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Development of the Baboon Fetal Adrenal Gland: Regulation of the Ontogenesis of the Definitive and Transitional Zones by Adrenocorticotropin*

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ABSTRACT

Throughout gestation, the primate fetal adrenal gland is comprised of the fetal zone, which expresses the P-450 17α-hydroxylase-C17,20 lyase (P-450c17) enzyme that catalyzes the synthesis of C_{19} steroids used for placental estrogen production. The development of the transitional zone comprised of cortical cells that express the P-450c17 and the 3β-hydroxysteroid dehydrogenase-isomerase (3βHSD) enzymes for cortisol production, and the definitive zone, which expresses 3βHSD, but not P-450c17, for mineralocorticoid synthesis, does not occur until relatively late in gestation. Although ACTH is considered essential to fetal adrenal growth and function, the role that ACTH has in the development of the transitional and definitive zones, is less clear. To answer this question, the width of these zones was determined by immunocytochemical expression of P-450c17 and/or 3βHSD in fetal adrenal glands obtained on day 100 (mid) of gestation (term = day 184) from baboons in which ACTH was administered to the fetus on days 95–99 of gestation or on day 165 (late) of gestation from baboons in which fetal ACTH was suppressed by treatment of the mother and fetus with betamethasone on days 150–164 of gestation. At midgestation, the fetal adrenal was comprised almost exclusively of fetal zone cells and a small definitive zone (38 $\pm$ 2 μm in width), but was essentially devoid of a transitional zone (7 $\pm$ 2 μm). Treatment with ACTH enhanced ($P < 0.05$) the width of the transitional zone (67 $\pm$ 4 μm), but not the size of the definitive zone (10 $\pm$ 4 μm). In late gestation, the width of the definitive zone, although 2-fold greater than that on day 100, was smaller ($P < 0.05$) than that of the transitional zone (120 $\pm$ 15 μm), which greatly exceeded that at midgestation. Treatment with betamethasone in late gestation eliminated the transitional zone, but had no effect on the size of the definitive zone (120 $\pm$ 8 μm). These findings indicate that the development of the baboon fetal adrenal transitional zone late in gestation is dependent on fetal pituitary ACTH. In contrast, the ontogenesis of the definitive zone at midgestation and its growth in late gestation occur in the relative absence of ACTH. (*J Clin Endocrinol Metab* **84:** 3831–3835, 1999)

THROUGHOUT gestation, the fetal adrenal cortex of the human (1), rhesus monkey (2, 3), and baboon (4) is comprised primarily of the fetal zone, which expresses the P-450 17α-hydroxylase-C17,20-lyase (P-450c17) enzyme essential for the production of the C19 androgen dehydroepiandrosterone and its sulfate that serve as requisite precursors for placental estrogen production (5). In contrast, the transitional zone, which expresses both the 3β-hydroxysteroid dehydrogenase-isomerase (3βHSD) and the P-450c17 enzymes for the production of cortisol, and the definitive zone, which expresses 3βHSD, but not P-450c17, for the production of aldosterone (1, 6), do not develop in the fetal adrenal gland until relatively late in gestation. In the baboon (7, 8) and rhesus monkey (9), ACTH of fetal pituitary origin (10) is essential to the developmental expression in late gestation of the messenger ribonucleic acids (mRNAs) and proteins for 3βHSD and P-450c17 in and *de novo* cortisol production by the fetal adrenal (11, 12). It would appear, therefore, that ACTH is important to development of the transitional zone, although this remains to be confirmed by analysis of zone-specific expression of 3βHSD and P-450c17. Moreover, it is not known whether fetal pituitary ACTH is also required for development of the definitive zone. To answer these questions, the current study compared the cellular expression of 3βHSD and P-450c17 in fetal adrenal glands obtained at midgestation from baboons in which the fetus was administered ACTH and in late gestation from animals treated with betamethasone, which we previously demonstrated inhibited fetal pituitary POMC mRNA/ACTH (8) expression.

