Summer 1997

Interaction of ACTH and Estradiol in the Regulation of Growth, Differentiation, and Steroidogenic Maturation of the Baboon Fetal Adrenal Gland

Maria Gomez Leavitt
Old Dominion University

INTERACTION OF ACTH AND ESTRADIOL IN THE REGULATION OF GROWTH, DIFFERENTIATION, AND STEROIDOGENIC MATURATION OF THE BABOON FETAL ADRENAL GLAND

by

Maria Gomez Leavitt
B. S. June 1979, Universidad Complutense, Madrid, Spain
B.A. May 1984, Old Dominion University, Norfolk, VA
M.S. May 1991, Old Dominion University, Norfolk, VA

A Dissertation Submitted to the Faculty of
Old Dominion University and Eastern Virginia Medical School
in Partial Fulfillment of the Requirement for the Degree of

DOCTOR OF PHILOSOPHY

BIOMEDICAL SCIENCES

OLD DOMINION UNIVERSITY
and
EASTERN VIRGINIA MEDICAL SCHOOL
August 1997

Approved by:

Gerald J. Pepe, Ph.D., (Director)

Reinhart B. Billiar, Ph.D., (Member)

Russell L Prewitt, Ph.D., (Member)

Ronit Rafaelof-Phair, Ph.D., (Member)

R. James Swanson, Ph.D., (Member)

ABSTRACT

INTERACTION OF ACTH AND ESTRADIOL IN THE REGULATION OF GROWTH, DIFFERENTIATION, AND STEROIDOGENIC MATURATION OF THE BABOON FETAL ADRENAL GLAND

Maria Gomez Leavitt
Old Dominion University and Eastern Virginia Medical School, 1997
Director: Dr. Gerald J. Pepe

The present study determined whether the growth of, as well as ACTH receptor and P-450 enzyme messenger ribonucleic acid and/or protein levels in the baboon fetal adrenal are dependent upon fetal pituitary ACTH during mid and late gestation and the mechanism by which placental estrogen modulates ACTH actions. Administration of betamethasone (3mg/day) to baboon mothers on days 60-99 of gestation and to the fetus (0.6 mg/, n=4) or to the fetus (0.6mg) and mother (6 mg/ml; n=4) every other day between days 150-164 of gestation (term=184 days) decreased ($P<0.05$) fetal pituitary proopiomelanocortin (POMC) mRNA levels by approximately 50%. Associated with this decline in pituitary POMC, the major 3.4 kb mRNA transcript for the adrenal ACTH receptor was reduced more than 80% at mid and late gestation in fetal adrenals of betamethasone-treated baboons compared to their age matched controls. Immunohistochemical studies confirmed the localization of sterodogenic enzymes $\Delta^5$3β-hydroxysteroid dehydrogenase-isomerase (3β-HSD) and cytochrome P450 17α-hydroxylase, 17/20-lyase (P450c17) to the definitive zone (3β-HSD (+), P450c17 (-)), transitional zone (3β-HSD (+) and P450c17 (+)), and fetal zone (P450c17 (+), 3β-HSD (-)). At term, betamethasone treatment resulted in a reduction in the size of the fetal zone

and eliminated the transitional zone. However, the definitive zone although reduced in size was maintained in betamethasone-treated animal. These data indicate that ACTH induces the differentiation of the transitional zone and thus the potential for the fetal adrenal to synthesize cortisol.

To test the hypothesis that estrogen modulates the role of ACTH on fetal adrenal development, the aromatase inhibitor CGS 20267 was administered to baboon mothers between days 100 and 164 of gestation (n=3). CGS treatment suppressed maternal and fetal peripheral serum estradiol to less than 2% of control levels, and enhanced ($P<0.05$) fetal adrenal weight by 75% but decreased fetal cortical cell size. In contrast, the intensity and immunoexpresion of P450c17 was enhanced and present in virtually all cells. We conclude that the ontogenic development of the fetal adrenal is prevented in estrogen-depleted baboons. Simultaneous administration of CGS and estrogen restored all aspects of fetal adrenal development. To test if estrogen-mediated modulation of adrenal development reflected a direct action of estrogen, we determined whether the adrenal expressed the estrogen receptor (ER). At term ER was observed in the definitive zone adjacent to the capsule but was not observed in either transitional or fetal zone cells. Treatment with CGS or CGS and estrogen had no effect on the ER expression. Betamethasone treatment, increased the intensity and width of the zone of cells expressing the ER. The betamethasone effect may be mediated by a glucocorticoid receptor (GR) which was detected in both the definitive and fetal zones. The results of the present study demonstrated that reduced placental estrogen appears to enhance preferentially ACTH-induced growth of the adrenal fetal zone preventing the formation of the definitive zone, while having no significant effect on ER expression of the definitive zone.

ACKNOWLEDGMENTS

I would like to thank:

My father, who spent many hours of his life teaching his children and emphasizing the importance of the learning process. Thanks for sharing with us your love for nature and for making me aware of the intricacy and beauty of all forms of life.

My mother, who in her domestic chores showed me the importance of perseverance and commitment to our vocation. Thanks for your care and unselfish love to your six children.

My husband, who patiently has supported all of my studies and work in this country for the past nineteen years. Thanks for the freedom and love I found with you.

My three children, Elizabeth, Matt and Joseph, from whom I have stolen many hours of attention to pursue my dream. Thank you for being the way you are.

My mentor, Gerald J. Pepe, Ph.D., for offering me work in his laboratory and to develop a fascinating project.

Membes of my committee: Russell L Prewitt, Ph.D., Ronit Rafaelof-Phail, Ph.D., and R. James Swanson, Ph.D., for their help and guidance during this research work.

Member of my committee: Reinhart B. Billiar, Ph.D., for his guidance, teaching and friendship. He provided the incentive and motivation to lead this research into new and exciting frontiers.

This project would have not been possible without your support and enthusiasm.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

Δ^4	Androstenedione
βm	Betamethasone
11βHSD	11β-Hydroxysteroid Dehydrogenase
16-OH DHA	16-OH Dehydroepiandrosterone
17-OHP_4	17α-Hydroxyprogesterone
3βHSD	$\Delta^5$3β-Hydroxysteroid Dehydrogenase-isomerase
ACTH	Adrenocorticotropic Hormone
bm	Betamethasone
C19 steroids	19-carbon Steroids
cAMP	Cyclic AMP
CGS 20267	Letrozole
CREB	cAMP Response Element Binding Protein
CRH	Corticotropin Releasing Hormone
DAB	3,3'-diaminobenzidine
DHA	Dehydroepiandrosterone
DHAS	Dehydroepiandrosterone Sulfate
DOC	11-Deoxycorticosterone
DZ	Definitive Zone
E	Cortisone
E_1	Estrone

E_2	Estradiol
E_3	Estriol
ER	Estrogen Receptor
F	Cortisol (4-pregnen-11β-17α,21-triol-3,20-dione)
FZ	Fetal Zone
GR	Glucocorticoid Receptor
H_2O_2	Hydrogen Peroxide
HDL	High Density Lipoproteins
HMGCoA	3-hydroxy-3-methyl-glutaryl Coenzyme A
HPAA	Hypotalamic-pituitary-adrenal-axis
IgG	Immunoglobulin G
im	Intra Muscular
LDL	Low Density Lipoproteins
MER-25	Ethamoxytriphetol
NGS	Normal Goat Serum
P_4	Progesterone
$P450_{17\text{-}OH}$	Cytochrome P450 17α-hydroxylase, 17/20-lyase
P450c17	Cytochrome P450 17α-hydroxylase, 17/20-lyase
P-450scc	Cytochrome P450 Cholesterol Side Chain Cleavage
P_5	Pregnenolone
PBS	Phosphate Buffered Saline
PCNA	Proliferating Cell Nuclear Antigen

PKA	Protein Kinase A
PMSF	Phenylmethylsulfonyl Fluoride
poly(A)$^+$	Polyadenylated
POMC	Proopiomelanocortin
SDS	Sodium Dodecyl Sulphate
SSC	0.3 M NaCl, 0.03 M sodium citrate-$2H_20$
SSPE	0.375 M NaCl, 0.025 M NaH_2P0_4-H_20, and 2.5 mM EDTA–Na_2,
TNK	100mM Tris-HC, 550mM NaCl, 10mM KCl, 2% bovine serum albumin, 0.1% Triton X-100, 1% normal goat serum,
TZ	Transitional Zone

CHAPTER I

BACKGROUND AND INTRODUCTION

The fetal adrenal cortex

Function during primate pregnancy

In most mammalian species, products of the fetal adrenal gland play an important role in regulating maturation of various organ systems in the fetus (Pepe and Albrecht, 1990) on which homeostatic mechanisms to respond to stress (Challis et al., 1996; Matthew and Challis, 1995) and survival after birth are dependent. Preceding parturition, fetal organs undergo accelerated maturation which facilitates the transition from intrauterine to extrauterine life. Cortisol (F) (4-pregnen-11β-17α,21-triol-3,20-dione) produced by the fetal adrenals is one of the chemical messengers involved in the stimuli for maturation of the lungs (Mendelson CR and Boggaram V, 1991; Farrell PM and Zachan RD, 1973; Weaver TE and Whitsett, 1991; Kotas and Avery, 1971; NIH Consensus Conference, 1995), the marked increase in the accumulation of glycogen in the liver (Greengard O and Dewey, 1970; Grengard O, 1973; Seron-Ferre and Jaffe, 1981; Eisen HJ et al., 1973) and the induction of several enzymes in the brain, retina, pancreas and gastrointestinal tract of the fetus (Moscona AA and Piddington R, 1966; Giannopoulos G 1975, Piddington R and Moscona AA, 1967 Moog F, 1971) late gestation. In most species investigated to date, late pregnancy is characterized by rising concentrations of F in the fetal circulation (Liggins GC, 1994). In sheep, F concentrations rise sharply prepartum and induce parturition presumably by increasing the placental enzyme, 17α-hydroxylase C17-20-lyase (P450c17) (Liggins GC et al., 1973; Seron-Ferre M and Jaffe

RB, 1981) which results in decreased placental progesterone secretion, increased placental estrogen secretion and thus an consequently increased uterine prostaglandin production. In human and nonhuman primates fetal F levels increase more slowly and the role of F in the initiation of labor is less convincing (Liggins GC, 1976; Albrecht ED et al., 1989; Casey ML and MacDonald PC, 1988; Muller-Huebachcortisoneat al., 1972); nevertheless, fetal adrenal *de-novo* F production appears to be a factor in determining the timing of parturition (Turnbull AC and Anderson ABM, 1984; Sucheston M and Cannon MS, 1969). In addition to these roles for fetal adrenal F, it is well established that the fetal adrenal gland in primates, including humans, is important to the synthesis and secretion of androgen precursors essential to the production of estrogen by the placenta (Diczfalusy E, 1964; Diczfalusy E, 1974: Siiteri PK and MacDonald PC, 1966; Oakey RE, 1970).

