazone daily for three months exhibited normal ovulatory cycles (versus ~5% of PCOS women receiving placebo; 27), while no PCOS women receiving 30mg pioglitazone daily for five months showed signs of ovulation (62). Metformin, a biguanid that enhances insulin sensitivity and inhibits hepatic glucose output, given in doses ranging from 1500–2550mg/day, and in durations of 3.5 to 12 months, improved ovulatory function in 41–96% of patients (30,31,32,63). In a recent systematic review and meta-analysis of randomized controlled clinical trials, metformin was found to be clinically effective for treating anovulation in PCOS women (24). Regarding the predictors of failure to ovulate by PCOS women in response to insulin sensitizer treatment (i.e., greater degrees of anovulation, obesity, insulin resistance and hyperandrogenism), the single risk factor for ovulatory failure in our PiOUNRESP PA female monkeys receiving pioglitazone was chronic anovulation rather than excessive hyperandrogenism or obesity.

Improving insulin action and diminishing circulating insulin levels are thus key physiological changes associated with partial, but significant, amelioration of menstrual dysfunction in both women with PCOS and in PA female rhesus monkeys. The latter finding advances our understanding of fetal programming of PCOS by showing that there may be a common pathophysiological mechanism for anovulation in PCOS women and in their nonhuman counterparts, PA female rhesus monkeys.

In previous studies, PA female monkeys exhibited lower peak and AUC serum progesterone values during spontaneous luteal phases (64), indicating possible luteal phase defects resembling those found in some PCOS women (65,66). In the present study, pioglitazone corrected such luteal phase progesterone abnormalities in ovulatory PiORESP PA monkeys by increasing serum AUC progesterone values through prolonging luteal phase duration and by normalizing follicular phase and overall menstrual cycle durations. Such pioglitazone-induced improvements in ovulatory menstrual cycle function may reflect improved follicle development from enhanced gonadotropin stimulation of the follicle and/or of its subsequent corpus luteum since luteal phase defects in women can be due to abnormal gonadotropin secretion (67,68,69). This study was not designed, however, to identify abnormalities in pulsatile gonadotropin release. Alternatively, a direct stimulatory effect of pioglitazone on corpus luteum function is possible since pioglitazone is a potent agonist for the PPAR γ nuclear hormone receptor (70). Cultured bovine luteal cells exposed to an endogenous ligand for PPAR γ , 5-deoxy-Delta[12,14] prostaglandin J[2] (PGJ2; a prostaglandin metabolite) increase progesterone production (71).

In contrast, PPAR γ ligands, such as PGJ2 and fatty acid metabolites (56), inhibit progesterone synthesis in non-luteinized, granulosa cells (72), similar to findings in other *in vitro* model systems (73). Such dichotomous effects provide insight into pioglitazone-induced changes in progesterone responsiveness to hCG in this study, which was reduced in both PA and control monkeys during the nonluteal phase, possibly from pioglitazone-mediated diminution of theca cell 3 β -hydroxysteroid dehydrogenase II (3 β HSD II) activity (73). Such contrasting findings between follicular and luteal phase outcomes of pioglitazone treatment may represent altered intracellular signaling in luteal cells versus theca cells, since luteinization alters LH receptor-mediated PKA activation in many species (74,75), and regulates CREB isoform expression in rhesus monkeys (76).

The remaining pioglitazone-mediated changes in steroid hormone levels in PioRESP PA female monkeys may represent pioglitazone-induced improvements in follicle development rather than direct PPAR γ effects on ovarian steroidogenesis. This explanation would provide the most parsimonious account for several of the steroid hormone and menstrual cycle changes in PioRESP PA female monkeys, including pioglitazone-mediated increases in [1] circulating levels of 17 α OHP, DHEA and estradiol (the latter aromatized from increased androgens; 77), [2] diminished androgenicity (reduced androstenedione/DHEA; increased estradiol/testosterone, unchanged androstenedione/testosterone) and enhanced estrogenicity (increased estradiol/testosterone; decreased progesterone/estradiol), and [3] normalized duration of the follicular phase.