Materials and Methods

Animals

Fetal adrenal glands were obtained from baboons (*Papio anubis*) via cesarean section on day 100 (mid) or day 165 (late) of gestation (term = day 184). Baboon fetuses of midgestation were untreated (n = 5) or treated with ACTH (n = 4; Cortrosyn, Organon, Inc., West Orange, NJ) administered via maternal transabdominal im injection once daily to the fetus (25 μg/100 μL saline) on days 95–99 of gestation. In late gestation, baboons were untreated (n = 5) or treated with betamethasone (Celestone Soluspan, Schering AG, Chicago, IL) administered to the mother (6 mg) and fetus (0.6 mg) every other day between days 150–164 of gestation (n = 3).

Immunocytochemistry

Baboon fetal adrenals were fixed in 10% formalin and embedded in paraffin. Sections (4 μm) through the central portion of the gland were

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mounted onto SuperFrost microscope slides (Fischer Scientific, Arlington VA), deparaffinized by heating at 60 C (15 min) followed by three washes in xylene, dehydrated in graded ethanols (8), and treated with H_2O_2 in methanol (0.3% for 3βHSD and 3.0% for P-450c17) to block endogenous peroxidase.

For analysis of 3βHSD, alternate sections were preblocked for 30 min with 5% normal goat serum (Vector Laboratories, Inc., Burlingame, CA) in phosphate-buffered saline and incubated (4 C) overnight with a rabbit polyclonal antibody to antihuman 3βHSD (supplied by Dr. Ian Mason, University of Edinburgh, Edinburgh, Scotland) diluted 1:5000 in 5% normal goat serum. After treatment with biotinylated goat antirabbit IgG (Vector Laboratories, Inc.), sections were incubated with avidin DH and horseradish peroxidase (Vectastain Elite Kit, Vector Laboratories, Inc.) and color developed with imidazole and diaminobenzidine. Sections were lightly counterstained with Gill's hematoxylin (Fisher Scientific), and an average of six randomly selected areas (157 × 130 μm)/section (n = 4–8 sections/fetal adrenal) were examined using an Optiphot-2 microscope attached to a video-based Image 1 Analysis System (Universal Imaging Corp., West Chester, PA) to determine the width (microns) of the cell layer expressing 3βHSD (7, 8).

For analysis of P-450c17, alternate adrenal sections were placed in Coplin jars containing 10 mmol/L sodium citrate buffer (pH 6.0; Sigma Chemical Co., St. Louis, MO) and microwaved (model JE 1540, 900W, General Electric) for 5.5 min. After cooling for 30 min, sections were washed, preblocked as for 3βHSD, and incubated (4 C) overnight with a rabbit polyclonal antibody to antihuman P-450c17 (supplied by Dr. M. Waterman, Vanderbilt University School of Medicine, Nashville, TN) diluted 1:2000. After incubation with biotinylated goat antirabbit IgG, avidin, and biotinylated horseradish peroxidase (Vector Laboratories, Inc.), sections were incubated with H_2O_2, imidazole, and diaminobenzidine and examined by light microscopy as described for 3βHSD. The mean width of cells adjacent to the capsule not expressing P-450c17 was determined by image analysis.

As described by Mesiano *et al.* (6), the mean width (microns) of the layer of cells expressing 3βHSD was defined as the total width of the definitive and transitional zones. The width of this cell layer not expressing P-450c17 was defined as the definitive zone (3βHSD positive; P-450c17 negative). This value was then subtracted from the total width of the 3βHSD cell layer to determine the width of the transitional zone (3βHSD and P-450c17 positive). The fetal zone was defined as those cortical cells that expressed P-450c17 but not 3β-HSD.

Statistics

The effects of development, betamethasone, and ACTH on the width of the definitive or the transitional zones were compared by ANOVA with multiple comparison of the means by the Newman-Keuls statistic. Student's *t* tests for dependent observations were used to compare the respective widths of the definitive and transitional zones in all groups.