Development of the fetal adrenal gland: fetal and definitive zone cells

During development, the fetal adrenal gland lacks the typical zonation that the adult adrenal presents. Thus, in human and nonhuman primates the fetal adrenal gland is composed of two morphologically distinct zones: an inner fetal cortical zone, which comprises between 80 and 90% of the gland during the majority of gestation and an outer definitive or adult zone, that occupies approximately the remaining 20% of the gland late in gestation (Pepe GJ and Albrecht ED, 1990; Seron-Ferre M and Jaffe RB, 1981). This type of zonation and adrenal development is not present in fetal adrenal gland of non-primates. The fetal zone is composed of large eosinophilic cells with large pale nuclei and distinct cell outlines that have the ultrastructural characteristics of steroid-secreting cells whereas, the definitive zone is composed of small basophilic cells with dark staining nuclei (Seron-Ferre M and Jaffe RB, 1981). Structural and functional evidence have been

described by Sucheston (Sucheston ME and Cannon MS, 1968;) and Jaffe and colleagues (Mesiano S et al.,1993) for a third zone, the transitional zone. This zone, which appears in human and nonhuman primates late in gestation lies between the definitive and fetal zone, it is comprised of large acidophilic cells with indistinct cell boundaries and is believed to be the site of F synthesis.

During the course of gestation in human (Lanman JT, 1953; Neville AM and O'Hare MH, 1982) and nonhuman primates (Pepe GJ et al., 1977; Kerr GR et al., 1969; McNulty et al., 1981; Seron-Ferre M et al., 1983; Albrecht and Pepe GJ, 1988), the fetal adrenal gland grows rapidly, with marked growth occurring primarily during the last one-third of intrauterine development. In humans, the weight of both fetal adrenal glands is less than 100 mg at 10 weeks gestation and increases to approximately 2000 mg by week 20 (Swinyard CA, 1941). Between 20 and 30 weeks of gestation the gland doubles in size, with the ontogenesis of adult-type zonation (Sucheston ME, 1968). By term, the human fetal adrenal weighs approximately 4000-8000 mg (Swinyard CA, 1941), about twice the weight of adult adrenals, and represents 0.5 percent of total body weight, compared with 0.005 percent in the adult (Miller WL and Tyrrell JB, 1995). Similar increases in fetal adrenal weight occur in the baboon (Pepe GJ, Titus JA et al., 1985) and the rhesus monkey (McClellan M, 1981). The increase in weight is achieved mainly by an increase in the size of the fetal zone with minimal maturation of the outer cortex until late in gestation. Thus, by day 150 of gestation in the rhesus monkey (term=165) a presumptive zona glomerulosa and a zona fasciculata are present but these "adult type zones" still only occupy less than 20% of the cortex (McClellan M et al., 1981). In the human, the fetal zone of the adrenal apparently undergoes rapid involution during early postnatal life. By

the second week after birth, adrenal weight has decreased by one-third, and the fetal zone in no longer detectable by age 1 year (Miller WL and Tyrrell JB, 1995). A similar process occurs in the baboon and rhesus monkey (McNulty WP et al., 1981), since distinct adult-type zones are observed in neonates by 6-12 months of age (Pepe GJ and Albrecht ED, 1985).

The factors regulating the remodeling of the fetal gland into the permanent mature cortex is remain to be elucidated. After birth in the nonhuman primate, proliferation of the definitive zone with absence of necrosis within the fetal zone was observed. This postnatal morphology in rhesus monkey was interpreted to represent the development of the zona fasciculata by transformation of the fetal zone (Holmes RL, 1968). In baboons (*Papio anubis*) it was suggested that the zona fasciculata develops by proliferation of new cells within its upper margins and not by transformation of preexisting cells in the fetal zone (Ducksay CA et al., 1991). In contrast, it was originally suggested (Sucheston ME and Cannon MS, 1968) that the fetal zone in humans disappears and that it is the definitive cortex which gives rise to the three zones of the adult adrenal gland (e.g. the glomerulosa, fasciculata and reticularis) during the first year of life. However, more recent studies have questioned this theory and it has been proposed that zones of the late human fetal adrenal cortex are similar to those of the adult, with the definitive zone being analogous to the nascent zona glomerulosa, the transitional zone to the zona fasciculata and the fetal zone to the zona reticularis (Mesiano S et al., 1993).

Steroid hormone production by the fetal adrenal gland

Substrate for fetal adrenal steroid production: Low density lipoprotein pathway

The principal secretory products of the human fetal adrenal gland are the C19 androgens, dehydroepiandrosterone (DHA), and DHA sulfate (DHAS) the C21 glucocorticoid and F. Based primarily on *in vitro* studies utilizing human fetal adrenal tissues obtained early in gestation, it is apparent that cholesterol derived from circulating LDL produced in the fetal liver is a major substrate for fetal adrenal steroidogenesis (Simpson ER et al., 1979; Carr BR et al., 1980; Carr BR et al., 1980; Carr BR et al; 1980; Carr BR et al., 1981; Carr BR et al., 1982; Parker Jr CR et al., 1980; Simpson ER et al., 1985; Simpson ER et al., 1985; Mason JI and Rainey WE 1987; Mason JI et al., 1983;). Thus, the quantity of DHAS and F synthesized by human fetal adrenal cells in culture increased as a function of the concentration of LDL present in the culture media (Carr BR et al.,1980; Mason JI and Rainey WE 1987). Consistent with these results, Solomon et al (1967) demonstrated using *in situ* perfusion that neither circulating acetate nor free cholesterol were efficient precursors for human fetal steroidogenesis. Similar conclusions have been deduced from *in vivo* studies in baboon (Pepe GJ and Albrecht ED, 1984) and rhesus monkeys (Ducsay CA et al., 1985) in which less than 5% of placental progesterone and pregnenolone secreted into the fetus were utilized for F production between mid and late gestation. Pregnenolone does not appear to be a quantitatively significant precursor in the human as well, because the total amount of this precursor estimated to be used for steroidogenesis is less than 1% of the fetal production rate of DHAS (Simpson ER et al., 1985; Simpson ER et al., 1985).

Fetal adrenal uptake of LDL occurs by receptor-mediated endocytosis. Carr and colleagues (Carr BR et al., 1980; Carr BR et al., 1980; Carr BR et al., 1980) examined the uptake and degradation of radiolabeled LDL by human fetal tissue fragments in the presence or absence of ACTH. Fetal adrenal LDL uptake was saturable, and presumably mediated by a population of high-affinity, low-capacity binding sites. As in the adult adrenal, where less than 10 percent of LDL enters the cell by a receptor-independent mechanism (Brown MS et al., 1979; Gwynne JT et al., 1982), LDL binds to fetal gland receptors that are internalized in coated vesicles and is then separated from its receptor, which is recycled back to the plasma membrane. Acid proteinases degrade LDL apolipoproteins to amino acids, and acid lipases free cholesterol from esters (Miller WL et Tyrrell JB, 1995). ACTH stimulates LDL receptor synthesis, the uptake of LDL cholesterol (Golos TG and Strauss JF III, 1988), and degradation of LDL by fetal adrenal tissue fragments (Carr BR et al., 1980; Carr BR et al., 1981;). To investigate the nature and regulation of the receptors involved in the endocytosis of LDL, the binding of LDL to membrane fractions prepared from adrenals of normal and anencephalic human fetuses was determined (Carr BR et al.,1981; Ohashi M et al., 1981). In tissues from both sources, binding was of high affinity. However, compared to values in tissues from anencephalics, not only were binding and degradation greater in normal fetuses, but only in normal tissue were the latter processes stimulated by ACTH. Although there was substantial binding of high density lipoproteins (HDL) by human fetal adrenals (Ohashi M et al., 1981), HDL was not degraded nor regulated by ACTH. Moreover, HDL does not stimulate or sustain human adrenal steroidogenesis (Gwynne JT and Strauss JF III, 1982; Ohashi M et al., 1981; Strauss JF III and Miller WL, 1991). Because no more than 20% of

cholesterol in the fetus is derived from the maternal compartment (Lins DS et al., 1977), Simpson et al. (Simpson ER et al., 1985; Simpson ER et al., 1985) have suggested that the majority of circulating LDL-cholesterol arises from *de novo* synthesis within the fetus. In support of this fetal role, extensive cholesterol production by human fetal liver, testes and adrenal has been demonstrated (Carr BR and Simpson ER, 1982).

Substrate for fetal adrenal steroid production: De novo biosynthesis.

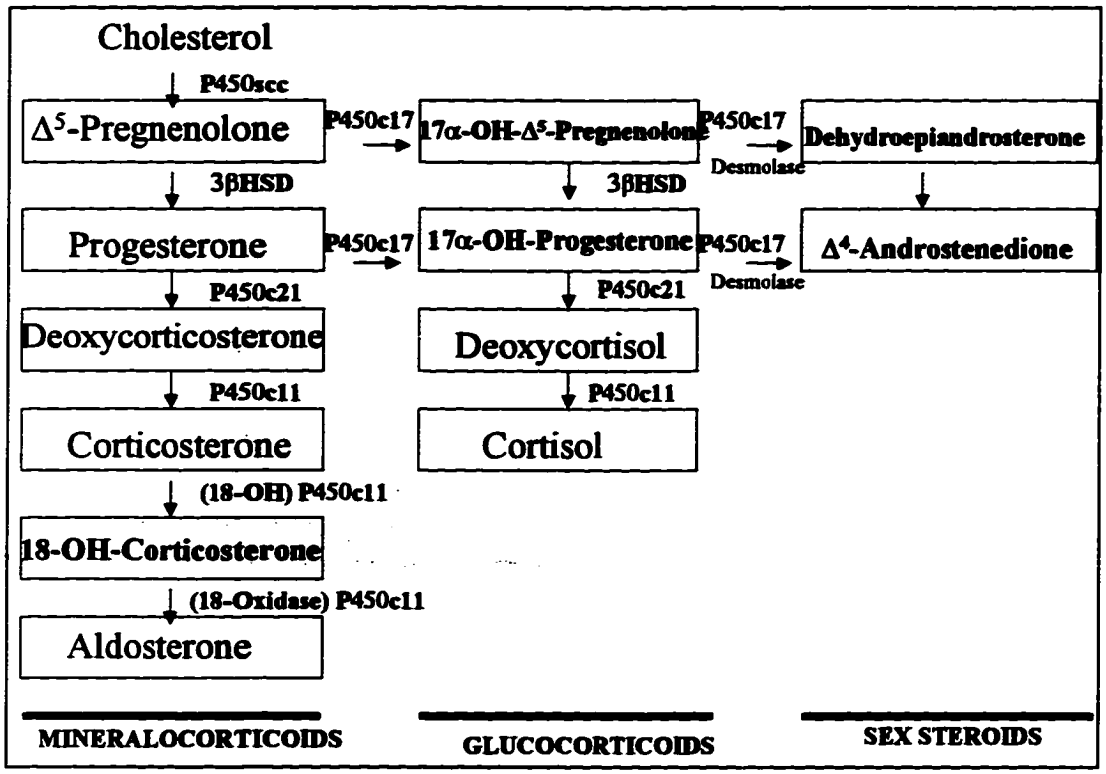
Despite the importance of LDL-cholesterol, it has also been demonstrated (Carr BR et al., 1981; Mason JI and Rainey WE, 1987; Carr et a., 1981) that the adrenal readily synthesizes cholesterol from acetyl CoA in response to trophic stimulation by ACTH. Moreover, *de novo* cholesterol production catalyzed by the rate limiting enzyme 3-hydroxy-3-methyl-glutaryl coenzyme A (HMGCoA) reductase, could account for as much as 30% of the daily secretion rate of DHAS and F by the human fetal adrenal (Sippell WG et al., 1981). As in the adult, fetal *de novo* cholesterol synthesis was only an important source of substrate in certain situations. Thus, Mason and Rainey (Mason JI and Rainey WE, 1987) demonstrated that in the absence of LDL-cholesterol, steroidogenesis by human fetal adrenal cells in culture could be maintained almost entirely by the *de novo* pathway. Mason and Rainey (Mason JI and Rainey WE, 1987) have shown that the principal molecular species of fetal adrenal HMG-CoA reductase in both cultured and fresh adrenal tissue was a 97KDa protein. The appearance of this protein in fetal adrenal cell cultures correlates with *in vitro* measures of enzyme activity. Moreover, because loss of enzyme protein associated with culture of fetal adrenal cells in the absence of ACTH was prevented by concomitant treatment with ACTH, it appeared that HMGCoA reductase activity in fresh adrenal tissue was maintained by ACTH. Indeed, *de novo*

cholesterol synthesis in adrenals of anencephalics was significantly lower than that in tissue of normal fetuses (Carr et al., 1981). In adult adrenal, HMGCoA reductase is inactivated by phosphorylation and is activated by dephosphorylation in response to intracellular cAMP elicited by the binding of ACTH to its receptor (Gwynne JT and Strauss JF III 1982). By contrast, in human fetal adrenal, HMGCoA reductase is probably synthesized in an inactive form in response to ACTH. Thus, in the fetus ACTH may not activate HMGCoA reductase by stimulating dephosphorylation but rather, as demonstrated in adult bovine adrenals cells, ACTH may promote increased rates of accumulation of HMGCoA reductase protein (Rainey WE et al., 1986).

