Regulation of Baboon Fetal Pituitary Prolactin Expression by Estrogen¹

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ABSTRACT

We previously showed that fetal adrenal fetal zone growth was increased and the number of follicles in the fetal ovary reduced in baboons in which estradiol was suppressed by treatment with the aromatase inhibitor letrozole between mid and late gestation periods. Because adrenal/ovarian development was restored in animals treated with letrozole and estradiol, and both tissues express estrogen receptor, we proposed that estrogen regulates fetal adrenal/ovary development via a direct action. However, because prolactin can modulate fetal adrenal and adult pituitary/ovarian function, the current study determined whether estrogen action involved estradiol-regulated changes in fetal prolactin/luteinizing hormone (LH) expression. Fetal prolactin levels and the number of prolactin-positive fetal pituitary cells (per 0.37 mm²) were increased ($P < 0.01$) between mid (6 ± 1 ng/ml; 15.8 ± 2.4) and late (257 ± 28 ng/ml; 57.3 ± 5.1) gestation, reduced ($P < 0.01$) in late-gestation fetuses in which estradiol was suppressed ($>95\%$) by letrozole (61 ± 11 ng/ml; 19.3 ± 2.0), and minimally but not significantly increased by letrozole and estradiol (99 ± 11 ng/ml; 32.7 ± 5.2). In contrast, the number of LH-positive fetal pituitary cells decreased ($P < 0.01$) between mid (48.8 ± 9.5) and late (17.4 ± 3.2) gestation, remained elevated ($P < 0.01$) in estrogen-suppressed animals (56.6 ± 5.1), and was partially but not significantly decreased by letrozole-estradiol (28.8 ± 5.2). We conclude that estrogen regulates fetal pituitary prolactin and LH expression and fetal prolactin levels. However, because prolactin and LH expressions in estrogen-suppressed fetuses were inversely related to previously demonstrated changes in adrenal/ovarian development, we propose that estrogen regulates the fetal ovary and adrenal gland directly and not via action on the fetal pituitary gland.

baboon, estradiol, estrogen, fetus, pituitary, prolactin

INTRODUCTION

Using the baboon as an experimental model for studies of human reproductive biology, our laboratories have shown that estrogen plays a central role in regulating placental and fetal development [1–3]. Thus, the increase in estrogen production in

late gestation enhanced the placental 11 β -hydroxysteroid dehydrogenase enzyme system, catalyzing conversion of cortisol to cortisone, which decreased the amount of maternal cortisol transported to the fetus. This change activated the fetal pituitary-adrenocortical axis and the ontogenesis of and cortisol production by transitional-zone cells of the fetal adrenal gland [4–6]. Estrogen also acted to restrain growth of the fetal zone, the site of androgen synthesis. Thus, in baboons in which estrogen production during the second half of gestation was suppressed by daily administration of the aromatase inhibitor letrozole, the volume of the fetal zone of the fetal adrenal gland and production of dehydroepiandrosterone sulfate were markedly increased [7]. Estrogen also acts to regulate the development of primordial follicles in the baboon fetal ovary [8]. Thus, in estrogen-suppressed baboons, the number of follicles formed by late gestation was reduced by 50% and restored to normal by concomitant treatment with letrozole and estradiol [9].

It is unlikely that the alteration in fetal ovarian and adrenal development in estrogen-suppressed animals reflects a change in pituitary trophic support. Thus, we showed that fetal serum gonadotropin levels were inversely correlated with follicle development, and thus were higher at mid than at late gestation. Moreover, gonadotropin levels remained elevated in late gestation in estrogen-suppressed baboons and decreased in animals treated with letrozole and estradiol [10]. In addition, mRNA levels of fetal pituitary ACTH precursor proopiomelanocortin were not significantly altered in late gestation in estrogen-suppressed fetuses [7]. Therefore, because the baboon fetal adrenal gland and ovary express estrogen receptor α (ER α ; ESR1) and ER β (ESR2) in a cell zone-specific manner [11–13], we have proposed that estrogen regulates fetal adrenal cellular development and fetal ovarian folliculogenesis, processes critical for adrenocortical and reproductive function in adulthood [8, 14], by exerting cell-specific direct actions.

