Fetal programming of adrenal androgen excess: lessons from a nonhuman primate model of polycystic ovary syndrome

David H Abbott[1,2,3], **Rao Zhou**[1], **Ian M Bird**[1,3], **Daniel A Dumesic**[2,4], and **Alan J Conley**[5]

[1]*Department of Ob/Gyn, University of Wisconsin, Madison, WI 53715, USA*

[2]*Wisconsin National Primate Research Center, University of Wisconsin, Madison, WI 53715, USA*

[3]*Endocrinology-Reproductive Physiology Training Program, University of Wisconsin, Madison, WI 53715, USA*

[4]*Reproductive Medicine and Infertility Associates, Woodbury, MN, 55125*

[5]*Population Health and Reproduction, School of Veterinary Medicine, University of California-Davis, Davis CA 95616, USA*

Abstract

Adrenal androgen excess is found in adult female rhesus monkeys previously exposed to androgen treatment during early gestation. In adulthood, such prenatally androgenized female monkeys exhibit elevated basal circulating levels of DHEAS, typical of PCOS women with adrenal androgen excess. Further androgen and glucocorticoid abnormalities in PA female monkeys are revealed by acute ACTH stimulation: DHEA, androstenedione and corticosterone responses are all elevated compared to responses in controls. Pioglitazone treatment, however, diminishes circulating DHEAS responses to ACTH in both prenatally androgenized and control female monkeys, while increasing the 17-hydroxyprogesterone response and reducing the DHEA to 17-hydroxyprogesterone ratio. Since 60-min post-ACTH serum values for 17-hydroxyprogesterone correlate negatively with basal serum insulin levels (all female monkeys on pioglitazone and placebo treatment combined), while similar DHEAS values correlate positively with basal serum insulin levels, circulating insulin levels may preferentially support adrenal androgen biosynthesis in both prenatally androgenized and control female rhesus monkeys. Overall, our findings suggest that differentiation of the monkey adrenal cortex in a hyperandrogenic fetal environment may permanently upregulate adult adrenal androgen biosynthesis through specific elevation of 17,20-lyase activity in the zona fasciculata-reticularis. As adult prenatally androgenized female rhesus monkeys closely emulate PCOS-like symptoms, excess fetal androgen programming may contribute to adult adrenal androgen excess in women with PCOS.

Keywords

fetal programming; androgen excess; zona reticularis; polycystic ovary syndrome

Introduction

In contrast to many mammals, the primate adrenal cortex is well endowed with androgen biosynthetic machinery located within two developmentally specific adrenocortical zones: the

Corresponding author: David H. Abbott, Ph.D., Department of Obstetrics and Gynecology, and National Primate Research Center, University of Wisconsin, 1223 Capitol Court, Madison, Wisconsin 53715, USA. Telephone: 608-263-3583, Fax: 608-263-3524, e-mail: abbott@primate.wisc.edu.

fetal zone and the zona reticularis (1,2). While the androgen-producing fetal zone develops during gestation and regresses shortly after birth, the androgenic zona reticularis, the innermost cortical zone, develops later in life and functionally matures at adrenarche (3-5). Both adrenocortical zones produce the adrenal androgen dehydroepiandrostrone (DHEA) and its sulfoconjugate DHEAS, while androstenedione is produced in relatively small amounts due to the reduced expression of 3beta-hydroxysteroid dehydrogenase II (3beta-HSD II; 2).

The androgenic predilection of the zona reticularis stems from the preferential conversion of 17-hydroxypregnenolone to DHEA (with onward sulfoconjugation to DHEAS), rather than to 17-hydroxyprogesterone (and onward conversion to cortisol), because of (1) diminished expression of 3beta-HSD II, together with relatively high expression of (2) the androgenic biosynthetic enzyme P450c17 (17alpha-hydroxylase/17,20-lyase), (3) the catalytic accessory protein, cytochrome b5, required for efficient 17,20-lyase activity, and (4) an obligate electron donor for P450c17 activity, NADPH cytochrome P450 oxido-reductase (1,2;Figure 1). Implicit in this steroidogenic activity is the fact that primates, in contrast to rodents, derive most of their androgen biosynthesis via the delta-5 pathway steroidogenic pathway (Figure 1) because 17-hydroxyprogesterone, as an inefficient substrate for primate 17,20-lyase, is negligibly converted to androstenedione (1,2).