Chemistry of steroid hormones

The conversion of cholesterol to pregnenolone (P_5) in mitochondria is the first and rate-limiting step in the synthesis of steroid hormones from cholesterol. This involves three distinct chemical reactions: 20a-hydroxylation, 22-hydroxylation, and scission of the cholesterol side chain to yield P_5 and isocaproic acid. As recently review by Miller (Miller WL, 1988), a single enzyme cytochrome P450 cholesterol side-chain cleavage (P450scc), mediates this process. P_5 is then transported outside the mitochondria before further steroidogenesis occurs. Once P_5 is produced from cholesterol it may be converted to 17-hydroxypregnenolone or to progesterone by $\Delta^5$3β-hydroxysteroid dehydrogenase-isomerase (3βHSD). This single microsomal enzyme catalyzes both 3β-hydroxysteroid dehydrogenation and the isomerization of the double bond from the Δ^5 steroid to the Δ^4 steroid (Δ represents double bond). Both 3βHSD and isomerase activities are found in a single 42-KDa protein (Thomas JL et al., 1989). There are at least two forms of human 3β-HSD, encoded by different genes. The 3β-HSD type I gene is expressed in the

placenta, skin, mammary gland, and possibly other peripheral tissues (Lorence MC et al., 1990; Lachance Y et al., 1990; Lorence et al., 1990). A distinct 3β-HSD type II gene is expressed in the adrenals and gonads (Lachance Y et al., 1991; Rheaumecortisoneet al., 1991). Both P_5 and progesterone (P_4) may undergo 17α-hydroxylation to 17α-hydroxypregnenolone and 17α-hydroxyprogesterone (17-OHP_4), respectively. These 17-hydroxylated steroids may then undergo scission of the C-17,20 carbon to yield the 19-carbon steroids (C19 steroids), DHA and androstenedione (Δ^4A). These two reactions are mediated by a single enzyme, cytochrome P450 17α-hydroxylase, 17/20-lyase (P450c17). As P450c17 has both 17α-hydroxylase activity and C-17,20-lyase activity, it is a key branch point in steroid hormone synthesis, catalazing either glucocorticoids (17α-hydroxylation but not C-17,20 cleavage) or androgens (both 17α-hydroxylation and C-17,20 cleavage) synthesis. After the formation of progesterone and 17-OHP_4, these steroids can be hydroxylated at C-21 position to yield 11-deoxycorticosterone (DOC), which does not have a 17OH group, and 11-deoxycortisol (S), respectively. This step involves the 21-hydroxylation by the microsomal enzyme P-450c21. 11-deoxycortisol is further hydroxylated within the mitochondria by the P450c11 enzyme to form F. The nature of the 21-hydroxylating step has been of great clinical interest because congenital adrenal hyperplasia most frequently results from 21-hydroxylase deficiency. In human fetuses affected with this genetic disorder, the dysfunctional adrenal can produce enough androgen to virilize a female fetus to a male phenotype (Miller WL and Levine LS, 1987; New MI et al., 1981; Saenger P, 1984). A summary of steroid biosynthesis in adrenal gland with the major secretory products and enzymes is presented in the following diagram:



Enzyme regulation of fetal adrenal steroidogenesis

It is well established that the expression and activity of the enzyme 3β-HSD responsible for the synthesis of $\Delta^4$3-ketosteroids, e.g. F, from pregnenolone is minimal during the majority of intrauterine development in the fetal adrenal of human (Lanman JT, 1953; Neville AM and O'Hare MH, 1982) and nonhuman primates (Pepe GJ, Titus JA et al., 1977; Kerr GR et al., 1969; McNulty WP et al., 1981; Seron-Ferre M and al., 1983; Albrecht ED and Pepe GJ, 1988; Simonian MH and Gill GN, 1981; Mesiano S et al., 1993; Coulter CL et al., 1996). When, however, human fetal definitive zone explants from the first and second trimester are cultured in the presence or absence of

adrenocorticotropic hormone (ACTH) and others agonists of the protein kinase A pathway, the capacity for F secretion is significantly induced as is the activity of 3β-HSD (Doody KM et al., 1990; Beitins IZ et al., 1973; Beitins IZ et al., 1972; Boltecortisoneet al., 1964; Branchaud CT et al., 1978). Consequently, adrenals of fetuses early in gestation are competent to respond to ACTH but may not have been exposed to sufficient ACTH *in vivo* during this developmental period.

Recent immunohistochemical evaluation of the cellular localization and ontogeny of steroidogenic enzymes has provided additional insight into the regulation of steroidogenesis *in utero*. As reported by Jaffe et al (1996), P450scc is expressed in all cells of the adrenal cortex in both the mid and the term rhesus fetus. In contrast, 3β-HSD was localized primarily in the definitive zone cells from mid gestation fetuses, whereas at term it was localized in both the definitive and transitional zone cells. P450c17 expression was detected in the transitional and fetal zones. Coexpression of 3β-HSD and P450c17 enzymes in the transitional zone (or also termed the inner definitive zone) by term gestation in the primate adrenal indicates the capacity of these cells to synthesize *de novo* F. These results are in agreement with those of Doody KM et al. (1990), where ACTH only increased secretion of F by fetal definitive zone cells, indicating that 3β-HSD is not present in fetal zone cells (Doody KM et al., 1990). These findings are also supported by *in vivo* observations where a rise in the serum concentrations of F in the baboon fetus (Pepe GJ and Albrecht ED, 1984) and in the umbilical artery in humans (Murphy BEP, 1982; Murphy BEP and Diez d'Aux RC, 1972; Smith ID and Shearman RP, 1974) and rhesus monkey (Mitchell et al., 1982 ; Pepe GJ et al., 1977; Pepe GJ, Albrecht ED 1984; Waddell BJ et al., 1988) occurs late in gestation. Studies in the baboon (Pepe GJ and

Albrecht ED, 1984) have confirmed that the increase in F levels reflects production by the fetal adrenal and is not the result of an increase in placental transfer of maternal F nor an alteration in fetal F metabolic clearance.

In contrast to expression late in gestation of 3β-HSD, the activities, proteins and genes for the enzymes catalyzing conversion of cholesterol to pregnenolone (P450scc) and hydroxylations (P450c17,21,11) at steroid carbons 17,21, and 11 appear to be present earlier in gestation (Pepe et al., 1977; Sholl SA, 1981; Sholl SA, 1982; Sholl SA, 1983; Voutilainen R and Miller WL, 1986; Di Blasio et al., 1987; Jaffe et al., 1988; Murphy BEP, 1969; Murphy BEP, 1972; Smith ID and Shearman RP, 1974). However, despite the apparent abundant levels of C-17,-21, and -11 hydroxylases throughout gestation, the primate fetal adrenal (Pepe GJ and Albrecht ED, 1990; Pepe GJ et al., 1988) appears to have a very limited capacity to utilize exogenous P_4 (Ducsay CA et al., 1985; Winkel CA et al., 1980; Pepe et al., 1979) or P_5 (Pep GJ et al., 1977; Pepe GJ and Albrecht ED, 1980) as substrate for F. Thus, although it was originally suggested that the fetal adrenal utilizes placental P_4 for corticoid synthesis (Solomon S et al., 1967; Voutilainen R et al., 1978; Branchaud et al., 1985), it has been shown in rhesus monkeys (Ducsay CA et al., 1985) and baboons (Pepe GJ and Albrecht ED, 1980) less than 6% of placental P_4 and P_5 secreted into the fetus prior to term is used to synthesize F .

Therefore, based on the *in vivo* enzymatic profile and developmental changes of the fetal adrenal, tree distinct steroidogenic zones can be described near term. The outer definitive zone, the major site of aldosterone synthesis, the inner definitive zone, the site of F synthesis, and the fetal cortical zone, where androgen synthesis occurs. Thus, the ability to synthesize F *de-novo* late in gestation coincides with the appearance and increased

expression of 3β-HSD protein as well as its messenger ribonucleic acid (mRNA) (Doody KM et al., 1990; Mesiano et al., 1993; Coulter CL et al., 1996; Ducsay CA et al., 1991;).

Regulation of fetal adrenal functional development.

Overview

Although the steroidogenic pattern as well as the growth and differentiation of the definitive and fetal cortical zones of the human and nonhuman primate fetal adrenal gland are clear features of intrauterine development, our understanding of the factors regulating these processes is incomplete. It has been noted that between mid to late gestation fetal adrenal size and weight in anencephalic human (Johannisson E, 1979), and rhesus monkey (Novy MJ et al., 1977) fetuses are considerably reduced compared with normal. In human anencephalic fetuses, the fetal adrenal gland develops normally up to 14-17 weeks of gestation, but after 20 weeks rapid maturation and disappearance of the transient zone results at birth in a markedly smaller (Johannisson E, 1979; Sucheston ME and Cannon MS, 1969). Moreover, plasma levels of ACTH are low in anencephalic fetuses (Seron-Ferre M and Jaffe RB, 1981). When the pituitary-adrenal axis is suppressed in normal fetuses are treated with glucocorticoids which presumably suppressed the fetal pituitary late in gestation in humans (Easterling WE et al., 1966; Ballard PL et al., 1980; Simmer HH et al., 1974; Gray ES and Abramovich D, 1980), rhesus monkeys (Challis et al., 1974), and baboons (Townsley JD 1976), the fetal adrenal atrophies and shows reduced steroidogenesis. Similar observations have been made in the lamb in which the maturation of immature fetal fascicular cells was prevented by removal of the fetal pituitary (Liggins GC et al.,1973; Robinson PM et al.,1983). Because treatment with ACTH reversed most of the effects of pituitary ablation, it appears that in the sheep fetal pituitary ACTH is

critical to fetal adrenal development during the second half of gestation. Collectively, these findings suggest that factors of fetal pituitary origin are important to fetal adrenal growth and maturation during intrauterine development in the primate. Since adrenal develops normally up to 20 weeks of gestation in anencephalic fetuses, extrapituitary factors perhaps of placental and/or maternal origin may also be important to adrenal maturation, at least early in gestation. Therefore, the regulation of the fetal adrenal appears to be multifactorial in nature.