It is well established, however, that prolactin, the fetal serum levels of which increase with advancing gestation in the human [15, 16] and nonhuman primate [17], can have an impact on adult ovarian function directly and/or indirectly via inhibition of pituitary gonadotropins [18]. Moreover, there are several studies showing that prolactin regulates aspects of fetal adrenal development, including growth [15] and androgen production by the fetal zone [19, 20]. Although it is well established that estrogen regulates prolactin in the adult [18, 21], whether estrogen exhibits a comparable role on the fetal pituitary gland is equivocal. For example, fetal prolactin levels were not decreased in women in whom estrogen levels were reduced by treatment with synthetic corticosteroid [22]. Therefore, in the current study we determined whether fetal serum levels and fetal pituitary expression of prolactin, as well as fetal pituitary expression of luteinizing hormone (LH), were regulated by estrogen, and thus might account for the previously demonstrated alterations in fetal adrenal and ovarian development elicited in estrogen-suppressed baboons.

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FIG. 1. Mean ($\pm$ SEM) serum levels of estradiol in maternal uterine (A) and umbilical (B) venous blood samples obtained on Days 95–120 (mid) and Days 162–175 (late) of gestation in untreated baboons, and on Days 162–175 of gestation in animals treated with letrozole (115 μ g/kg body weight per day) or letrozole and estradiol benzoate (115 μ g/kg body weight per day each) administered subcutaneously to the mother beginning on Day 100 (term = Day 184). Values ($n = 9$ –32 per group) with different lowercase letters differ from each other at $P < 0.01$ (ANOVA and Student-Newman-Keuls multiple-comparison statistic).

MATERIALS AND METHODS

Animals

Female baboons (*Papio anubis*) weighing 10–15 kg were housed individually in metabolic cages in air-conditioned quarters under standardized conditions as described previously [23]. Females were paired with males for 5 days at the anticipated time of ovulation as estimated by menstrual cycle history and turgescence of the external sex skin, and pregnancy was confirmed by ultrasound. Baboons were used strictly in accordance with U.S. Department of Agriculture regulations and the National Institutes of Health Guide for the Care and Use of Laboratory Animals (publication 85–23, 1985). The experimental protocol employed was approved by the Institutional Animal Care and Use Committees of Eastern Virginia Medical School and the University of Maryland School of Medicine.

Baboons were either untreated and studied on Days 95–120 ($n = 13$; 8 with female fetus; $n = 5$ with male fetus) or on Days 162–175 ($n = 33$; 15 female and 18 male) of gestation (term = Day 184) or were treated beginning on Day 100 of gestation and continuing to the day of delivery with the aromatase inhibitor letrozole (CGS 20267; 4,4-[1,2,3-triazol-1-yl-methylene]bis-benzonitrile; Novartis Pharma AG, Basel, Switzerland) administered subcutaneously in sesame oil to the mother (115 μ g/kg body weight per day; $n = 25$; 12 female and 13 male) or with letrozole and estradiol benzoate (each at 115 μ g/kg body weight per day; $n = 19$; 6 female and 13 male), essentially as described previously [24]. At 1- to 3-day intervals between Days 80 and 175 of gestation, baboons were sedated with ketamine-HCl (10 mg/kg BW; Parke Davis, Detroit, MI), and a maternal saphenous blood sample (4–7 ml) was collected.

On Days 95–120 or 162–175 of gestation, all baboons (i.e., untreated or treated with letrozole with or without estradiol) were sedated with ketamine and anesthetized with isoflurane, and blood samples were obtained from maternal saphenous and uterine veins and from the umbilical artery and vein. After collecting a sample of amniotic fluid, the fetus and placenta were delivered, and the fetus was killed with an overdose of pentobarbital. The fetal pituitary gland was isolated, weighed, placed in sterile $15 \times 15 \times 5$ mm Tissue Tex II Cryomolds (Miles Scientific, Elkhart, IN) containing OCT Embedding Medium (Miles Scientific), frozen on dry ice, wrapped in parafilm, and stored at -80°C .

Assay of Estradiol and Prolactin

The levels of estradiol and prolactin in serum and of prolactin in amniotic fluid samples were determined by radioimmunoassay using an automated chemiluminescent Immulite procedure (Diagnostic Products Corp., Los Angeles, CA) previously described for analysis of estradiol [24]. The antibody used for determination of prolactin was highly specific and exhibited minimal cross-reactivity with other pituitary or placental peptides, and samples assayed at different dilutions exhibited linearity. The interassay and intraassay coefficients of variation were $<15\%$. The sensitivity of the assay was 15 pg/ml for estradiol

and 0.5 ng/ml for prolactin. Finally, we previously showed that serum levels of prolactin remained low and relatively unchanged over a 2-h period in nonpregnant baboons sedated with ketamine or anesthetized with halothane [25].