Old World monkeys (i.e., rhesus macaques (*Macaca mulatta*) from the Indian subcontinent), Great Apes and humans exhibit analogous maturation of the androgenic adrenal zona reticularis (6), with all undergoing adrenarche, or increased adrenal DHEA and DHEAS secretion (2) coincident with greatly reduced expression of 3beta-HSD II within the zona reticularis. Monkeys differ from Great Apes and humans, however, with respect to the developmental timing of adrenarche. In contrast to progressive differentiation of the zona reticularis from neonatal to adolescent stages in Great Apes and humans (7,2), such progressive maturation of the zona reticularis in rhesus monkeys and baboons occurs during late fetal to neonatal life coincident with regression of the fetal zone (2,5). Rhesus monkeys, therefore, have close similarities to adrenal androgen physiology found in humans making them appropriate animal models for investigating pathophysiological mechanisms underlying adrenal androgen excess.

This chapter focuses on a potential fetal programming etiology for adrenal androgen excess in polycystic ovary syndrome (PCOS), a hyperandrogenic, infertility and metabolic disorder found in 6-7% of reproductive-aged women (8-10). PCOS accounts for 82% of women presenting with androgen excess (11) and between 20% and 30% of PCOS women exhibit adrenal androgen excess, manifest by elevated circulating levels of DHEA, DHEAS and androstenedione (12-15), as well as specific DHEA and androstenedione hyperandrogenic responses to ACTH (16). Moreover, DHEAS levels, are generally elevated in most women with PCOS, suggesting a prevalent adrenal contribution to this hyperandrogenic condition because of the unique adrenal origins of the conjugated steroid (14). While hyperandrogenic adrenal responses to ACTH in PCOS women are not necessarily accompanied by other remarkable adrenal abnormalities of the zona glomerulosa and zona fasciculata, or the hypothalamic-pituitary-adrenal axis (16,17), some PCOS studies suggest a generalized hypersecretion of adrenal steroids (15), or ACTH hyperresponsiveness to corticotropin releasing hormone (CRH) (18,19). The latter, however, may reflect diminished somatostatin release in PCOS women (20), particularly since somatostatin treatment reduces CRH-mediated ACTH release in PCOS women (21).

To explore a fetal programming origin for adrenal androgen excess in PCOS, adrenal androgen abnormalities were examined in adult female rhesus monkeys exposed *in utero* to androgen excess. These prenatally androgenized female rhesus monkeys closely mimic the reproductive, infertility and metabolic phenotypes found in PCOS women (22) and show a similar degree of adrenal androgen excess.

Prenatally androgenized female rhesus monkeys as models of adrenal androgen excess

Prenatally androgenized female rhesus monkeys are generated by exposure to fetal male levels of testosterone during early or late gestation. Their pregnant mothers receive subcutaneous injections of 10-15 mg testosterone propionate (TP) for up to 41 consecutive days, starting on gestation days 40-44 (early) or 100/110 (late) in approximately 165-day term pregnancies (23). Such experimentally induced androgen excess generates PCOS-like phenotypes when prenatally androgenized females reach adulthood (22). Early gestation exposed prenatally androgenized female monkeys show irregular or absent ovulatory menstrual cycles, ovarian hyperandrogenism, enlarged polyfollicular ovaries and luteinizing hormone (LH) hypersecretion, in addition to insulin resistance, diminished insulin secretion, increased incidence of type 2 diabetes, visceral adiposity and hyperlipidemia (22,24-27). Female monkeys similarly exposed to androgen excess during late gestation also exhibit an adult PCOS-like phenotype, but without obvious abnormalities in LH and insulin secretion, or in insulin action (22).