In summary, six to seven months of daily pioglitazone therapy improved ovulatory function in a majority of PA female rhesus monkeys through either direct action of this PPAR γ receptor ligand on ovarian function, leading to improved follicular development, or through PPAR γ -mediated improvements in insulin sensitivity, causing decreased circulating insulin levels. These data support our hypothesis that hyperinsulinemia from insulin resistance plays a central role in the pathogenesis of anovulation in this fetal programming model for PCOS, and emphasize the possible fetal origins of PCOS in women.

Acknowledgements

this work was supported by NIH grants R01 RR013635, P51 RR000167, T32 AG000268, and P50 HD044405. This research was conducted at a facility constructed with support from Research Facilities Improvement Program grant numbers RR15459 and RR020141. We thank EJ Peterson, JM Turk, K Hable, S DeBruin, DK Barnett and RD Medley for technical assistance; F Wegner, D Wittwer, S Jacoris and Assay Services of the National Primate Research Center, University of Wisconsin-Madison (NPRC) for hormone assay expertise; D Florence DVM, I Bolton DVM, K Brunner DVM and D Werner-Kelln for veterinary care; D Wade and S Maves for animal care.

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Figure 1.

Serum steroid levels in PA (placebo phase: solid bars, pioglitazone treated phase: dotted bars) and control (placebo phase: open bars, pioglitazone treated phase: dashed bars) adult female rhesus monkeys following 200 IU recombinant hCG i.m. at 0h (backtransformed means and upper 95% confidence limits). Progesterone, A: $p < 0.05$ vs placebo, a: $p < 0.05$ vs 0h (control and PA combined), b: $p < 0.05$ vs 48h (control and PA combined), c: $p < 0.05$ vs 24h or 48h (control and PA combined); 17αOHP, A: $p < 0.01$ pioglitazone vs placebo (PA only, all time point combined), a: $p < 0.05$ vs 0h, b: $p < 0.05$ vs 24h; DHEA, A: $p < 0.003$ vs placebo (control and PA all time points combined); Androstenedione, A: $p < 0.07$ vs control female monkeys (all treatment and all time points combined), a: $p < 0.05$ vs 0h (all females all treatment phase

combined); Testosterone, a: $p < 0.05$ vs 0h (all females and all treatment phases); Estradiol, A: $p < 0.001$ vs placebo (control and PA female monkeys combined), a: $p < 0.05$ vs 0h (all female monkeys all treatment phases combined).

Figure 2.

The ratios of hormones from the hCG test in PA (placebo phase: solid bars, pioglitazone treated phase: dotted bars) and control (placebo phase: open bars, pioglitazone treated phase: dashed bars; backtransformed means and upper 95% confidence limits). Androstenedione/DHEA, A: $p < 0.01$ vs placebo (control and PA females combined); a: $p < 0.0004$ vs 0 min; b: $p < 0.01$ vs 24h (all treatments and females combined). Estradiol/Testosterone, A: $p < 0.001$ vs placebo (all females and all timepoints combined); a: $p < 0.006$ vs 0h; b: $p < 0.004$ vs 24 h. Progesterone/Estradiol, A: $p < 0.0009$ vs placebo (all females and all timepoints combined); a: $p < 0.001$ vs 0h; b: $p < 0.02$ vs 24h; c: $p < 0.005$ vs 48h.

Figure 3.

Serum LH:FSH ratio in PA (placebo phase: solid circle, pioglitazone treated phase: solid square) and control (placebo phase: open circle, pioglitazone treated phase: open square) adult female rhesus monkeys following 20µg GnRH i.v. at 0 min (backtransformed means and 95% confidence limits). * : $p < 0.03$, PA versus controls (all times and treatments combined). In addition, pioglitazone values are increased ($p < 0.05$) versus placebo (both female groups and all times combined).

Table 1

Age, body weight, body mass index (BMI) and baseline menstrual and endocrine parameters in five control and eight prenatally androgenized (PA) female rhesus monkeys given daily oral dosing of pioglitazone (3mg/kg/day) or placebo for 6–7 months. These characteristics in PA monkeys are also separated by whether pioglitazone diminished menstrual duration (pioglitazone responsive (Pio_{RESP}) PA monkeys) or failed to diminish menstrual cycle duration (pioglitazone unresponsive (Pio_{UNRESP}) PA monkeys).