Immunocytochemistry

Fetal pituitary expression of prolactin and LH was determined by peroxidase immunocytochemistry using rabbit polyclonal antibodies to human prolactin and human LH and Avidin-Biotin Cytochemicals provided by the manufacturer (Biomedica Corp., Foster City, CA) and following procedures described previously [5]. Briefly, frozen fetal pituitary glands were positioned in a Leica Reichart-Jung (Bethesda, MD) precision microtome cryostat (-15°C), sectioned (7 μ m), and mounted onto Superfrost Plus (Fisher Scientific, Arlington, VA) microscope slides, which were sequentially numbered, placed in slide boxes, and stored at -80°C . Fetal pituitary sections ($n = 10$ –12 per animal) in three to seven animals in each of the experimental groups were brought to room temperature (5 min), fixed for 5 min in Zamboni medium, delipidated in methanol containing 2% H_2O_2 (5 min), blocked with 5% normal goat serum in PBS (60 min), rinsed twice in PBS (3 min), and treated with preimmune serum (control) or primary antibody at concentrations recommended by the manufacturer. After incubation at room temperature for 60 min, sections were rinsed twice with PBS and incubated for 30 min with biotinylated anti-rabbit secondary antibody. After washing with PBS, sections were treated (30 min) with streptavidin peroxidase reagent and washed and incubated (3 min) with chromogen substrate solution. After two washes with distilled water, sections were counterstained with hematoxylin, rinsed with distilled water, cover slipped, and baked for 10 min (80°C) for permanent preservation of immunostained slides.

To quantify the number of anterior pituitary cells expressing prolactin or LH, brightfield images were captured from immunostains using an Olympus DP70 digital camera attached to an Olympus BX41 microscope (Optical Elements Corp., Melville, NY) and were analyzed for diaminobenzidine chromogen-positive staining using Metamorph image analysis (Molecular Devices Corp., Downingtown, PA). Using a grid encompassing the entire anterior pituitary of each immune-stained section (three to four sections per animal), every third sequential area ($223 \times 167 \mu\text{m}$; 8–20 areas per section) comprising approximately 350–600 pituitary cells was analyzed. The number of prolactin- or LH-positive cells was calculated as the percent of the pituitary area expressing prolactin or LH times the number of pituitary cell nuclei per area.

Statistics

Animals were studied only at mid or late gestation, and thus differences in hormone concentrations and pituitary prolactin expression (mean $\pm$ SEM) between treatment groups were analyzed by ANOVA, with posthoc comparison of the means using the Newman-Keuls statistic. Estradiol levels

TABLE 1. Mean ($\pm$ SEM) fetal, placental, and pituitary weights at mid (Days 95–120) and late (Days 162–175) gestation in untreated baboons and at late gestation in baboons treated as described in the legend to Figure 1.

Treatment	Gestation stage	Fetal body weight (g)	Pituitary weight (mg)	Placental weight (mg)
None	Mid	228 $\pm$ 14 ^a	10 $\pm$ 1 ^a	92 $\pm$ 5 ^a
None	Late	822 $\pm$ 20 ^b	25 $\pm$ 1 ^b	183 $\pm$ 7 ^b
Letrozole	Late	919 $\pm$ 19 ^b	27 $\pm$ 2 ^b	241 $\pm$ 9 ^c
Letrozole + estradiol	Late	808 $\pm$ 35 ^b	25 $\pm$ 2 ^b	178 $\pm$ 10 ^b

^{a,b,c} Values ($n = 13$ –33/group) with different superscript letters differ from each other at $P < 0.01$.

FIG. 2. Mean ($\pm$ SEM) serum levels of prolactin in maternal saphenous (A) and umbilical arterial (B) blood samples obtained on Days 95–120 (mid) and Days 162–175 (late) of gestation in untreated baboons, and on Days 162–175 of gestation in animals treated with letrozole or letrozole and estradiol administered subcutaneously to the mother beginning on Day 100 (term = Day 184), as outlined in the legend to Figure 1. Values ($n = 9$ –32 per group) with different lowercase letters differ from each other at $P < 0.01$ (ANOVA and Student-Newman-Keuls multiple-comparison statistic).

in umbilical vein were log-transformed prior to analysis to satisfy ANOVA assumptions. Comparisons of hormone levels in umbilical artery and umbilical vein or in male and female fetuses were performed using dependent or independent Student *t*-tests, respectively.