Both types of adult prenatally androgenized monkeys demonstrate ovarian androgen excess. For example, 24 hours after an intramuscular injection of 200 IU of recombinant human chorionic gonadotropin (rhCG), both early and late gestation exposed prenatally androgenized female monkey groups exhibit elevated circulating testosterone levels compared to controls (22). Early gestation exposed prenatally androgenized monkeys also show an elevated ovarian 17-hydroxyprogesterone response to rhCG (28) together with elevated basal testosterone levels (29,27). These females with more obvious signs of fetal programming of adult hyperandrogenism were thus studied to determine whether they had adrenal androgen excess comparable to that found in PCOS women.

Potential adrenal hyperandrogenism in infant prenatally androgenized monkeys exposed to fetal androgen excess during early gestation

In rhesus monkeys, early infancy coincides not only with regression of the androgen-producing fetal zone of the adrenal, but also with the simultaneous maturation of the androgenic zona reticularis (2,5). From at least birth to 1 month of age, prenatally androgenized female monkeys exhibit elevated circulating levels of androstenedione (DH Abbott, unpublished results), indicative of adrenal and/or ovarian androgen excess. Our preliminary findings suggest that adrenal androgen excess does contribute to this hyperandrogenism in infant prenatally androgenized females, given the elevated protein expression of adrenal P450 oxido-reductase and possibly cytochrome b5 (AJ Conley, DH Abbott, unpublished results). Interestingly, infant male rhesus monkeys exhibit an adrenal-dependent rise in circulating testosterone levels between 4-25 weeks of age (30) so that exposure to fetal androgen excess (fetal male or prenatally androgenized female) during early gestation permanently may enhance adrenal androgen biosynthesis following birth.

Adrenal hyperandrogenism in adult prenatally androgenized monkeys exposed to fetal androgen excess during early gestation

As a sign of functional adrenal hyperandrogenism, prenatally androgenized female monkeys exposed to androgen excess during early gestation exhibit elevated basal circulating levels of DHEA (31) and DHEAS (28). Elevated basal DHEAS levels are a primary indicator of adrenal androgen excess in women with PCOS (15,32) suggesting that prenatally androgenized monkeys may closely emulate their PCOS human counterparts in this adrenal abnormality. To examine prenatally androgenized monkey adrenal steroid dysfunction in more detail, we utilize

acute ACTH stimulation (at 0730h-0800h) administered 14.5-16 hours after dexamethasone treatment (Dex-ACTH) on days 2-6 of the menstrual cycle or during a 30-day anovulatory interval. We employ a high intravenous dose of ACTH (50μg; ~5.5μg/kg of human $ACTH_{1-39}$) to saturate the preferred P450c17 delta-5 pathway and to reveal any minor shifts in steroidogenesis from 17-hydroxypregnenolone to progesterone, via increased 3beta-HSD II activity (31;Figure 1). Such ACTH-mediated adrenocortical stimulation further illustrates the functional adrenal hyperandrogenism of prenatally androgenized female monkeys, with elevated serum DHEA and androstenedione responses to ACTH (Figure 2;31) accompanied by normal serum 17-hydroxyprogesterone and cortisol responses to ACTH. Note that the latter result suggests an additional, but normal, zona reticularis origin of circulating 17-hydroxyprogesterone, since P450c21 in the zona fasciculata would rapidly metabolize this product to deoxycortisol (Figure 1).

In these prenatally androgenized female monkeys, the relatively greater increase in serum DHEA compared to serum 17-hydroxyprogesterone, androstenedione and DHEAS in response to Dex-ACTH causes (1) an increased ratio of serum DHEA to 17-hydroxyprogesterone, and (2) diminished ratios of serum DHEAS to DHEA, and serum androstenedione to DHEA (31). Such adrenal androgen excess in early gestation exposed prenatally androgenized female monkeys corresponds with selectively increased 17,20-lyase activity in the zona fasciculata (elevated DHEA and androstenedione levels) as well as in the zona reticularis (elevated DHEA levels). This enhanced efficiency of 17,20-lyase activity in the prenatally androgenized monkey adrenal, however, occurs without enhanced 17alpha-hydroxylase activity, since basal and ACTH-induced levels of 17-hydroxyprogesterone (from the zona reticularis) and cortisol (from the zona fasciculata) are normal. A putative molecular basis for an isolated lyase activity increase in prenatally androgenized monkey adrenals could be increased serine phosphorylation of P450c17 (31) possibly facilitated by an increase of cytochrome b5 or P450 oxido-reductase, or most likely in view of the involvement of insulin sensitivity, a combination of all three. Additionally, conversion of excessive DHEA to DHEAS, via SULT2A1 sulfotransferase in the zona reticularis, and to androstenedione via 3beta-HSD II in the zona fasciculata (and to a greatly reduced extent in the zona reticularis; Figure 1), could provide one explanation for the elevated levels of these latter two androgenic adrenal steroids in the absence of an altered cortisol response.