Characteristic	Control females (n=5)	PA females (n=8)	Pio _{RESP} PA females (n=5)	Pio _{UNRESP} PA females (n=3)
<u>At initial selection for study¹:</u>				
Age (years)	19.7±1.5	19.9±0.8	19.9±0.9	19.9±1.7
Body weight (kg)	9.9±0.6	8.8±0.3	8.9±0.4	8.6±0.4
BMI (kg/m ²)	43.3±1.6	37.5±1.3 ^a	39.1±1.4	35.3±2.0 ^a
<u>Body weight (kg)¹:</u>				
Immediately before starting placebo	10.2±0.6	9.2±0.3	8.8±0.3	8.3±0.3
Immediately before cessation of placebo ^b	9.8±0.2	8.8±0.4	8.2±0.4	8.1±0.2
Immediately before starting pioglitazone	10.3±0.5	9.4±0.3	9.1±0.4	8.5±0.3
Immediately before cessation of pioglitazone ^b	9.8±0.2	8.5±0.3	7.8±0.3	7.6±0.2
<u>Baseline menstrual cycle and relevant serum hormone parameters²:</u>				
Incidence of menstrual cycle irregularities (>34 days)	0/5	7/8 ^c	4/5	3/3
Testosterone (nmol/L)	0.6[0.5, 0.9]	1.1[0.8, 0.6] ^d	1.1[0.7, 1.8]	0.7, 1.0 ³
LH (ng/ml)	0.9[0.7, 1.2]	1.1[0.8, 1.4]	1.0[0.8, 1.4]	1.6, 1.2 ³
FSH (ng/ml)	2.2[1.9, 2.6]	2.2[1.6, 3.1]	2.3[1.5, 3.6]	3.6, 1.7 ³
LH:FSH ratio	0.4[0.3, 0.5]	0.5[0.4, 0.6]	0.4[0.3–0.6]	0.4, 0.7 ³

¹ Mean±SEM

² Backtransformed mean[95% confidence limits]

³ No comparable baseline sample was collected from one of the Pio_{UNRESP} PA females

^a p<0.02 versus controls

^b p<0.0007 versus immediately before starting treatment (all females, including unresponsive PA females, and treatments combined, and no significant effects between treatments)

^c p<0.005 versus controls, Fisher's Exact test

^d p<0.07 versus controls

Table 2

Gestational timing of fetal androgen excess in PA and control female rhesus monkeys receiving daily oral dosing of 3mg/kg pioglitazone or placebo for 6–7 months in relation to mean menstrual cycle duration (days) on treatment.

Female Monkey		Gestational age at onset of TP	Duration of TP	Mean menstrual cycle duration on placebo	Mean menstrual cycle duration on pioglitazone
Control females					
1	2076	-	-	27	28
2	79152	-	-	30	32
3	2082	-	-	28	25
4	2083	-	-	27	30
5	AI58	-	-	26	23
Pio _{RESP} PA females					
1	79135	41	25	45	36
2	79169	40	15	40	25
3	81147	40	35	35	30
4	81150	40	35	31	28
5	84106	114	25	38	34
Pio _{UNRESP} PA females					
1	84085	115	25	40	57
2	81067	60	15	56	94
3	80146	40	25	>120	>120

Table 3
Insulin and glucose parameters derived from the FSIGTT for control and PA female rhesus monkeys during placebo and pioglitazone treatment.