RESULTS

Serum Estradiol Levels and Body Weights

As demonstrated previously [24], maternal peripheral serum estradiol levels increased from approximately 1 ng/ml on Days 85–120 of gestation to 2.50–3.50 ng/ml by Day 165 (data not shown). Within 48–72 h of administration of letrozole beginning on Day 100, maternal estradiol levels declined to 100–150 pg/ml and remained suppressed through Day 165. In baboons treated with letrozole and estrogen, levels of estradiol were restored to normal (data not shown). Maternal peripheral serum estradiol levels probably reflect placental secretion. Thus, mean ($\pm$ SEM) estradiol levels in uterine vein (Fig. 1A) were approximately 70% greater ($P < 0.05$) on Day 165 (3.50 ± 0.28 ng/ml) than on Day 100 (2.05 ± 0.13 ng/ml). Moreover, uterine venous estradiol levels were reduced by approximately 85% ($P < 0.05$) on Day 165 in animals treated with letrozole (0.50 ± 0.05 ng/ml) and increased ($P < 0.05$) with treatment with letrozole and estrogen to a level (1.88 ± 0.29 ng/ml) comparable to that at mid gestation (Fig. 1A). As shown in Figure 1B, placental estrogen secretion into the fetus, and thus estradiol levels in umbilical vein, were also increased ($P < 0.05$) approximately 3-fold between mid (0.22 ± 0.08 ng/ml) and late (0.83 ± 0.08 ng/ml) gestation, reduced ($P < 0.05$) by treatment with letrozole (0.05 ± 0.01 ng/ml), but not significantly changed by letrozole and estradiol (0.07 ± 0.02 ng/ml). Similar changes in umbilical arterial estradiol were noted, although mean levels at mid (0.11 ± 0.06 ng/ml) and late (0.52 ± 0.13 ng/ml) gestation in untreated baboons and in late gestation following treatment with letrozole (0.04 ± 0.01) or letrozole and estradiol benzoate (0.04 ± 0.1) were lower ($P < 0.05$) than respective values in umbilical vein, presumably reflecting fetal metabolism of estrogen.

TABLE 2. Mean ($\pm$ SEM) prolactin levels (ng/ml) in umbilical arterial blood samples of male and female fetuses at mid (Days 95–120) and late (Days 162–175) gestation in untreated baboons and at late gestation in baboons treated as described in the legend to Figure 1.

Treatment	Gestation stage	Male	Female
None	Mid	4 ± 1	8 ± 2
None	Late	302 ± 49	204 ± 17
Letrozole	Late	74 ± 25	56 ± 8
Letrozole + estradiol	Late	108 ± 12	74 ± 19

Fetal body and pituitary weights, as well as wet weight of the placenta, increased ($P < 0.01$) 3- to 4-fold between Days 100 and 165 of gestation (Table 1). Treatment with letrozole and/or estrogen had no effect on fetal body or pituitary weight; however, placental weight in estrogen-suppressed animals was 35% greater ($P < 0.01$) than that in untreated baboons and was restored to normal by treatment with letrozole and estradiol.

Serum Prolactin Levels

Maternal peripheral and umbilical arterial levels of prolactin are shown in Figure 2. Because umbilical arterial prolactin levels in male and female fetuses were not significantly different (Table 2), values in respective treatment groups were combined for statistical purposes (Fig. 2B). Prolactin levels in maternal saphenous serum (Fig. 2A) were similar at mid (337 ± 62 ng/ml) and late (391 ± 40 ng/ml) gestation, reduced ($P < 0.01$) by >76% in late gestation in animals in which placental estrogen was suppressed by letrozole (77 ± 18 ng/ml), and restored ($P < 0.01$) to normal in animals treated with letrozole and estradiol (387 ± 62 ng/ml). Prolactin concentrations in the fetus were 40-fold greater ($P < 0.01$) at Day 165 (257 ± 28 ng/ml) than at Day 100 (6 ± 1 ng/ml; Fig. 2B). In baboon fetuses in which placental estrogen was suppressed by letrozole, levels of prolactin at Day 165 in the umbilical artery (61 ± 11 ng/ml) were approximately 80% lower ($P < 0.01$) than in untreated animals. Treatment with letrozole and estradiol increased fetal prolactin to a mean level (99 ± 11 ng/ml) that was not significantly different from that in animals treated with letrozole and was lower ($P < 0.01$) than that in untreated baboons. Levels of prolactin in umbilical venous samples were similar to values in umbilical artery, and thus were also increased ($P < 0.05$) between mid (5 ± 1 ng/ml) and late (244 ± 24 ng/ml) gestation, decreased ($P < 0.05$) by 70% in late gestation in animals treated with