To account for the increased corticosterone response to ACTH combined with adrenal hyperandrogenism in prenatally androgenized female monkeys, however, additional enzymatic changes in adrenal steroid biosynthesis and metabolism must exist beyond enhanced 17,20-lyase in the zona fasciculata and zona reticularis. While pregnenolone is the preferred substrate for cortisol biosynthesis in primates, a small amount of progesterone byproduct is the basis for corticosterone biosynthesis by the zona fasciculate (Figure 1, 2,6,34,35). The corticosterone response is produced primarily in the primate zona fasciculata (2,6), and is elevated following ACTH stimulation in prenatally androgenized females, so we must assume 3beta-HSD II activity is elevated in the zona fasciculata of these monkeys. This hypothesis agrees with a 3-4% decrease in the efficiency of cortisol biosynthesis in the zona fasciculata (derived from the increased ratio of corticosterone to cortisol at 60 min after ACTH administration; 34) of prenatally androgenized female monkeys, as well as an ~14-fold increase in corticosterone versus an ~8-fold increase in cortisol following ACTH administration (31). While enhanced 3beta-HSD II activity exists in some PCOS women with adrenal androgen excess (36), it is unclear whether this phenomenon translates into corticosterone hyperresponsiveness to ACTH in humans.

Insulin is associated with adrenal androgen excess in prenatally androgenized female monkeys

As in PCOS (37), insulin is associated with the pathophysiological mechanism of reproductive and endocrine abnormalities in prenatally androgenized female rhesus monkeys, as evidenced by a 6-month study of pioglitazone treatment, a thiazolidinedione-based insulin sensitizer. Pioglitazone treatment not only diminishes insulin resistance and reduces circulating insulin levels, but also normalizes menstrual cyclicity and diminishes ovarian hyperandrogenism in most prenatally androgenized female monkeys (27), similar to its effects in PCOS women (38,39).

Similar to its effects in PCOS women (40,41), pioglitazone treatment of prenatally androgenized female monkeys diminishes aspects of adrenal androgen excess. Serum DHEAS responses to ACTH are reduced in both prenatally androgenized and control female monkeys (Table 1), analogous to findings of reduced basal serum DHEAS levels in PCOS women undergoing treatment with troglitazone (42). In prenatally androgenized female monkeys alone, pioglitazone normalizes ($p<0.02$) the serum DHEA to 17-hydroxyprogesterone ratio at 60 min following ACTH administration (prenatally androgenized, placebo: 22.0 [16.9, 28.7] ng/ml, pioglitazone: 8.1 [6.1, 10.8]; control, placebo: 7.3 [1.9, 28.8], pioglitazone: 7.7 [2.0, 26.7]; backtransformed logarthmic mean [95% confidence interval]). Serum DHEA responses to ACTH, however, remain elevated at their previously reported values (compare Table 1 to Figure 2) in prenatally androgenized compared to control female monkeys regardless of whether they receive pioglitazone or placebo treatment.