Results

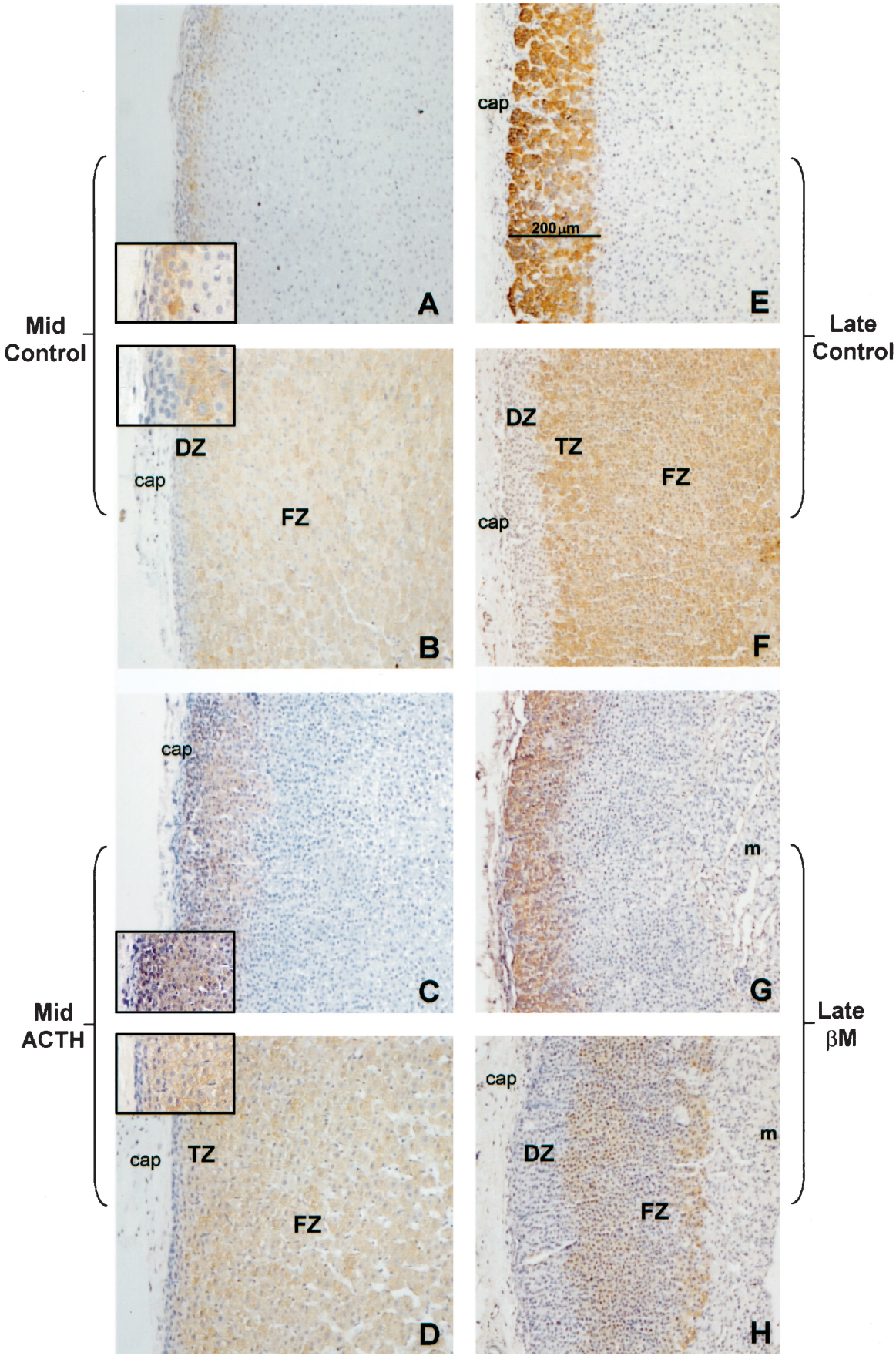
At midgestation, the expression of 3βHSD was minimal and confined to a relatively narrow layer of cells adjacent to the capsule (Fig. 1A). Although P-450c17 was abundantly expressed in the cortex at midgestation (Fig. 1B), the protein was not expressed in the narrow layer of cells expressing 3βHSD (Fig. 1, *inset*). After treatment of the fetus for 5 days with ACTH, the width of the cell layer expressing 3βHSD was increased (Fig. 1C), and a significant component of these cells also expressed P-450c17 (Fig. 1D). These results are illustrated in Fig. 2, and cellular expression of P-450c17 and/or 3βHSD are described as the mean (±SE) width of the adrenal cortex comprised of the definitive (3βHSD-positive; P-450c17-negative) and transitional (3βHSD- and P-450c17-positive) zones. At midgestation the fetal adrenal is comprised almost exclusively of fetal zone cells and a small definitive zone (38 ± 2 μm), but is essentially devoid of a transitional zone (7 ± 2 μm). Treatment of the fetus with ACTH at this time in gestation enhanced ($P < 0.05$) the width of the transitional zone (67 ± 4 μm), but not the definitive zone (10 ± 4 μm), the width of which appeared to be less than that in adrenals of untreated controls.