TABLE 3. Mean ($\pm$ SEM) prolactin levels (ng/ml) in amniotic fluid at mid (Days 95–120) and late (Days 162–175) gestation in untreated baboons and at late gestation in baboons treated as described in the legend to Figure 1.

Treatment	Gestation stage	Prolactin
None	Mid	72 ± 12^a
None	Late	274 ± 26^b
Letrozole	Late	346 ± 36^b
Letrozole + estradiol	Late	340 ± 56^b

^{a,b} Values ($n = 9$ –30/group) with different superscript letters differ from each other at $P < 0.01$.

FIG. 3. Representative photomicrographs of prolactin immunocytochemical expression in fetal anterior pituitary on (A) Days 95–120 ($n = 5$; 3 female and 2 male) and (B) Days 162–175 ($n = 7$; 5 female and 2 male) of gestation in untreated baboons and on Days 162–175 in animals treated with (C) letrozole ($n = 5$; 3 female and 2 male) or (D) letrozole and estradiol ($n = 4$; 2 female and 2 male), as described in the legend to Figure 1. Frozen sections (10–12 per animal) were incubated with an antibody to prolactin, stained with diaminobenzidine, and counterstained with Gill hematoxylin; the prolactin (brown precipitate) was examined by light microscopy. Specificity was determined by an absence of staining in sections of term fetal posterior pituitary incubated with prolactin antibody (E). Original magnification $\times 400$.

letrozole (75 ± 15 ng/ml), and only minimally increased by treatment with letrozole and estradiol (95 ± 14 ng/ml).

Concentrations of prolactin in amniotic fluid (Table 3) were increased approximately 4-fold between mid (72 ± 12 ng/ml) and late (274 ± 26 ng/ml) gestation, but they were not altered in late gestation following treatment with letrozole (346 ± 36 ng/ml) or letrozole and estrogen (340 ± 56 ng/ml).

Pituitary Prolactin Expression

Serum levels of prolactin in umbilical artery appear to reflect secretion from the fetal pituitary. Thus, as seen in Figure 3, immunocytochemical expression of prolactin in the fetal anterior pituitary at midgestation was very light and only detected in a few pituitary cells (Fig. 3A). In contrast, expression of prolactin near term was abundant and detected in the cytoplasm in several fetal pituitary cells (Fig. 3B). In baboons treated with letrozole (Fig. 3C), similar to the marked reduction in serum prolactin levels, fetal pituitary expression of prolactin was very light, and in many sections was not detectable. Although fetal pituitary prolactin expression appeared to be more abundant in baboons treated with letrozole and estrogen (Fig. 3D), this was not uniformly observed in all tissue sections. Specificity was confirmed by absence of immunostaining in sections of fetal posterior pituitary obtained at late gestation (Fig. 3E).

Consistent with the immunocytochemistry, image analysis showed that the percent of the anterior pituitary that was positive for prolactin was approximately 4-fold greater ($P < 0.01$) at Day 165 ($11.3\% \pm 1.0\%$) than at Day 100 ($2.8\% \pm 0.3\%$), reduced ($P < 0.01$) at Day 165 in estrogen-suppressed baboons ($3.8\% \pm 0.4\%$), and partially but not significantly restored by treatment with letrozole and estradiol ($6.4\% \pm 0.8\%$). However, the number of anterior pituitary cells (e.g., lactotrophs, gonadotrophs, etc.) per unit area of pituitary examined was similar in sections obtained at Day 100 (542 ± 30) and Day 165 (492 ± 25) and on Day 165 in baboons treated with letrozole (502 ± 24) or letrozole and estradiol (503 ± 31). Thus, as shown in Figure 4, in both male and female fetuses the apparent number of pituitary cells expressing prolactin at mid gestation (15.8 ± 2.4) was increased ($P < 0.01$) approximately 4-fold by Day 165 (57.3 ± 5.1), reduced ($P < 0.01$) at Day 165 in estrogen-suppressed baboons (19.3 ± 2.0), and partially but not significantly restored by treatment with letrozole and estradiol (32.7 ± 5.2).