Role of fetal pituitary ACTH

During the past decades, it has generally been accepted that ACTH is the principal trophic hormone regulating the fetal adrenal gland (Jacobs RA et al., 1994; Gulyas BJ et al., 1977; Carr BR and Simpson ER, 1981). Both immunocytochemical and biochemical studies have shown that ACTH is present in the human pituitary gland after ten weeks of gestation (Baker BL and Jaffe RB, 1975; Begeot M et al., 1977; Silman et al., 1976). Mean plasma ACTH concentrations of 240 pg/ml have been found in cord blood samples obtained at 14-34 weeks in fetuses delivered by hysterectomy. A decrease to 160 pg/ml in plasma ACTH concentrations occurs after 34 weeks. However, these single point determinations of fetal ACTH may not accurately reflect hypothalamic-pituitary function *in vivo* because of the multiple sites of secretion, stress and potential pulsatile nature of pituitary ACTH release (Pepe GJ et al., 1994). In the sheep, immunoreactive ACTH becomes detectable in fetal blood on about day 100 of gestation and steadily increases at a rate of approximately 5pg/ml between days 100 to 140 of gestation (Saez JM et al., 1984; Jones CT et al., 1977; Jones CT et al 1980; Challis JRG and Brooks AN, 1989). Progressive gestational increases in fetal pituitary POMC mRNA and ACTH output by the

baboon (Pepe GJ et al., 1994;) and human (Siler-Khodr TM, 1974) have also been described. However, recent studies using *in vitro* and *in vivo* experimental paradigms to study the relative importance of fetal pituitary ACTH upon fetal adrenal growth, differentiation, and steroidogenesis have yielded equivocal results. For example, it has been shown in cultures of human fetal adrenal cells that ACTH either inhibits (Simonian MH and Gill GN, 1981), activates (Diblasio AM et al., 1990), partially activates (Jaffe RB et al., 1977) or has no effect (Simonian MH and Gill GN, 1981) on cell proliferation and/or steroidogenesis. Stimulatory effects of ACTH on proliferation of the adult-type definitive cells was reported after culture of collagenase-dispersed human fetal adrenal cells obtained early in gestation. However, ACTH caused involution and subsequent disappearance of the large fetal cortical zone cells under the same experimental conditions (Kahri AI et al., 1976; Fujieda K, 1982). Jaffe et al (1977) demonstrated ACTH stimulation of F secretion and binding in the definitive zone cells of human adrenals in early gestation but inconsistent ACTH induced secretion of DHAS from the fetal zone cells. In different culture conditions, addition of ACTH increased the rate of growth and the cytoplasmic size (i.e. hypertrophy) of the definitive cells over an 8-day period but had no apparent effect on fetal zone cells (Kahri AI et al., 1976; Kahri AI et al., 1974). These results contrast with studies demonstrating that ACTH induced proliferation and enhanced steroidogenesis of cultures of fetal zone cells obtained from fetuses at 18-20 of weeks of gestation (Diblasio AM et al., 1990). Moreover, 3βHSD expression, used as a marker to asses adrenal corticosteroid-producing cell proliferation, was increased in response to ACTH in human definitive and fetal zone cells *in vitro* (Mesiano S and Jaffe RB, 1993; Mason et al., 1993). It has also been shown that ACTH inhibits mitosis of adult human

and bovine adrenocortical cells in culture (Hornsby PJ and Gill GN, 1977; Hornsby PJ and Gill GN, 1978) as well as human fetal definitive and cortical cells cultured for more than 6 days (Simonian MH and Gill, 1981). An important limitation of primary cultures of midgestational fetal adrenal cortical cells is that they undergo "maturation" and other phenotypic changes *in vitro* including the capacity to synthesize F in the presence of added ACTH (Kahri AI and Halinen H, 1974). Simonian and Gill (1981) demonstrated that initially, fetal and definitive zone cells in culture increased their secretion of their characteristic steroids, DHAS and F, respectively; however, after 3-4 days of exposure to ACTH the pattern of steroid secretion by the fetal zone cells shifted to F production, indicating induction of 3βHSD activity in these cells. Therefore, primary cell culture of fetal adrenal gland may not be the best model to evaluate the role of ACHT on fetal and definitive zone function and development *in vivo*.

Western blot analysis of fetal adrenals, obtained during the first and second trimester of human pregnancy demonstrated the absence of 3βHSD protein in the fetal zone and its exclusive presence only in the definitive zone cells (Doody KM et al., 1990). The absence of 3βHSD protein in the fetal zone explained the low ACTH-stimulated F secretion in these cells relative to the 4 to 5-fold increase in ACTH induced F production in culture of definitive zone cells. In partial agreement with lack of 3βHSD protein in the fetal zone, *in situ* localization of 3βHSD mRNA in adrenal gland sections from midgestation human fetuses is not detected in definitive nor fetal zone cells (Mesiano et al., 1993; Dupontcortisoneet al., 1990). However, Parker et al., (1995) using immunohistochemistry, detected expression of 3βHSD in definitive zone cells and occasionally in fetal zone cells in early human gestation. In mid gestation 3βHSD

expression was low in definitive zone cells and virtually absent from the fetal zone. Throughout the third trimester expression of 3βHSD was localized in the definitive zone with some immunoreactivity near the central medullary vein of the adrenal (Parker et al., 1995).

To investigate the ontogeny of adrenal steroid hydroxylases with reference to ACTH concentration in sheep, John et al.(1987), conducted an experiment concluding that, although pituitary ACTH may be important to the increase in P450c17 measured late in gestation, ACTH may not be required for the initial expression of the P-450 hydroxylases in the human adrenal gland. Similar conclusions have been obtained using fetal bovine adrenal cells (Lund et al.,1988). Fetal adrenal adenylate cyclase activity was not detectable in cultured human adrenals from anencephalics, although enzyme activity was very high in both the definitive and cortical zones of glands obtained from normal fetuses. Collectively, these results demonstrate that the role of ACTH *per se* on growth (hyperplasia and /or hypertrophy) and 3βHSD, a marker of functional maturation of definitive zone and steroidogenic capacity, remain to be determined.

In the adult adrenal, ACTH stimulates the synthesis and release of steroids by interacting with cell-surface receptors that stimulate adenylate cyclase and the production of cAMP (Jefcoate CR et al., 1986 ; Nicholson WE et al., 1978; Kojima I et al., 1985;). Cyclic AMP then brings about a change in the activity of different protein kinases, including cAMP-dependent protein kinase A (PKA). One of the roles of PKA is to phosphorylate the cAMP response element binding protein (CREB). CREB is a member of a family of DNA-binding proteins known as bZIP proteins that consist of distinct DNA-binding and transactivation domains (Meyer ET et al., 1993). CREB protein binds as a

homodimer to the cAMP response element (CRE), a palindromic octamer (TGACGTCA) that usually lies upstream of the basal promoter of a significant number of genes. The DNA-binding phosphoprotein CREB is responsible for regulation of gene transcription in response to hormonal stimulation of the cAMP pathway (Meyer TE and Habener JF, 1993). cAMP also autoregulates its own expression by modulating three cAMP response elements responsible for cAMP induction of transcription (Meyer et al., 1993; Walker WH et al., 1996). It is not completely understood which specific proteins are phosphorylated in response to ACTH to activate steroidogenesis. However, accumulation of cAMP does precede steroidogenesis, and binding of cAMP to the regulatory subunit of protein kinase A correlates well with stimulation of steroidogenesis (Hayashi K et al., 1979)

As in the adult adrenal, *in vitro* studies of human adrenal cortical cells from midgestation fetuses has shown that these cells do contain ACTH receptor mRNA and that ACTH receptor expression is upregulated by its own ligand. This upregulation is apparently mediated in the human (Lebrethon et al., 1994; Mesiano et al., 1996) and baboon (Davies WA et al., 1993) fetal adrenal by a PKA, and not a PKC, mechanism. Recently, our laboratory (Albrecht et al, 1996) demonstrated that ACTH receptor mRNA levels in the baboon fetus increase 13-fold from early to mid gestation and then unexpectedly declined by 70% in term adrenal. This biphasic monomodal developmental expression contrasts with previous data demonstrating a progressive increase in adrenal weight (Albretch et al., 1996), the ontogenic increase in activity of steroidogenic enzymes (Pepe GJ and Albrecht ED, 1991) and an increase in *de novo* F production near term (Pepe GJ et al., 1990) consistent with action of ACTH in both fetal and definitive zone cells. To address this apparent paradox, these authors have recently demonstrated that in

contrast to the developmental increases in growth and function of the definitive zone, there is a decline in the output per cell of fetal zone-specific DHA, between mid and late gestation (Albrecht ED and Pepe GJ, private communication). Although the secretion of DHAS increased with advancing gestation, the absolute levels of DHA and DHAS were lower than those of F near term. The increase in size of the fetal adrenals, apparently accounts for the progressive increase in total adrenal C19-steroid output. These findings suggest that there is an uncoupling of development between the fetal zone and the definitive zone in the second half of pregnancy, again indicating a multifactorial regulation of fetal adrenal maturation.

In situ hybridization and immunohistochemical studies of rhesus monkey adrenal gland from mid and late gestation (Coulter et al.,1996) demonstrated that stimulation of pituitary ACTH secretion increased 3βHSD expression in both the definitive and transitional zone. The ACTH-induced increase in P450c17 mRNA in the transition zone exceed that in the fetal zone. In contrast, the relative abundance of P450scc detected in the definitive, transitional and fetal zones was not altered by increased ACTH secretion. Collectively, these data suggest that the regulation of adrenal growth and maturation including increased expression of 3βHSD and F production late in gestation may be more complex than initially thought. A major objective of the present work is to study the developmental role and specific action of pituitary ACTH on fetal adrenal growth (hypertrophy and/or hyperplasia) and cellular differentiation *in vivo* and the onset of 3βHSD expression in the baboon fetal adrenal gland and how estrogen may modulate this process.

Indirect regulation of pituitary ACTH production by estrogen modulation of placental metabolism of placental metabolism and of maternal F and cortisone