Together, these pioglitazone-mediated changes in adrenal steroidogenesis predominantly implicate a specific reduction in 17,20-lyase activity following the pioglitazone-mediated decline in circulating insulin levels. Such a notion is consistent with the negative correlation between 60-min post-ACTH 17-hydroxyprogesterone levels and basal insulin (Figure 3a), the positive correlation between 60-min post-ACTH DHEAS levels and basal insulin (Figure 3b) and the overall negative correlation between 0-min pre-ACTH 17-hydroxyprogesterone and DHEAS levels (Figure 3c), in all female monkeys combined. Decreased serine phosphorylation of P450c17 (33, 43), decreased cytochrome b5 activity (44, 43), decreased concentrations of the electron-donating redox partner for 17,20-lyase, P450 oxido-reductase (45, 46), or a combination of all three may provide a molecular basis for the relatively diminished 17,20-lyase activity observed in pioglitazone-treated prenatally androgenized female monkeys. It is unlikely that such diminished adrenal androgen excess is a direct result of pioglitazone action. Pioglitazone, at therapeutic doses, increases P450 oxido-reductase expression (47, 48), increases melanocortin 2 (ACTH) receptor expression and cortisol responses to ACTH (49), and downregulates P450c17 and 3betaHSD expression (50).

The finding of pioglitazone-mediated decrease in 17,20-lyase activity agrees with compensatory hyperinsulinemia from insulin resistance playing a causal role in adrenal hyperandrogenism in PCOS (51,52), and with insulin-enhanced adrenal androgen responses to ACTH in PCOS (53). Moreover, if enhanced 17,20-lyase activity extends into the adrenal zona fasciculata, it can also contribute to proportionately increased DHEA biosynthesis, perhaps even at the expense of 17α-hydroxyprogesterone and cortisol production, as found in the highly inbred Angora goat (54). Such an expansive function for 17,20-lyase in the zona fasciculata might also contribute towards the lack of concomitant increase in 17α-hydroxyprogesterone and cortisol (Figure 1) following ACTH in prenatally androgenized versus control female monkeys.

Insulin stimulation of P450c17 in the adrenal cortex of PCOS women, if similar to its action in theca cells from polycystic ovaries, acts through the PI3-kinase branch of the insulin

signaling pathway, to synergize with additional co-activation by cAMP/kinase A (55). Such insulin overstimulation of P450c17 under circumstances of insulin resistance at the level of glucose metabolism, would suggest that steroidogenesis and glucose metabolism are regulated by alternate signaling events distal to PI-3 kinase activation (55), and would explain the absence of insulin resistance in terms of steroiodgenic enzyme regulation in the ovary (56) as well as the adrenal.

Potential fetal programming origins of adrenal androgen excess

In the adrenal cortex, androgens are implicated in the regulation of their own biosynthesis. For example, adrenocortical cells express androgen receptors (57,58), flutamide (an androgen receptor antagonist) diminishes adrenal androgen biosynthesis in PCOS women (59,60), while testosterone can directly affect adrenal androgen biosynthesis (61). Taken together, a hyperandrogenic environment during fetal life may influence adrenal cortical differentiation in a manner that enhances fetal zone androgen biosynthesis.

Alternatively, insulin appears to upregulate androgen biosynthesis in the adrenal cortex of normal (61) and PCOS women (53), and in the polycystic ovary (36). Since both fetal (29) and infant (62) prenatally androgenized female rhesus monkeys exhibit enhanced insulin secretion, the fetal zone and infant zona reticularis may be susceptible to increased insulin-mediated serine phosphorylation of P450c17, thus preferentially enhancing 17,20-lyase activity by increasing the affinity of P450c17 for its electron donor, P450 oxido-reductase (33,43). Therefore, fetal androgen excess may induce relative insulin hypersecretion in exposed female fetuses and infants, which in turn programs adrenal hyperandrogenism.

Conclusions and therapeutic considerations

Androgen excess, during early to mid-gestation in female rhesus monkeys, causes a remarkably close phenocopy of adrenal hyperandrogenism found in 20-30% of PCOS women. The ability to experimentally induce adrenal androgenism by fetal programming further suggests that specific changes in adrenal gene expression, perhaps from epigenetic alterations of gene methylation (63,64), may contribute to adrenal androgen excess beyond genotypic determination (65). While the molecular mechanisms underlying adrenal pathophysiology in PCOS women and prenatally androgenized female monkeys are incompletely understood, isolated enhancement of adrenocortical 17,20-lyase activity appears to be a common attribute, as evidenced by the elevated expression of P450 oxido-reductase, and possibly cytochrome b5, found in the adrenal cortex of our prenatally androgenized female monkeys (AJ Conley and DH Abbott, unpublished results). It remains to be determined whether such molecular underpinnings of adrenal androgen excess are induced by the direct actions of testosterone, its androgenic or estrogenic metabolites, or by the hyperandrogenic induction of fetal and infant insulin hypersecretion. Regardless, our findings of fetal programming of adrenal androgen excess in a nonhuman primate model for PCOS suggest that such hyperandrogenism may be a consequence of fetal environmental influences rather than a primary, intrinsic defect.