Pituitary LH Expression

In contrast to prolactin, immunocytochemical expression of fetal pituitary LH (Fig. 5) in female fetuses appeared to be higher at mid (Fig. 5A) than late (Fig. 5B) gestation, a decline that was prevented in estrogen-suppressed fetuses (Fig. 5C) and

FIG. 4. Overall mean ($\pm$ SEM) number of fetal pituitary cells per 0.037 mm² expressing prolactin in fetal pituitary sections of male (circles) and female (squares) baboons examined by immunocytochemistry, as described in the legend to Figure 3, on Days 95–120 (mid; $n = 5$) and Days 162–175 (late; $n = 7$) of gestation in untreated baboons, and on Days 162–175 of gestation in animals treated with letrozole ($n = 5$) or letrozole and estradiol benzoate ($n = 4$). Values with different superscript letters differ from each other at $P < 0.01$ (ANOVA and Student-Newman-Keuls multiple-comparison statistic).

that did occur in animals treated with letrozole and estrogen (Fig. 5D). In male fetuses, however, fetal pituitary expression of LH appeared to be similar at mid and late gestation and unaltered by treatment with letrozole with or without estrogen (data not shown). These immunocytochemical observations were confirmed by image analysis (Fig. 6). Thus, in female fetuses the apparent number of pituitary cells expressing LH at midgestation (48.8 ± 9.5) was decreased approximately 65% at Day 165 (17.4 ± 3.2), a decline that was prevented ($P < 0.01$) in estrogen-suppressed baboons (56.6 ± 5.1) but not (P

FIG. 6. Mean ($\pm$ SEM) number of fetal pituitary cells per 0.037 mm² expressing LH in fetal pituitary sections from male ($n = 3$ –4 per group) and female ($n = 3$ –4 per group) fetuses examined by immunocytochemistry, as described in the legend to Figure 5, on Days 95–120 (mid) and Days 162–175 (late) of gestation in untreated baboons, and on Days 162–175 of gestation in animals treated with letrozole or letrozole and estradiol benzoate. Values with different superscript letters differ from each other at $P < 0.01$ (ANOVA and Student-Newman-Keuls multiple-comparison statistic).

< 0.05) in baboons treated with letrozole and estradiol (28.8 ± 5.2). In male fetuses, however, pituitary LH expression was similar at mid (19.5 ± 4.8) and late (15.5 ± 2.6) gestation and in late gestation following treatment with letrozole (18.5 ± 6.1) or letrozole and estradiol (21.6 ± 3.7 ; Fig. 6).

DISCUSSION

The results of the current study demonstrate that there was a developmental increase in fetal serum levels of prolactin during the second half of gestation in the baboon and that the latter

FIG. 5. Representative photomicrographs of immunocytochemical LH expression in anterior pituitary gland of female fetuses on (A) Days 95–120 ($n = 4$) and (B) Days 162–175 ($n = 4$) of gestation in untreated baboons, and on Days 162–175 in animals treated with (C) letrozole ($n = 4$) or (D) letrozole and estradiol ($n = 3$). Frozen sections (10–12 per animal) were incubated with antibody to LH, stained with diaminobenzidine, and counterstained with Gill hematoxylin; the prolactin (brown precipitate) was examined by light microscopy. Original magnification $\times 400$.

reflects enhanced production by and, presumably, secretion from the fetal pituitary. Thus, both the number of anterior pituitary cells per unit area that expressed prolactin and the weight (e.g., volume of cells) of the pituitary were each increased approximately 3- to 4-fold between mid and late gestation. Although fetal prolactin levels have also been shown to be elevated with advancing gestation in the human [15, 16] and rhesus monkey [17], the current study is the first to show that this increase is regulated by estradiol, the levels of which rise between mid and late gestation in the baboon, as they do in humans and other nonhuman primates [26]. Moreover, it appears that this action of estradiol is elicited at the pituitary and/or the hypothalamus that has been shown to drive pituitary lactotroph function in the sheep [27] and rodent fetus [28]. Thus, in the current study fetal prolactin levels and expression by the pituitary were markedly reduced in estrogen-suppressed animals and only partially but not significantly increased by treatment with letrozole and estradiol. The incomplete restoration of prolactin presumably reflects the fact that fetal estradiol levels were increased to only 10%–15% of normal by treatment with letrozole and estrogen, the latter because of placental metabolism of maternally administered hormone [29, 30]. In support of this suggestion, maternal prolactin levels in late gestation were reduced in estrogen-suppressed animals and totally restored in letrozole-estrogen-treated baboons in which maternal estradiol levels were also increased to normal. In addition, in immature rat pituitary cell cultures, estrogen has been shown previously to increase prolactin secretion and the number of prolactin-secreting cells in a dose-dependent manner [31].