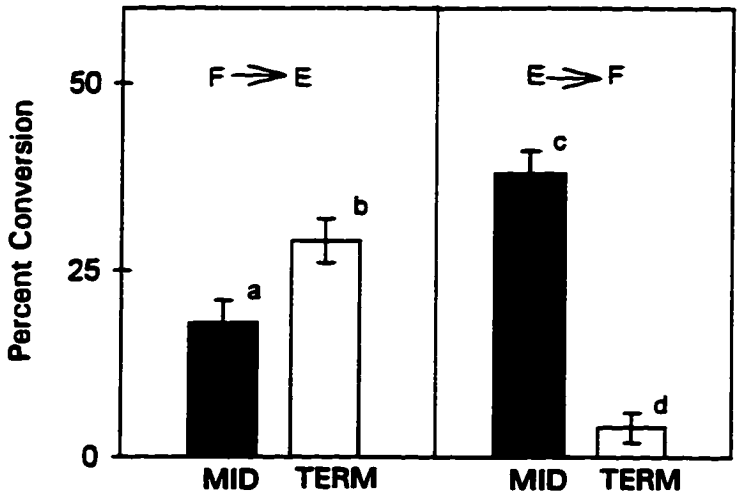
F is measurable in the fetal circulation throughout pregnancy in the human (Murphy BEP, 1982) and nonhuman primates (Pepe GJ and Albrecht ED, 1984; Jaffe RB et al., 1978). Because of the enzymatic profile of the fetal adrenal, the fetus must obtain its F from the maternal circulation at least in early gestation. Indeed studies in the human (Beitins IZ et al., 1973; Migeon CJ et al., 1957) and rhesus monkey (Kittinger GW, 1974, Michell BF et al., 1982) indicate that there is extensive movement of F across the placenta, between the fetal and maternal circulation throughout gestation. In a comprehensive study, Pepe and Albrecht (1984), compared transplacental interconversion of F and cortisone (E) in the baboon *in vivo*. At mid gestation, reduction of the biologically inactive metabolite cortisone to its biologically active form F, exceeded the reverse reaction whereas by term oxidation of F to cortisone was seven fold greater than the conversion of cortisone to F. Since the total umbilical F concentration did not vary between mid and late gestation, these authors calculated that near term the baboon fetuses must produce approximately 60% of F *de novo*. In human, the maternal ratio of infused [14 C]F *in vivo* was four times greater than that for endogenous F suggesting that near term the human fetus must produce approximately 75% of its F *de novo* (Beitins IZ et al., 1973). Pepe and Albrecht (Pepe GJ and Albrecht ED, 1985) concluded that the differences in transplacental F - cortisone interconversion between mid and late gestation may be of physiological significance to the maturation and development of fetal-pituitary-adrenal axis at term. Based on these observations, they proposed that the placenta, via metabolism of F - cortisone, regulates the qualitative and quantitative patterns of these corticosteroids

reaching the fetal pituitary gland at mid and term gestation. Thus preferential conversion of maternal cortisone to F in placenta when fetal production of F is low has a negative feed back effect on fetal pituitary secretion of ACTH, thereby limiting fetal adrenal *de novo* F synthesis through most of pregnancy. By term gestation, however, increased oxidation of maternal F to biologically inactive cortisone by the placenta, reduces maternally-derived fetal serum F levels, allowing stimulation of fetal pituitary secretion of trophic hormone and the timely onset of *de novo* F production by the fetal adrenal gland.

The principal secretory androgen of the human fetal adrenal gland throughout gestation is DHAS and to a lesser extent its nonconjugated form DHA (Seron-Ferre M and Jaffe RB, 1981). The production of adrenal androgens from P_5 requires 17α-hydroxylation, thus the major source of DHAS in the fetus is the adrenal fetal zone. In the fetal rhesus monkey the serum levels and daily production rates of DHAS increase steadily during gestation and parallel fetal cortical growth (Seron-Ferre M et al., 1973). DHAS is extensively metabolized to 16-OH DHA by the fetal liver in the human but not in the baboon. Both DHAS and 16-OH DHAS can be hydrolyzed by the human placenta to the non-conjugated form (Boltecortisoneet al., 1964). After hydrolysis, 16-OH DHA is aromatized by the placenta to estriol, quantitatively the major estrogen present in pregnant woman, whereas DHA is aromatized to estrone and estradiol (Seron-Ferre M and Jaffe RB, 1981). Thus, fetal adrenal androgens are precursors for placental production of estrogens in humans (Simmer HH et al., 1964; Chang RJ et al., 1976; Easterling WE, 1966) and nonhuman primates (Pepe GJ and Albrecht ED, 1985; Kling et al., 1972; Townsley JD et al., 1977; Walsh SW, 1979; Jaffe et al., 1981; Lopez-Bernal et al., 1980; Seron-Ferre M et al., 1983). The interdependence of fetus, placenta and mother in the

Seron-Ferre M et al., 1983). The interdependence of fetus, placenta and mother in the formation of estrogens has led to the concept of the "fetoplacentomaternal unit" (Pepe GJ and Albrecht ED, 1990; Seron-Ferre M and Jaffe RB, 1981).

Pepe and Albrecht have extensively studied the role of estradiol (E_2), the production of which increases with advancing gestation in the human (Diczfalusy E, 1974; Siiteri PK and MacDonald PC, 1966) and in the baboon (Albrecht ED and Townsley JD, 1978; Kling OR et al., 1972; Albrecht ED et al., 1980), on the regulation of placental corticosteroid metabolism. In earlier studies they showed that administration of the antiestrogen ethamoxytriphetol (MER-25) to baboons between days140 and 170 of gestation (term=day184) resulted in a pattern of F - cortisone metabolism across the placenta near term which is normally seen at mid-gestation. An alternative approach used to study the role of estrogen on transplacental F - cortisone metabolism was to suppress estrogen production by fetectomy, or to enhance estrogen production by premature administration of the aromatizable C19-steroid precursor androstenedione. Removal of the fetus significantly reduced serum E_2 levels and prevented the change in placental F - cortisone metabolism which normally occurs in intact animals between mid and late gestation (Pepe GJ and Albrecht ED, 1987). Premature increase in estrogen levels at mid gestation (Pepe et al., 1988) to values similar to those at term resulted in a preferential increase in placental oxidation of F - cortisone, a pattern similar to that seen in term animals. This interconversion pattern in the placenta is presented diagrammatically below:

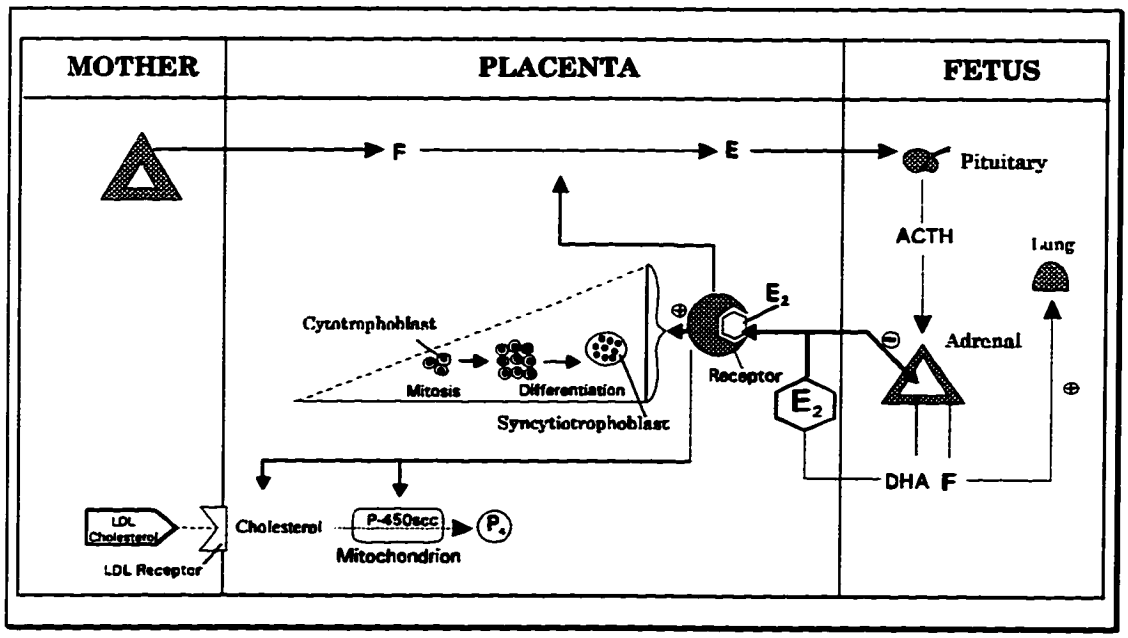


Based on these observations, Pepe and Albrecht concluded that estrogen regulates the change in placental F -cortisone metabolism, decreases fetal maternally-derived F concentration, and thus indirectly, activates the fetal hypothalamic pituitary axis which results in the timely onset of *de novo* F production by the fetal adrenal gland.

Recent studies have now confirmed that the interconversion of F and cortisone is catalyzed by two different 11β-hydroxysteroid dehydrogenase (11βHSD) enzymes (Pepe JG and Albrecht ED, 1995; Stewart PM et al., 1995; Stewart PM et al., 1994); 11βHSD-1, an NADP(H)-dependent dehydrogenase/oxo-reductase with low affinity for F , and 11βHSD-2 a high affinity, NAD-dependent dehydrogenase only oxidizing F to cortisone (Albiston AL et al.,, 1994; Sewart PM and Mason JI, 1995; Whorwood CB et al., 1995). Classic enzyme kinetics (Pepe GJ and Albrecht ED, 1985; Baggia S et al., 1990; Pepe GJ et al., 1996, Baggia S et al., 1990) demonstrated that baboon placental NAD(H)-

dependent 11βHSD oxidase activity at midgestation was increased 4-fold in animals in which placental estrogen production was increased by treatment of the mother with aromatizable androstenedione or estradiol and decreased at term in baboons in which placental estrogen production was depleted by fetectomy. *In vitro* studies (Pepe GJ et al., 1996) demonstrated that the baboon placenta expressed the mRNA for 11βHSD-1 at mid gestation favoring the formation of cortisone to F, presumably catalyzed by this 11βHSD-1 enzyme protein. Although 11βHSD-1 mRNA or protein has not been consistently detected in human placenta (Stewart PM et al., 1994; Stewart PM et al., 1995; Whorwood CB et al., 1995; Sun K et al., 1996; Lakshmi V et al., 1993), the mRNA and protein for 11βHSD-2 have always been detected (Stewart PM et al., 1994; Brown RW et al., 1996; Krozowski Z et al., 1995). In recent studies Pepe and Albrecht demonstrated that both the mRNA and protein for both 11βHSD enzymes are expressed in baboon and human placenta. Moreover, levels of mRNA and protein increased for both enzymes with advancing gestation. Up-regulation of 11βHSD-1 seems to contradict previous results where transplacental conversion of cortisone to F *in utero* declined with advancing gestation. To address these conflicting results, Pepe and Albrecht (Pepe et al., 1996) have recently proposed that changes in transplacental corticosteroid metabolism may result from an estrogen dependent change in the compartmentalization of 11βHSD-1 and 11βHSD-2 within the syncytiotrophoblast. Collectively, these observations and earlier studies on adrenal function support the concept that initiation of fetal adrenal maturation near term is modulated by an estrogen-regulated change in the expression and location of the two 11βHSD enzymes. Thus between early and midgestation, because placental

metabolism favores reduction of cortisone to F, bioactive F arrives in the fetus and blocks fetal ACTH production and adrenal definitive zone 3βHSD-catalyzed *de novo* F synthesis; however by term with increasing estrogen resulting in an up-regulation and compartmentalization of the two 11βHSD enzymes, bioinactive cortisone is preferentially delivered across the placenta into the fetus permitting activation of the fetal pituitary-adrenocortical system. (ACTH cannot cross the primate placenta; thus only ACTH secreted by the fetal pituitary reaches the fetal adrenal). This hypothesis is presented diagrammatically below (adapted from Albecht ED and Pepe GJ, PHS 398, 1997):



In support of this concept of the activation of baboon hypotalamic-pituitary-adrenal-axis (HPAA) by E_2 these authors demonstrated an increase in fetal pituitary POMC mRNA and ACTH peptide content between mid and late gestation (Pepe GJ et al.,

1994), which parallels the ontogenic increase of the adrenal enzymes P450c17 and 3βHSD (Pepe GJ et al., 1991; Davies WA et al., 1993) and the subsequent initiation of *de novo* F synthesis near term within the fetus. Similar changes in the pituitary POMC and ACTH content also occurred when the levels of E_2 were prematurely increased (Pepe GJ et al., 1990).