Therapeutic use of low-dose flutamide reliably diminishes adrenal androgen excess in PCOS women (66). Unfortunately, the use of flutamide to investigate possible direct effects of androgen excess in the fetal female environment on adrenal androgen biosynthesis is difficult due to its permanent effects on somatosensory and cognitive function in rhesus monkeys exposed during fetal development (67,68). On the other hand, insulin sensitizers alone (PCOS women: 40,41; prenatally androgenized monkeys: 27) or in combination with flutamide (69), also ameliorate adrenal androgen excess in both PCOS women and adult female prenatally androgenized monkeys, without inducing hepatoxicity. Therefore, non-teratogenic insulin sensitizers such as metformin (70), in addition to their potential benefits in reducing pregnancy

complications in PCOS (71), may prove useful in exploring how androgen-induced fetal hyperinsulinism programs adrenal hyperandrogenism. Future studies are required to determine whether insulin sensitizer treatment of pregnant PCOS women, or pregnant TP-treated monkeys, will prevent postnatal adrenal hyperandrogenism and other phenotypic characteristics of PCOS in their daughters and confirm a specific programming mechanism.

Acknowledgements

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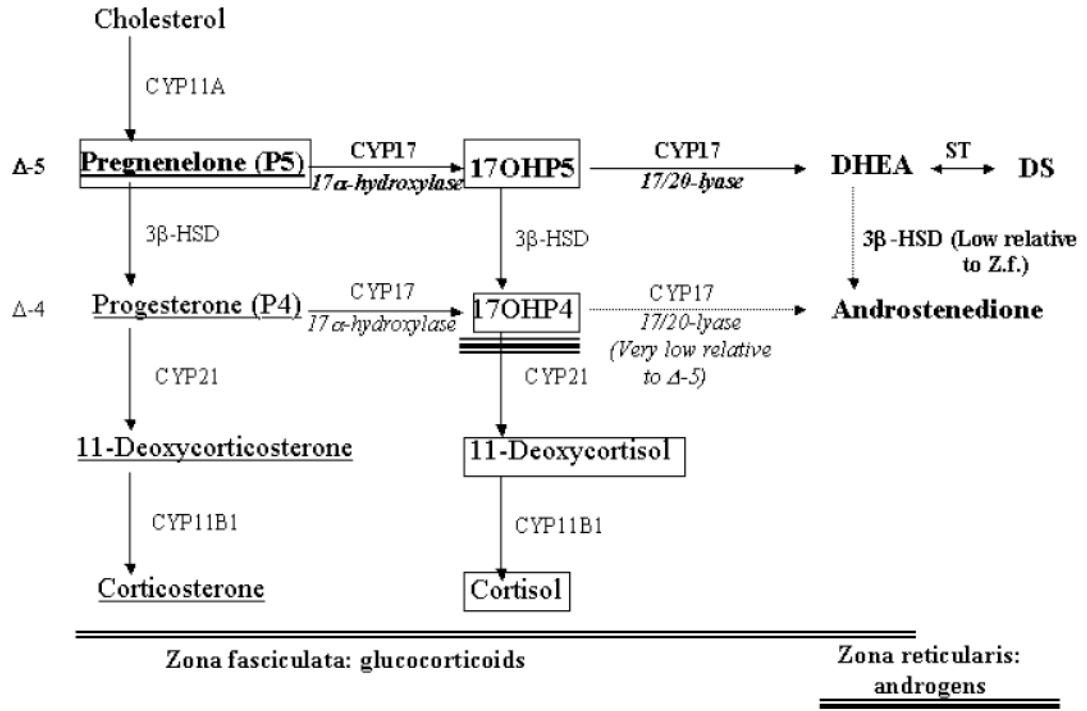
Figure 1.