Compared with findings at midgestation, the expression of 3βHSD was significantly increased in fetal adrenals of late gestation (Fig. 1E). Although a significant component of these 3βHSD-positive cells adjacent to the capsule did not express P-450c17 (Fig. 1F), a relatively larger layer of 3βHSD-positive cells more internal did express the P-450c17 enzyme. Thus, as summarized in Fig. 2, the width of the definitive zone (79 ± 5 μm), although 2-fold greater ($P < 0.05$) than that at midgestation, was smaller ($P < 0.05$) than that of the transitional zone (120 ± 15 μm), which greatly exceeded ($P < 0.05$) that at midgestation. Administration of betamethasone reduced the width of the layer of cells expressing 3βHSD. However, the width of the 3βHSD cell layer in which P-450c17 was absent was not reduced (Fig. 1, G and H). In contrast, there were virtually no 3βHSD-positive cells that also expressed P-450c17. Thus, betamethasone treatment eliminated the transitional zone, but not the definitive zone (120 ± 8 μm), the width of which appeared to be greater than that in adrenals of untreated baboons during late gestation. The size of the fetal zone also appeared to be reduced by treatment with betamethasone (Fig. 1H).

Discussion

The results of the present study indicate that the width of the zone of the baboon fetal adrenal cortex expressing both 3βHSD and P-450c17 was negligible at midgestation when fetal ACTH levels are low (7, 10), enhanced by treatment of the fetus at midgestation with ACTH, and increased in late gestation when fetal ACTH levels are elevated (8, 10). Moreover, in baboons in which fetal pituitary POMC mRNA/ACTH expression was suppressed in late gestation by treatment with betamethasone (8), the development of cortical cells expressing both 3βHSD and P-450c17 was prevented. These developmental and betamethasone-induced changes in 3βHSD and P-450c17 were accompanied by similar alterations in fetal cortisol production, which we previously demonstrated was negligible at midgestation, increased significantly at term (12, 13), and was virtually eliminated in betamethasone-treated baboons in late gestation (8). Collectively, these *in vivo* experimental observations indicate that the development of the transitional zone and the ontogenesis of fetal adrenal cortisol production in the baboon fetal adrenal gland in late gestation are dependent on fetal pituitary ACTH. A similar role for ACTH has been proposed by Coulter *et al.* (9), who demonstrated that the number of fetal adrenal cells expressing 3βHSD and presumed to be cells of the transitional zone based on location and histologic appearance was increased after treatment of the late gestation rhesus monkey fetus with metyrapone.

The results of present study also indicate that the size of the definitive zone, like that of the transitional zone, was increased between mid- and late gestation. However, in contrast to the transitional zone, treatment with ACTH at midgestation or suppression of endogenous ACTH by betamethasone in late gestation did not alter the width of the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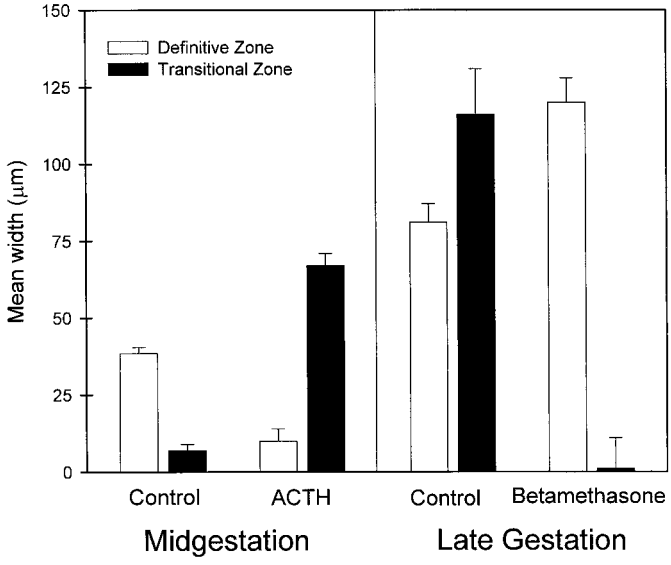