It is well established that prolactin receptor is expressed in several fetal tissues, including the adrenal [32–34]. Moreover, we have shown that prolactin increased fetal adrenal androgen production both *in vitro* [19] and *in vivo* [20]. However, as shown in the current study, in the relative absence of estrogen, prolactin expression/levels in late gestation were markedly suppressed. Therefore, the increase in growth of the fetal zone of the fetal adrenal and androgen production observed previously in estrogen-depleted baboons [7] most likely reflects absence of estrogen, and not of prolactin. This suggestion is supported by additional studies in which we showed that estradiol suppressed fetal androgen levels *in vivo* [35] and adrenocorticotrophic hormone-stimulated androgen production by baboon fetal adrenal cells *in vitro* [36, 37]. With regard to the ovary, it is well established that human ovarian function is compromised primarily by hyperprolactinemia [18]. Although prolactin receptor knockout animals are infertile, the infertility appears to result primarily from problems with implantation and/or failure of oocyte development after ovulation, as well as inappropriate progesterone secretion by the corpus luteum [38, 39]. It is also unlikely that the decrease in ovarian follicle formation previously demonstrated in estrogen-suppressed fetal baboons [8, 9] reflects an action of prolactin. Collectively, these findings and our previous studies showing that ESR1 and ESR2 are expressed in the fetal ovary [12] and adrenal gland [11] support our hypothesis that estrogen regulates fetal ovarian folliculogenesis and fetal adrenal zone-specific cellular development via eliciting a direct action on these organ systems.

We showed previously that the estrogen-dependent ontogenesis in fetal ovarian follicle formation did not appear to reflect an action of fetal pituitary gonadotropins [10]. Thus, fetal follicle-stimulating hormone levels were negatively correlated with ovarian folliculogenesis, declined between mid and late gestation, and remained elevated in estrogen-suppressed animals in which follicle formation was markedly

reduced [10]. The results of the current study show that in female but not male fetuses, fetal pituitary LH expression also declined between mid and late gestation and was prevented in estrogen-suppressed animals; these findings further support this hypothesis. Moreover, these findings show that the decline in fetal pituitary gonadotropin expression in the female with advancing gestation is regulated by estradiol, but this is not true in the male, as demonstrated recently in our laboratories (E. D. Albrecht and G. J. Pepe, unpublished data).

The results of the current study also showed that there was a developmental increase in prolactin levels in amniotic fluid between mid and late baboon gestation, and that the latter was not modulated by estrogen. Prolactin in amniotic fluid is primarily of decidual origin [40–42], and levels in amniotic fluid have also been shown to increase between early and late gestation in women. Progesterone is a potent stimulator of decidual prolactin expression in both the human [43, 44] and the baboon [45]. We showed previously that progesterone levels were unaltered in baboons treated with letrozole [24], which would explain the absence of change in amniotic fluid prolactin levels in estrogen-suppressed baboons in the current study.

In summary, the results of the current study showed that fetal prolactin levels and the number of fetal pituitary cells expressing prolactin were increased between mid and late gestation in untreated baboons, reduced in fetuses in which estradiol levels were suppressed by treatment with letrozole, and partially but not significantly restored by concomitant treatment with letrozole and estradiol. In contrast, female fetal pituitary LH expression was higher at mid than at late gestation, increased in estrogen-suppressed animals, and partially decreased with letrozole-estradiol treatment. We conclude that estrogen regulates developmental changes in fetal prolactin levels and fetal pituitary expression of prolactin and LH. However, because expression of prolactin and LH in estrogen-suppressed fetuses was inversely related to the changes in adrenal and ovarian development demonstrated previously in these animals, the results of the current study support our hypothesis that estrogen regulates fetal ovarian folliculogenesis and fetal adrenal development via a direct action and not via action on fetal pituitary gonadotroph/lactotroph function.

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