Role of pituitary/placental factors in addition to ACTH

It has also been suggested that ACTH and other peptides of fetal pituitary origin and/or perhaps placental tissues, interact to modulate adrenal growth. It is well known that in human anencephalic fetuses, the fetal adrenal gland develops normally up to 14-17 weeks of gestation, i.e. in the absence of pituitary ACTH (Benirschke K, 1956; Gulyas BJ et al., 1977; Gray ES et al., 1980), supporting the suggestion that extrapituitary factors perhaps of maternal and/or placental origin, e.g. hCG (Seron-Ferre M et al., 1978), PRL (Pepe GJ and Albrecht ED, 1985; Pepe GJ and Albrecht ED, 1985) or various growth factors (Pepe GJ and Albrecht ED, 1980), are important to early adrenal maturation. In addition to the actions of peptide factors, it has also been suggested that the high concentrations of circulating E_2 in the human fetus modulate F and DHAS secretion by fetal definitive and cortical cells, respectively. Indeed, Fujeida et al (1982) and Mesiano et al (1993) demonstrated that at concentrations ranging from 1-10 μmol/L, E_2 inhibited ACTH-stimulated F production and increased DHA production by cultured human fetal zone cells. Although E_2 alone had no effect on steroidogenesis or on basal expression of steroidogenic enzymes, E_2 treatment increased the abundance of ACTH-stimulated mRNA encoding P450scc and P450c17. The abundance of mRNA for 3βHSD was not affected. These data suggest that E_2 modifies ACTH-regulated steroidogenic capacity of cultured

human fetal adrenal cells to favor DHAS rather than F synthesis. These findings contrast with *in vitro* studies in the baboon in which short-term incubates of fetal cortical cells with estrogen suppressed the stimulatory effects of ACTH with respect to the formation of the Δ^5-steroids DHA and DHAS. However, production of F , which was minimal, was not affected (Albrecht ED and Pepe GJ, 1987; Pepe GJ et al., 1989). The apparent different effects elicited by estrogen in these *in vitro* studies may be related to the degree of adrenal maturation since the fetal adrenal is known to rapidly differentiate in culture. The possible effects of ontogeny may also be crucial in light of the fact that fetal adrenal 3βHSD activity increases only very late in gestation (Pepe GJ et al., 1990) and at a time during which estrogen production in human (Tulchinski et al., 1982;) and baboons (Albrecht ED and Townsley, 1978) is maximal.

Pepe and colleagues, have confirmed the inhibitory effects of estrogen on the fetal adrenal response to ACTH at midgestation using an *in vivo* experimental paradigm (Pepe GJ et al., 1988). Thus, the ability of the fetal adrenal to increase DHA production in response to an acute infusion of ACTH was abolished in baboons in which placental estrogen production was increased prematurely at midgestation. Collectively, results in the baboon (Albrecht ED and Pepe GJ, 1987) support the hypothesis that estrogen produced by the placenta from androgen precursors of fetal adrenal origin feeds back selectively on the fetal cortical zone to suppress the formation of C_{19}-steroid estrogen precursors, i.e. DHA and DHAS, but not F production by definitive zone cells at midgestation.

Direct effect of estrogen on target tissues

The binding of steroid hormones to specific, high affinity binding proteins, i.e. receptors, in tissues acts to coordinate complex events involved in development,

differentiation, and physiological regulation. Transduction of the hormonal signal is mediated through the binding of receptor-hormone complexes to target sites in the promoter regions of regulated genes (hormone-responsive elements), thereby repressing or activating the level of specific gene transcription (Gronemeyer H, 1992; Parker MG, 1993; Dean DM and Sanders MM, 1996;). Early studies use a translocation model to explain the mechanism of estrogen action, which suggested the binding of estrogen to a cytoplasmic receptor to form a receptor-estrogen complex. The binding was suggested to result in an estrogen-induced translocation of the estrogen receptor to the nucleus to regulate specific genes (Shyamala G and Gorski J, 1969; Gorski J et al., 1973). However, studies in the past decade have shown that the cytosolic receptor is not truly cytoplasmic but rather a nuclear receptor unoccupied with ligand that is in a state readily extractable from the nucleus (Welshons WV et al., 1984; King WJ and Greene GL, 1984). In contrast, the estrogen-occupied receptor has a much higher affinity for the nucleus and can only be extracted with high osmolar salt solutions. Immunodetection of ER with a variety of antibodies directed against different epitopes of the ER molecule, is now often used as a reliable method to detect the presence of the steroid receptor (Abbondanza C et al., 1993; Furlow et al., 1990; McClellan MC et al., 1984; Greene GL et al., 1980; Linstedt AD et al., 1986). Most studies today indicate the estrogen receptor, as well as the other gonadal steroid receptors, to be a nuclear protein even when it is not occupied by estrogen.

The steroid hormone receptors form a large family of transcription regulatory factors with varying degrees of sequence homology. Molecular cloning of the cDNA of the steroids receptors has led to rapid advances in our knowledge of these proteins. Thus, discrete functional domains for DNA binding, ligand binding and transcriptional activation

have been identified. However, significant gaps still exist in our understanding of the mechanism by which the receptor stimulates or represses gene transcription. For many years the mammalian uterus and oviducts have been used as the model for studying estrogen-dependent signal transduction pathways. These well characterized systems show that estrogen up-regulates ER and progesterone receptors at lower concentrations in rodents (Bergman MD et al., 1992; Kassis JA et al., 1984; Zhou Y et al., 1993; Zhou Y et al., 1995, Hsueh AJW et al ., 1974; Walters MR et al., 1979; Hillier SG et al., 1988; Kudolo GB et al., 1983), nonhuman primates (Brenner et al., 1974; Slayden OD et al., 1993; McClellan MC et al, 1984) and humans (Press MF et al., 1986). However, E_2 has an inhibitory effect at supraphysiological levels in rat uterus (Zhou Y et al., 1993, Shupnik MA et al., 1989) and MCF-7 breast cancer cells (Saceda M et al., 1988; Red LD et al., 1989).

A more recent advance in the understanding of ER regulation is that it is tissue specific and not modulated by circulating estrogen levels in all ER-containing tissues. Thus, tissue specific regulation of ER mRNAs and protein have been described in different species. In rat, the mRNA encoding for ER in uterus, liver and pituitary were regulated in different directions by E_2. In uterus, ER mRNA increases after ovariectomy, returning to base levels after E_2 replacement. In contrast, liver ER mRNA declines after ovariectomy, returning to normal levels after E_2 replacement. Pituitary ER mRNA decreases after ovariectomy, increasing after E_2 replacement to return subsequently to basal levels (Shupnik MA et al., 1989). Thus, in the rat elevated estrogen levels appear to reduce expression of ER in uterus while up-regulating its expression in liver and pituitary. In another study it was demonstrated that changes of ER in the hypothalamus are not in

phase with those of the uterus during the rat estrous cycle (Zhou Y et al., 1995). Moreover, in normal human breast epithelial and fibroblast cells cultured separately, E_2 increased the intensity of ER protein in the epithelial cells but had no effect on fibroblast ER (Malet CA et al., 1991). These observations suggest that the tissue specific pattern of ER protein and mRNA levels are regulated differently by the same levels of circulating estrogen. How this tissue-specific expression pattern of ER is controlled is not completely understood. Some recent studies have proposed tissue specific differential promoter utilization of the human estrogen receptor (Gradien K et al, 1995;), ligand-independent activation of the ER by tyrosine phosphorylation (Arnold SF et al., 1995; Auricchio F et al., 1995) or protein-protein interaction (Skipper JK). Additional studies also described ER binding to non-classical estrogen response elements in the rat prolactin gene (Murdoch FE et al., 1995).

There is little information concerning the regulation of the ER in the adrenal either during development or in adult life. In contrast with numerous studies of localization and upregulation of ER by E_2 in rhesus monkey endometrium (Slayden OD et al. 1993; Koji T and Brenner RM, 1993; McClellan MC et al, 1984) and oviducts (Brenner RM et al., 1974;), only one study examined concentration and distribution of ER in primate fetal and adult adrenals. (Hirst et al., 1992). In the fetal adrenal, nuclear immunolocalization of ER was present in a narrow definitive zone adjacent to the capsule but was absent throughout the fetal zone.

Recently, Albrecht and colleagues (Albrecht ED et al., 1996) have proposed a critical role for estrogen in the second half of pregnancy to diminish ACTH receptor expression in the baboon fetal gland. Thus, it has been proposed that estrogen normally

feeds back (i.e. baboon umbilical vein serum estradiol 0.2 ng/ml at mid gestation and 3.0 ng/ml in late gestation) to restrain ACTH-regulated growth and steroidogenesis in the fetal zone, while simultaneously enhancing via the placental activation of the 11β-HSD system (Albrecht et al., 1996) ACTH-regulated development of the definitive zone. This would imply that ER might also be present in the fetal zone cells.

Therefore, it has been proposed that estrogen has two different effects on the fetal HPAA; an indirect one to enhance fetal pituitary ACTH secretion which results in timely maturation of the definitive zone and F biosynthesis; and concomitantly, a direct action on the fetal cortical zone to modulate the degree of C_{19}-steroid production to ensure an appropriate level of placental E_2 production. The mechanism by which estrogen acts at the level of the fetal adrenal remains to be elucidated. In the present proposal, experiments are designed to examine the role of estrogen on expression of key steroidogenic enzymes, to determine if estrogen receptors are present in fetal and/or definitive zone cells and to evaluate the mechanism(s) by which estrogen modulates ACTH regulated adrenal steroidogenesis. The effect of decreasing estrogen levels will be compared and integrated with another method of decreasing fetal ACTH levels, i.e. administration of the synthetic glucocorticoid betamethasone.

Specific Aims

Development of the adrenal gland is linked closely in mammalian fetuses to important physiological events of pregnancy, including maintenance of gestation, induction of parturition and maturation of various fetal organs systems in preparation for extrauterine life (Pepe GJ and Albrecht ED, 1990). Thus, F , of fetal adrenal origin, is one of the chemical messengers involved in the stimuli to lung maturation, deposition of glycogen in

the liver, and induction of enzymes in the brain, retina, pancreas and gastrointestinal tract that normally are associated with late intrauterine life (Albrecht ED and Pepe GJ, 1988). In addition to these roles for fetal adrenal F , it is well established that the fetal adrenal gland in primates, including humans, is important to the synthesis and secretion of androgen precursors essential to the production of estrogen by the placenta (Albrecht ED et al., 1980). It is apparent, therefore, that aberrations in adrenal function in fetal life could have deleterious consequences to pregnancy maintenance as well as fetal and subsequently neonatal development. In humans and non human primates, including the baboon (*Papio anubis*), the fetal adrenal gland is composed of two principal zones, one zone comprised of fetal cortical cells and termed the fetal zone, and the other, comprised of adult type tissue and termed the definitive zone. The fetal zone constitutes about 80% of the volume of the gland during the majority of gestation and is the principal source of the 17α-hydroxylase C17,20 lyase cytochrome P450 enzyme ($P450_{17\text{-}OH}$) catalyzing the formation of the Δ^5C_{19} steroid hormones dehydroepiandrosterone (DHA) and DHA-sulfate (DHAS) (Pepe ED and Albrecht GJ, 1990; Petito SH et al., 1988). In contrast, the definitive zone cells not only express $P450_{17\text{-}OH}$ but are the only cells which express 3β-hydroxysteroid dehydrogenase/ isomerase (3βHSD). The 3βHSD enzyme is present in very low amounts during most of intrauterine development and thus DHA and not F is the quantitatively significant hormone secreted by the fetal adrenal. However, near term as a result of the activation of the fetal hypothalamic-hypophyseal axis leading to increased formation of pituitary ACTH, adrenal 3βHSD activity is increased, and in response to ACTH, definitive zone cells secrete F . It has also been demonstrated that estrogen suppressed the response of the baboon fetal adrenal to ACTH *in vitro* and *in vivo* with respect to the formation of

the DHA and DHAS (Albrecht ED and Pepe GJ, 1987; Albrecht ED and Townsley JD, 1978). Estrogen also suppressed DHA and DHAS, but not F , secretion *in vivo* by the adult adrenal of pregnant and nonpregnant baboons (Albrecht ED and Pepe GJ, 1995). It is apparent that the fetal and definitive zone cells of the developing adrenal gland are exposed to factors that modulate their steroidogenic response to ACTH. The studies outlined in this proposal are designed to test the overall hypothesis that ACTH and estrogen interact to regulate growth and steroid hormone biosynthesis within the baboon fetal adrenal cortex. Experiments will be utilized to examine the effects of estrogen deficient/repleted baboons or betamethasone treatment on fetal adrenal cellular hyperplasia and hypertrophy, differentiation (marker: 3βHSD, P450c17, P450scc), induction of ACTH receptor mRNA expression, and cellular remodeling (i.e. apoptosis). This will be done to test the following specific hypotheses:

Hypothesis I. Placental estrogen modulates ACTH-induced growth and maturation of the fetal definitive zone (e.g. 3βHSD, P450c17) while concomitantly acting to modulate ACTH action on fetal cortical cell hyperplasia and /or hypertrophy and synthesis of DHA. To test this hypothesis the following *in vivo* treatments will be employed:

A. Animals in which ACTH levels are altered:

a) animals in which fetal pituitary POMC/ACTH expression was suppressed at midgestation by administration of betamethasone, a synthetic glucocorticoid;

b) animals in which ACTH was restored at midgestation by administration of ACTH in addition to betamethasone;

c) animals in which fetal ACTH was prematurely increased at midgestation by administration of ACTH to the fetus;

d) animals in which fetal pituitary POMC/ACTH expression was suppressed at term gestation by administration of betamethasone to the fetus or to the fetus and mother.

B. Animals in which ACTH levels are altered will be compared with:

a) animals in which estrogen synthesis was blocked by administration of the aromatase inhibitor CGS 20267 throughout the second half of gestation.

b) animals treated simultaneously with CGS 20267 and estradiol throughout the second half of gestation to study the specifity of CGS effects.

Hypothesis II. Estrogen exerts its action(s) in the fetal adrenal gland via a receptor-mediated mechanism. To test this hypothesis, fetal adrenals of varying gestational ages and from the above treatment groups will be examined for the presence/absence of the estrogen receptor by immunocytochemistry. This will permit the study of the tissue distribution and the estimation of the content (semi-quantitative by cell number and labeling intensity) of the receptor.

CHAPTER II

MATERIALS AND METHODS

The baboon as a model for the study of the endocrinology of human pregnancy

Physical and anatomical considerations

The baboon is considerably larger than the extensively used macaque species. Yet, the animal is docile, easy to handle and extremely adaptable. Because of its size, blood and other tissue samples can be collected at discrete periods during gestation. As in humans, the baboon possesses a hemochorial and monodiscoid placenta and a fetoplacental unit in which adrenal androgen precursors are used for placental estrogen synthesis.

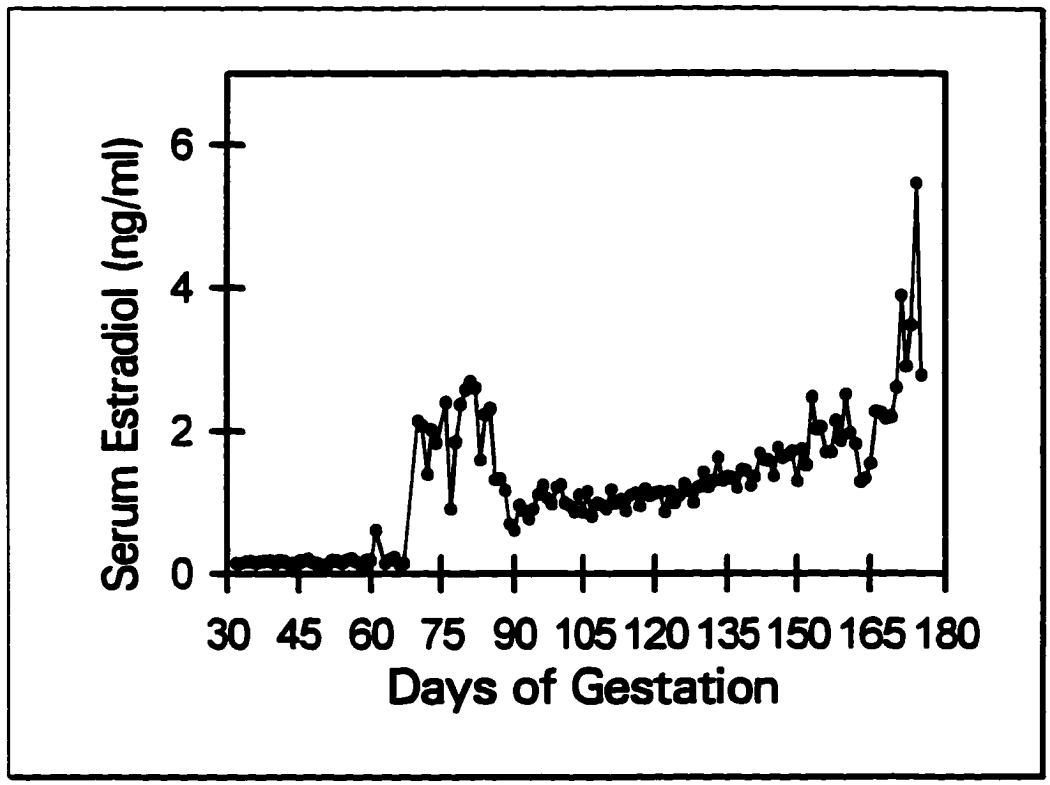
Endocrinological considerations

Progesterone and Estrogen

The maternal pattern of placental steroid hormone production exhibited in the pregnant baboon is similar in many important aspects to that in pregnant woman. The rate of P_4 production (Albrecht ED and Townsley JD, 1976; Goldzieher JW and Axelrod LR, 1969) and serum P_4 concentrations (Albrecht ED and Townsley JD, 1976) are elevated during pregnancy in baboons as in women, although the absolute amounts of P_4 are less in baboons. The metabolism of P_4 is also similar in these two species (Goldzieher JW and Axelrod LR, 1969) the major metabolite being pregnanediol (Merdatz IR and Beling CG, 1969; Goldzieher JW and Axelrod LR, 1969).

The concentrations and secretory pattern (Albrecht ED and Townsley JD, 1978) of estradiol (E_2) and estrone (E_1) in the maternal and fetal circulation of pregnant baboons are also similar to those in pregnant women. Moreover, the absolute amounts of E_2 present in the

peripheral circulation at term in baboons are in the same order of magnitude as those in women. In the baboon however, the major urinary estrogen is E_1 and not estriol (E_3). Maternal serum estradiol levels during the baboon pregnancy are shown below (adapted from Albrecht ED and Pepe GJ, PHS 398, 1997) :



Corticosteroids

The metabolism of F (cortisol) and cortisone in the adult female baboon are similar to that in the women. In both species, adrenals of adult animals secrete only F and 99% of serum levels of cortisone are derived from secreted F (Pepe GJ and Townsley JD, 1978). During pregnancy the pattern of F- cortisone metabolism is altered in both species (Pepe GJ and

Townsley JD, 1975); an increase in corticosteroid binding globulin is observed, without an increase in the production rate of F (Oakey RE, 1975; Pepe GJ et al., 1976). Examination of urinary metabolites of F in baboon neonates revealed a pattern very similar to that in human neonates; a low F production rate, reduced glucuronylation and an increase in production of highly polar compounds such as 6β-hydroxy F (Pepe GJ and Townsley JD, 1976). Development, function and morphological zonation of the fetal adrenal in the human and the baboon are similar and distinct from those in other species.

Animal Maintenance

A colony of baboons (*Papio anubis*) was established in 1978 at the Eastern Virginia Medical School in the Central Animal Facility. Female baboons weighing 13-16 kg, are housed individually in aluminum-stainless-steel primate cages (32.5" x 36" x 56", LC-1106 baboon cage, Research Equipment Co, Bryan, TX) in air-conditioned rooms with 12/12 h light/dark lighting schedule. Animals receive Purina monkey chow (Ralston Purina Co., St. Louis, MO) twice daily, fresh fruit and vitamins once a day and water *at libitum*. Baboons were cared for and used strictly in accordance with USDA regulations and the NIH Guide for the Care and Use of Laboratory Animals (Publication 85-23, 1985). The experimental protocols employed in this study were approved by the Institutional Animal Care and Use Committee of the Eastern Virginia Medical School.

The menstrual cycle of the baboon

The menstrual cycle of the baboons resembles that of the human in all major phases with the exception of a short definite estrus cycle in the baboon as in other nonhuman primates. In our colony the average length of the menstrual cycle, as determined by the first day of bleeding of one cycle up to the onset of bleeding in the following cycle is 29 days. Changes in

the perineum (sex skin) is used to determine various stages of the menstrual cycle. They are the proliferative, ovulatory, luteal, and involution phases. These stages of the cycle are correlated with changes in the perineum area and offers a visual indication of hormonal activity and sexual receptivity.

The changes in the sex skin can be described as: a) perineal turgescence b) maximum turgescence c) perineal deturgescence, and d) perineal rest (Hendrickx AG, 1967).

a) Perineal turgescence. At this stage the perineum begins to swell and the color deepens from a dull pinkish red to a brighter red. The turgescence of the perineum continues through the postmenstrual and preovulatory phases. This period lasts from 4 to 8 days.

b) Maximum turgescence. During this time period which is associated with maximum estrogen production, the perineum becomes fully distended and reaches its deepest and most intense color, a bright red. The entire perineum is devoid of wrinkles and has smooth, shiny appearance. The period of maximum turgescence encompasses the latter part of the preovulatory phase, the entire ovulatory phase, the transition period and in most instances the postovulatory phase. Usually there is a brief period of near-maximum turgescence followed by another brief period of about five days when the perineum reaches its maximum size. Ovulation seems to occur during the last half of the maximum-turgescence period. The entire period last between 5 to 15 days.

c) Perineal deturgescence. This period, in almost all cases, coincides with the postovulatory and luteal phases. Deturgescence of the perineum is brought about by the decrease in ovarian estrogen production and a concomitant increase in progesterone production by the newly formed corpus luteum. The onset of deturgescence is indicated by the beginning of wrinkles on

the outer border of the sex skin and by a change in color from a bright red to a pinkish red. The duration of this period is between 4 and 14 days.

d) Perineal rest. The time period during which the perineum is at rest coincides with the latter part of the luteal phase, the premenstrual phase if one exists, and the onset of the menstrual phase. The epithelial surface of the perineum is dull compared to the bright and shiny surface during maximum turgescence. This period lasts from 5 to 15 days.

e) Pregnancy. In the pregnant animal the perineum remains at rest throughout the gestation period although the color changes to a magental red (Hendrickx AG, 1967).

Females are paired with male baboons (25-40Kg) for five days at the anticipated time of ovulation. Pregnancy is confirmed 30 days post ovulation by absence of menstruation and prepartum thereafter.