Para-meters	Control monkeys (n=5)		PA monkeys (n=8)		Pioglitazone (n=5)		Pioglitazone (n=3)	
	Placebo	Pioglitazone	Placebo	Pioglitazone	Placebo	Pioglitazone	Placebo	Pioglitazone
G ₀ (mmol/L)	2.82 (2.77, 3.18)	3.54 (3.24, 6.52)	3.45 (3.35, 5.43) ^{**}	3.60 (3.05, 4.07) ^{**}	4.05 (3.35, 8.88) ^{**}	3.44 (2.97, 5.08) ^{**}	3.45 (3.35, 4.10)	3.60 (3.05, 4.07)
I ₀ (pmol/L)	251 (147, 428)	180 (112, 288) ^{a, g}	215 (143, 323) [*]	118 (83, 166) ^{* a}	261 (155, 441) [*]	129 (76, 222) ^{* g}	155 (89, 269)	103 (67, 160)
K _g (%/min)	8.00 (6.43, 9.58)	6.54 (4.88, 8.19)	7.11 (5.28, 8.94)	3.36 (2.68, 4.04)	5.16 (1.96, 13.56) [*]	3.15 (1.92, 5.16) [*]	6.34 (4.24, 8.44)	3.30 (1.77, 4.83)
S _g (*10 ⁻² /min)	3.07 (1.08, 5.43)	2.69 (2.26, 3.73)	2.88 (0.83, 6.12) ^{**}	2.31 (1.45, 6.72) ^{**}	4.01 (2.78, 5.24)	1.88 (1.42, 2.34)	1.15 (0.03, 4.97)	6.72 (2.29, 7.57)
S _i	3.25 (0.30, 4.62)	3.76 (2.18, 5.76) ^h	3.23 (0.52, 8.77) ^{**}	3.99 (1.99, 4.05) ^{**}	1.53 (0.19, 3.10) ^{**}	3.92 (2.91, 4.03) ^{**}	4.98 (3.36, 10.04)	3.99 (0.03, 14.83)
DI (min)	457 (253, 661)	444 (265, 622)	432 (290, 575)	263 (176, 350)	386 (153, 620)	259 (149, 370)	509 (466, 553)	268 (100, 437)
AIR _g (nmol/L)	1.49 (1.24, 1.73)	1.08 (0.68, 1.47)	0.90 (0.73, 1.08)	0.65 (0.49, 0.81)	0.86 (0.60, 1.13)	0.65 (0.38, 0.93)	0.97 (0.75, 1.20)	0.64 (0.47, 0.81)
AIR _{TOL} (nmol/L)	1.75 (1.23, 2.27)	1.04 (0.72, 1.36) ^b	1.34 (1.01, 1.67)	0.67 (0.51, 0.83) ^b	1.72 (1.22, 2.22)	0.90 (0.53, 1.26) ⁱ	1.41 (0.88, 1.95)	0.83 (0.54, 1.12)
Total free fatty acids ₋₁₅	418 (267, 653)	478 (358, 637)	726 (497, 1061) ^{* j}	502 (394, 641) ^{* j}	737 (451, 1207) ^k	521 (385, 705) ^k	710 (430, 1171)	478 (300, 760)
Total free fatty acids ₁₈₀	409 (309, 541)	515 (444, 598)	596 (471, 753) ^{* j}	618 (545, 701) ^{* j}	628 (438, 901) ^k	611 (538, 693) ^k	556 (428, 722)	627 (498, 789)

* data are shown as mean (95% confidence limits) of back-transformed logarithmic data

** analyzed by nonparametric analyses (Wilcoxon signed rank test or Mann-Whitney test, SAS), data are expressed as median (interquartile interval) All other data are expressed as mean (95% confidence limits). Units used: S₁ (*10⁻⁵/min/(pmol/L)), AUC_{INS} (*10³*(mmol/L)*min), AUC_{GLU} (*10³*(mmol/L)*min), fatty acids (μmol/L).

^a p<0.02 vs placebo (control and all PA females combined);

^b p<0.01 vs placebo (control and all PA females combined);

^c p<0.004 vs placebo;

^d p<0.04 vs placebo (control and all PA females combined);

^e p<0.01 vs placebo (control and all PA females combined);

^f p<0.04 vs control on placebo;

^g p<0.03 vs placebo (control and PiogRESP PA females combined);

- h* $p < 0.02$ vs placebo (control and PtoRESP PA females combined);
- i* $p < 0.03$ vs placebo (control and PtoRESP PA females combined);
- j* $p < 0.04$ vs controls (all time points and treatment phases combined);
- k* $p < 0.07$ (all time points and treatment phases combined).

Table 4

Mean($\pm$ SEM) glycosylated hemoglobin (%) in five control and eight prenatally androgenized (PA) female rhesus monkeys given daily oral dosing of pioglitazone (3mg/kg/day) or placebo for 6–7 months. These characteristics in PA monkeys are also separated by whether pioglitazone normalized menstrual duration (pioglitazone responsive (PiORESP) PA monkeys) or failed to normalize menstrual cycle duration (pioglitazone unresponsive (PiOUNRESP) PA monkeys).