Steroid biosynthesis in the mid- to inner adrenal cortical zones, zona fasciculata and zona reticularis, of Old World primates and humans. Steroids in bold represent the predominant pathway for androgen biosynthesis, steroids within boxes represent the predominant pathway for cortisol biosynthesis, and underlined steroids represent the predominant pathway for corticosterone biosynthesis. The larger arrows reflect the proposed enhanced 3β-HSD II and 17,20 lyase enzymatic function in PA female monkeys, while the lighter arrows reflect relative low enzymatic activity in both control and PA females. Double underlining reflects the extent of zona fasciculata enzymatic activity, while triple underlining reflects the extent of zona reticularis enzymatic activity. 17OHP5: 17α-hydroxypregnenelone; 17OHP4: 17α-hydroxyprogesterone; CYP11A: P450scc, CYP 17: P450c17, ST: SULT2A1 sulfotransferase; 3β-HSD II: 3β-hydroxysteroid dehydrogenase II, CYP21: P450c21, CY11B1: P450c11. Modified from (31).
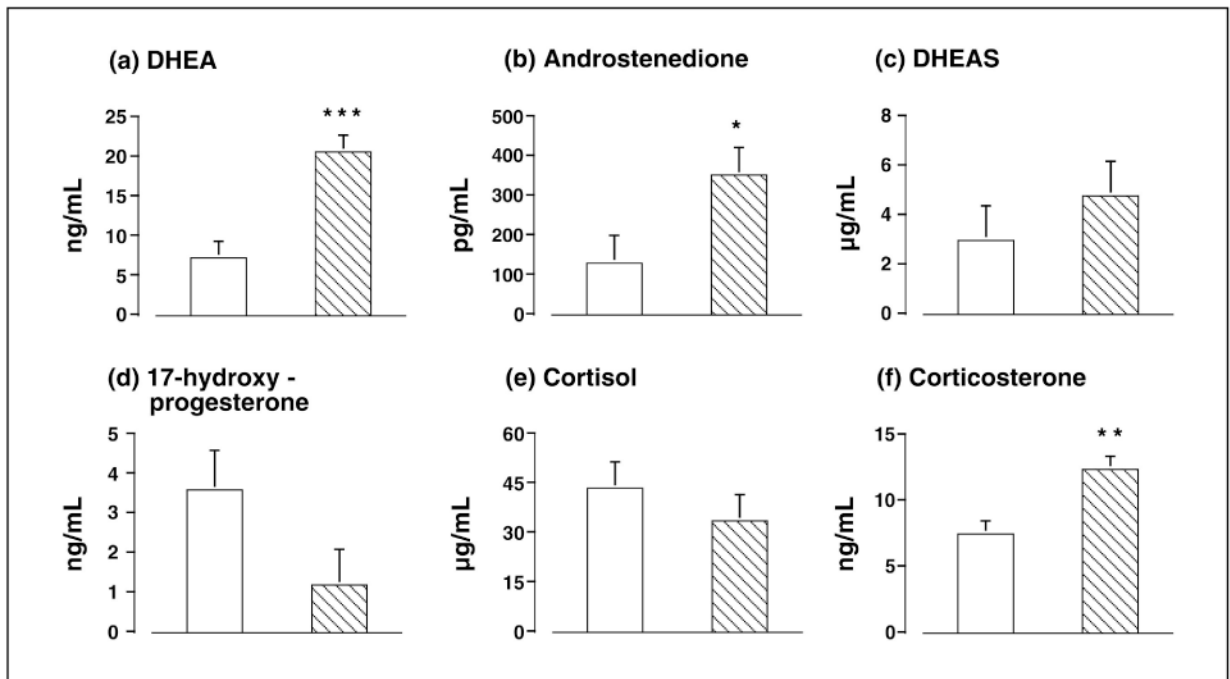
Figure 2.
The differential in circulating adrenal steroid values between 0 and 60 min following an intravenous injection of 50μg ACTH in adult control (open bars) and prenatally androgenized (hatched bars) female rhesus monkeys for adrenal androgen-related steroids (a) DHEA, (b) androstenedione, and (c) DHEAS, as well as adrenal glucocortoicoid-related steroids (d) 17-hydroxyprogesterone, (e) cortisol, and (f) corticosterone. Modified from (31).
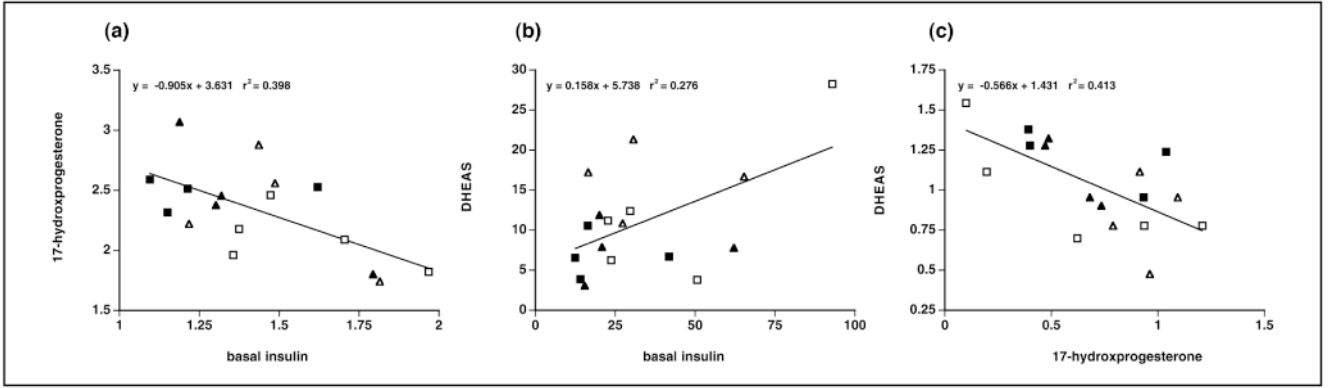
Figure 3.