FIG. 2. Mean (±SE) width of the definitive and transitional zones of the fetal adrenal in untreated baboons of mid- (day 100; n = 5) and late (day 165; n = 5) gestation and baboons in which the fetus received ACTH on days 95–99 of gestation (n = 4) or in which betamethasone (n = 3) was administered to the mother (6 mg) and fetus (0.6 mg) every other day on days 150–164 of gestation (term = day 184). The widths of the definitive (3βHSD-positive; P-450c17-negative) and transitional (3βHSD and P-450c17-positive) zones were quantified by image analysis of zone-specific expression of 3βHSD and/or P-450c17.

definitive zone. Hypophysectomy of the sheep fetus at mid- to late gestation also had no effect on the apparent width of the fetal adrenal glomerulosa (*i.e.* definitive zone), as based on histology, although the size of the developing fasiculata (*i.e.* transitional zone) was dramatically reduced (14). Histological (15, 16) and stereological (17) analyses of the human fetal adrenal have also shown that growth of the zona glomerulosa was not affected by fetal anencephaly, *i.e.* in the absence of ACTH. It appears, therefore, that the development of the definitive zone of the fetal adrenal is regulated by factors other than ACTH. Earlier studies in the adult rat also demonstrated that the width of the zona glomerulosa of the adrenal was not significantly affected by hypophysectomy, *i.e.* removal of pituitary ACTH (18). In the adult adrenal, glomerulosa cell function is regulated by angiotensin II via interaction with the AT_1 receptor. Interestingly, human fetal adrenal cortical cells primarily express the AT_2 receptor (19). The AT_1 receptors apparently are only expressed in a few layers of cortical cells at the periphery (presumed definitive zone) of the fetal adrenal gland (19). Studies of the regulation and role of AT_1 and AT_2 receptors in the primate fetal adrenal gland, however, remain to be performed. Activin and inhibin have also been postulated to modulate fetal adrenal development (1). Recently, we demonstrated that the baboon fetal adrenal expresses abundant quantities of inhibin α-subunit in fetal zone cells throughout the course of gestation (20). However, inhibin α-subunit was not detected in the outer layer of definitive zone cells expressing 3βHSD in late gestation. Although the role of inhibin on adrenal cellular maturation remains to be determined, *in vitro* studies suggest that the presence of α-subunit may serve to limit the production of activins (dimers of β-subunits), which have been shown to inhibit fetal adrenal cell proliferation as well as stimulate ACTH-dependent cortisol production (21). Finally, we previously demonstrated that estrogen of placental origin acts on the baboon fetal adrenal gland *in vitro* (22) and *in vivo* (23) to suppress ACTH-dependent dehydroepiandrosterone production by fetal zone cells, apparently by interaction with the estrogen receptor, which is expressed in the cells of the fetal zone (24). Interestingly, estrogen receptor was also expressed in relatively high levels in definitive zone cells, and therefore, estrogen may also act to coordinate the development and/or function of the baboon fetal adrenal definitive zone.

In summary, the current study demonstrated that the development of the baboon fetal adrenal transitional zone late in gestation is dependent on fetal pituitary ACTH. In contrast, the ontogenesis of the definitive zone at midgestation and its growth and development in late gestation occurred in the relative absence of ACTH, suggesting that factors other than ACTH regulate maturation of the primate fetal adrenal definitive zone.

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FIG. 1. Immunocytochemical expression of 3βHSD (A, C, E, and G) and P-450c17 (B, D, F, and H) in untreated baboons of mid- (day 100; A and B) and late (day 165; E and F) gestation and in baboons in which the fetus received ACTH on days 95–99 (C and D) or in which betamethasone (G and H) was administered to the mother (6 mg) and fetus (0.6 mg) every other day on days 150–164 of gestation (term = day 184). The cell layer expressing 3βHSD and P-450c17 is designated the transitional zone (TZ), that expressing 3βHSD but not P-450c17 is the definitive zone (DZ), and cells only expressing P-450c17 comprise the fetal zone (FZ). Magnification, ×100; 1.7 cm = 200 μm. *Inset,* ×200. cap, Capsularis; m, medulla.

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