Stage of treatment	Control females (n=5)	PA females (n=8)	PiORESP PA females (n=5)	PiOUNRESP PA females (n=3)
Immediately before starting placebo	6.4 $\pm$ 0.5	8.7 $\pm$ 1.3	9.3 $\pm$ 1.8	7.3 $\pm$ 0.1
Immediately before cessation of placebo	7.0 $\pm$ 1.4	8.9 $\pm$ 1.4	9.7 $\pm$ 1.6	7.3 $\pm$ 0.5
Immediately before starting pioglitazone	7.8 $\pm$ 1.5	9.1 $\pm$ 1.0	10.7 $\pm$ 1.7	8.4 $\pm$ 0.7
Immediately before cessation of pioglitazone	5.8 $\pm$ 0.5 ^{a b}	6.6 $\pm$ 0.3 ^b	6.8 $\pm$ 0.6 ^a	6.0 $\pm$ 0.1

^a p<0.05 vs immediately before starting pioglitazone treatment (control and PiORESP PA females combined);

^b p<0.007 vs immediately before starting pioglitazone (all females combined).

Table 5
Menstrual cycle parameters derived from circulating progesterone levels in control and PA female rhesus monkeys during placebo and pioglitazone treatment.

Parameters	Control monkeys (n=5)		PA monkeys (n=8)		Piogresp PA monkeys (n=5)		Piounresp PA monkeys (n=3)	
	Placebo	Pioglitazone	Placebo	Pioglitazone	Placebo	Pioglitazone	Placebo	Pioglitazone
Ovulation rate	1.02 (1.00, 1.04)	1.03 (0.97, 1.09)	0.71 (0.66, 0.76) ^a	0.76 (0.65, 0.87) ^a	0.75 (0.7, 0.8) ^h	0.93 (0.87, 0.99) ^j	0.70, 0.50	0.50, 0.30
Menstrual cycle duration	27 (26, 29)	28 (24, 31)	40 (35.8, 53.3) ^c	35 (28.5, 84.8) ^{**}	37.7 (35.4, 40.0) ^j	30.8 (28.8, 32.8) ^k	56.0 (40.0, 120.0)	94.0 (57.0, 120.0)
Follicular phase duration	12 (11, 13)	13 (9.5, 13.5)	26 (21, 42.5) ^{**d}	19 (13.5, 69.3) ^e	24.7 (21.7, 27.7) ^j	15.4 (13.9, 16.9) ^m	45.0 (21.0, 120.0)	78.0 (43.0, 120.0)
Luteal phase duration	16.0 (15.8, 16.2)	15.9 (15.2, 16.6)	15.4 (14.6, 16.2)	15.6 (15.0, 16.2)	14.6 (14.2, 15.0) ⁿ	15.8 (15.0, 16.6)	19.8, 14.7	14.0, 16.0
AUC P	125 (93, 169)	195 (116, 327) ^f	104 (68, 160) [*]	156 (114, 216) ^{*g}	92 (51, 172) [*]	149 (92, 264) ^{*o}	133, 137	81, 151

All data were tested for normality by Anderson-darling test before analysis.

* data are shown as mean (95% confidence limits) of back-transformed logarithmic data;

** analyzed by nonparametric analyses (Wilcoxon signed rank test or Mann-Whitney test) since data did not comply with normality when submitted to an Anderson-darling test, data are expressed as median (interquartile interval); All other data are expressed as mean (95% confidence limits) except for Piounresp PA monkeys when individual values are shown when data for only two animals are available. Units for cycle length, follicular length, and luteal length: days; AUC P (area under curve progesterone in luteal phase): (nmol/L)*days. One anovulatory PA is omitted from the analysis of ovulation rate, luteal length, and AUC P.

^a p<0.02 vs control, both treatments combined

^b p<0.05 vs placebo, PA females only

^c p<0.004 vs control on placebo;

^d p<0.004 vs control on placebo;

^e p<0.03 vs control on pioglitazone;

^f p<0.004 vs control on placebo;

^g p<0.02 vs PA on placebo.

^h p<0.017 vs control on placebo;

ⁱ p<0.05 vs placebo in PA;

^j p<0.003 vs control females on placebo;

^k p<0.035 vs PA females on placebo.

l $p<0.003$ vs control females on placebo;
 m $p<0.017$ vs PA females on placebo;
 n $p<0.025$ vs control females on placebo;
 o $p<0.02$ vs placebo (C and Proresp PA females combined).