Associations between serum values of (a) 17-hydroxyprogesterone at 60 minutes following an intravenous injection of 50μg ACTH and basal insulin, (b) DHEAS at 60 minutes following an intravenous injection of 50μg ACTH and basal insulin, and (c) DHEAS and 17-hydroxyprogesterone at 0 minutes following an intravenous injection of 50μg ACTH in control (open triangle: receiving placebo treatment (27), solid triangle: receiving pioglitazone treatment; 27) and PA (open square: placebo, solid square: pioglitazone) female monkeys. Data are expressed as $\log_{10}$ transformed serum values only in (a) and (c). Units used: (a) 17-hydroxyprogesterone, $\log_{10}$ ng/ml; insulin, $\log_{10}$ μU/ml; (b) DHEAS, μg/dl; insulin, μU/ml; and (c) DHEAS, $\log_{10}$ μg/dl; 17-hydroxyprogesterone, $\log_{10}$ ng/ml.

Table 1

Differences in mean±SEM circulating adrenal steroid values between 0 and 60 minutes following an intravenous injection of 50μg ACTH in the combined group of control and prenatally androgenized female rhesus monkeys receiving placebo or pioglitazone treatment (27).

Steroid	0-60 min placebo	0-60 min pioglitazone	p-value (<)
17α-hydroxyprogesterone (ng/ml)	2.3±1.0	3.6±1.0	NS
Cortisol (μg/dl)	34.9±3.8	36.7±3.9	NS
DHEA (ng/ml)	16.7±1.7	17.9±1.8	NS[#]
DHEAS (μg/dl)	6.9±1.5	2.2±1.6	0.05
Androstenedione (pg/ml)	213±52	345±78	NS
Corticosterone (ng/ml)	11.1±1.2	10.8±1.1	NS

[#] The difference in serum DHEA levels between 0 and 60 minutes post-ACTH injection for prenatally androgenized female monkeys exceeds that in control females, regardless of placebo or pioglitazone treatment (treatment values combined: prenatally androgenized, 20.0±1.8 ng/ml; controls, 14.5±1.8 ng/ml; $p<0.05$). NS= $p>0.05$