Blood Sampling

Between day 70 - 100 or day 130 - 170 of gestation blood samples were obtained from pregnant baboons at 1300 - 1430 h every alternate day. Animals were briefly sedated by intramuscular administration of 100 mg Ketamine HCL (Parke-Davis, Detroit, MI) and 5 ml blood samples (3 - 7 ml) were obtained from the maternal saphenous or antecubital vein via a 21 gauge scalp vein needle. Maternal and umbilical blood samples were also collected at the time of the cesarean section. Serum was stored at -20 °C until assayed for serum E_2, DHAS and cortisol concentrations determined by solid phase ^{125}I radioimmunoassay (Coat-A–Count, Diagnostic Products Corp, Los Angeles, CA).

Experimental groups

Animals in which ACTH levels are altered.

Group 1: animals received no treatment and were operated at midgestation (d100; term=184).

Group 2: animals in which fetal pituitary POMC/ACTH expression was suppressed at midgestation by administration of betamethasone, a synthetic glucocorticoid. Animals were treated with 3 mg betamethasone (Celestone Soluspan, Schering Corp., Chicago, IL) administered im to the mothers daily after ketamine HCl (10 mg/kg BW, Aveco Co., Ft. Dodge, IA) sedation between days 60 and 99 of gestation.

Group 3: animals in which ACTH was restored at midgestation by administration of ACTH in addition to betamethasone. Thus, the fetuses of betamethasone-treated (3 mg/day, days 60-99) animals were administered 25 μg ACTH (Cortrosyn, Organon Inc., West Orange, NJ) im in 100 μl saline on days 95-99 via maternal transabdominal injection after ketamine sedation and brief anesthetization (halothane, 1.0-1.5%; nitrous oxide, 0.5 liters/min; oxygen, 2.0 liters/min).

Group 4: animals in which fetal ACTH was prematurely increased at midgestation by administration of 25 μg ACTH (Cortrosyn, Organon Inc., West Orange, NJ) im in 100 μl saline on days 95-99 via maternal transabdominal injection after ketamine sedation and brief anesthetization (halothane, 1.0-1.5%; nitrous oxide, 0.5 liters/min; oxygen, 2.0 liters/min) to untreated baboons.

Group 5: animals received no treatment and were delivered by cesarean section near term (day164). These animals have elevated fetal pituitary POMC/ACTH expression compared to day 100 fetuses (Pepe GJ et al., 1994).

Group 6: animals in which fetal pituitary POMC/ACTH expression was suppressed at term gestation by administration of betamethasone to the fetus (0.6 mg/100 μl; n=4) or to the fetus (0.6 mg/100 μl) and the mother (6.0 mg/ml, im; n=4) every other day between days 150 and 164 of gestation (term = day 184); fetuse delivered on day165.

Animals in which ACTH levels are altered by betamethasone at term will be compared with:

Group 7: animals in which estrogen synthesis was blocked by administration of the aromatase inhibitor CGS 20267 (125 μg/Kg body weight/0.5 ml sesame oil/day, sc) between days 150 and 164 of gestation (term = day 184).

Group 8: animals treated simultaneously with CGS 20267 and estradiol benzoate (0.25-1.0 mg/day) between days 150 and 164 of gestation (term = day 184).

Operative procedure

Anesthesia.

The endocrine system is sensitive to the potential effects of various anesthetics. Therefore, it was essential to use anesthetic agents which produce the fewest possible side effects (Walter ML et al., 1987). Walker et al (1987) studied the effects of various anesthetics on serum concentrations of hormones secreted by the hypothalamus/ pituitary and the adrenals of female baboons. Administration of ketamine HCL (Ketalar, Parke-Davis, Morris Plains, NJ)

was found to maintain anesthesia and had no effect on serum levels of prolactin, F and dehydroepiandrosterone. Although dehydroepiandrosterone sulfate rose , this occurred after 75 min of ketamine administration. Halothane had no effect on hormones of the adrenocortical system whereas acetylpromazine increased prolactin secretion and also induced a marked rise in androgen production (Walter ML et al., 1987).

Accordingly, ketamine was used as an intravenous anesthetic in this study. Ketamine alone however, is not considered an effective agent in attaining analgesia to visceral pain (Walter ML et al., 1987). Halothane an inhalant anesthetic agent, in addition to depressing the secondary afferent system also depresses subcortical structures that regulate somatic and visceral functions. Therefore, in the present study ketamine HCL (Vetalar, Parke-Davis, Morris Plains, NJ) was used for sedation and halothane/nitrous oxide for induction of anesthesia.

Baboons were restrained by intramuscular injection of ketamine HCL (10mg/Kg BW). Twenty min after the onset of ketamine, animals were placed in a supine position and intubated with an endotracheal tube through which a mixture of gases (nitrous oxide; N_2O_2, 25%, 1 liter/min; and O_2, 75%, 3 liters/min) were passed. Between 0 and 30 min 1.5% halothane (Fluothane, Ayerst Laboratories, NY) and N_2O_2 were administered. From 30 min until termination of surgery, the concentration of the halothane was reduced to 1.0% in the presence of N_2O_2 carrier. Maternal heart rate was monitored throughout the period of anesthesia and maintained at 90 - 100 beats/min.

General surgical method.

At the indicated times of pregnancy animals were restrained and sedated as described and the uterus exposed by an abdominal incision. After a transverse incision was made in the

uterus, the umbilical cord was clamped and the fetus and placenta delivered removed. Fetuses were injected with an overdose of ketamine.

Preparation of adrenals for immunocytochemistry

Adrenals were weighed and one adrenal was immediately fixed in 10% buffered formalin (Sigma Chemical Co., St. Louis, MO) at 4°C overnight for subsequent localization of 3β-HSD, P450c17, estrogen receptor, apoptosis, glucocorticoid receptor and ACTH receptor mRNA by immunohistochemistry and *in situ* hybridization, respectively, and for determination of the number of fetal zone and definitive zone cells and the number of each cell type expressing the cell cycle marker, proliferating cell nuclear antigen (PCNA). After fixation, adrenals are dehydrated in ethanol, cleared in xylene, and embedded in paraffin blocks which are cooled at -20 C before sectioning.

Adrenal morphometry and immunocytochemistry

Sections (4 μm) of paraffin embedded adrenal glands were mounted onto Superfrost microscope slides (Fischer Scientific Co., Arlington, VA), deparaffinized by heating at 60 °C for 15 minutes followed by three washes in xylene for a total of 12 minutes. After dehydration in three washes of absolute ethanol for 6 minutes, two washes of 95% ethanol for 4 minutes and one wash of 75% ethanol for 2 minutes, slides were incubated in 0.4% to 3 % (v/v) H_2O_2 in methanol for 10 minutes to block endogenous peroxidase. After one wash in distilled H_20 for 5 minutes followed by one wash in PBS for 5 minutes, tissue sections were preblocked for 15 to 30 minutes with 5% normal goat serum in PBS (NGS; Vector Laboratories, Burlingame, CA)). After incubation (4C) overnight with anti-PCNA/cyclin monoclonal antibody PC-10 diluted 1:200 in 5% normal goat serum or with rabbit polyclonal antibody to anti-human 3β-HSD (generously supplied

by Dr. Ian Mason) diluted 1:5000 in 5% NGS, sections were washed twice in PBS for 10 minutes and incubated with biotinylated goat anti-mouse or goat anti-rabbit IgG (Vector Laboratories, Burlingame, CA)) for one hour. After two washes with PBS, tissue sections were incubated for 1 hour with Avidin DH and horseradish peroxidase H (Vectastain Elite Kit, Vector Laboratories, Burlingame, CA) for 45 minutes in PBS. Color development was achieved by incubation for 3 to 10 minutes with a solution containing imidazole 3,3'-diaminobenzidine and 0.1% (v/v) H_2O_2 in PBS. Sections were lightly counterstained between 2 to 3 minutes with Gill's hematoxylin (Fisher) and mounted in Biomount (Fisher). PCNA and 3β–HSD expression were analyzed by Image Analysis on an average of 6 randomly selected areas (157 μm x 130 μm)/slide of 4-8 fetal adrenal sections per animal using an Optiphot-2 microscope attached to a Video-Based Image 1 Analysis System (Universal Imaging Corp, West Chester, PA). The number of definitive and fetal cortical cells per 0.025 mm^2 was quantified by counting nuclei in 6 randomly selected sections and results compared with the number of cells in which nuclear expression of PCNA was 5-fold greater than background. The definitive zone was quantified as the width of the cell layer immunostaining for 3β–HSD and determined by examining 6 randomly selected regions of each adrenal section.

Evaluation of adrenal cells for apoptosis was performed using an Apoptag Plus *in situ* Apoptosis Detection Kit (Oncor, Gaithersburg, MD) and procedures for use with paraffin-embedded tissues supplied by the manufacturer. This assay labels the multitude of 3'-OH DNA ends generated by DNA fragmentation and typically localized in morphologically identifiable nuclei and apoptotic bodies. In contrast, normal or proliferative nuclei, which have a relatively insignificant number of 3'-OH DNA ends, do not stain with the kit.

Microwave retrieval

Sections (4 μm) of paraffin embedded adrenal glands mounted onto Superfrost microscope slides were deparaffinized by heating at 37C overnight and at 60 C for 30 minutes following by three washes in xylene for 30 minutes. After dehydration in 100% ethanol for 15 seconds, slides were incubated with 2% (v/v) H_2O_2 in methanol for 30 to 45 seconds, then washed in 95% ethanol for 20 seconds, followed sequentially by 70% ethanol for 20 seconds, distilled H_20 for 1 minute, and PBS for 5 minutes. Slides were placed in one of two plastic Coplin jars filled with 10 mM sodium citrate buffer, pH 6 (Sigma). The Coplin jars were situated in the center of a glass pan containing approximately 400 ml water, and were always placed in the same position in the microwave oven. When heating at power level setting 9 (General electric Model JE 1540, microwave oven maximum power 900 W), the boiling point of the buffer solution was reached in approximately 5.5 minutes. After cooling for 30 minutes, sections were washed in PBS and preblocked with TNK buffer (100mM Tris-HC, pH 7.6, 550mM NaCl, 10mM KCl, 2% bovine serum albumin, 0.1% Triton X-100, 1% normal goat serum) for 30 to 45 minutes at room temperature. After 48 hours incubation (4C) with monoclonal anti-human estrogen receptor antibody 6F11 (NCL-ER-6F11, batch # 115105, Novacastra Immunohistochemistry Ltd., Burlingame, CA) diluted 1:40 in TNK buffer or with polyclonal rabbit antibody to anti-human glucocorticoid receptor (PAI-511, lot # 019-123, Affinity Bioreagents, Inc., Neshanec Station, NJ) diluted 1:100 or with polyclonal rabbit antibody to anti-pig P450 c17 enzyme (generously supplied by Dr. Ian Mason, University of Edinburgh, UK) diluted 1:2000, sections were washed twice in PBS for 10

minutes and incubated with biotinylated goat anti-mouse or goat anti-rabbit IgG (Vector Laboratories, Burlingame, CA) diluted 1: 200 in TNK buffer containing 0.5 % of normal baboon serum for one hour. Localization of the primary antibody-biotinylated second antibody complex was then performed using Avidin DH and horseradish peroxidase H (Vectastain Elite Kit, Vector Laboratories, Burlingame, CA) for 45 minutes in TNK buffer with diaminobenzadine as the chromogenic substrate as described above.

When the estrogen receptor antibody, ER-6F11, was incubated overnight at 4C with 15-fold excess of the whole recombinant human ER molecule (Oncogene Research Products, Cambridge, MA), the nuclear staining was completely abolished indicating specificity of the ER monoclonal antibody. Specificity of the GR was demonstrated by preabsorbing the GR antibody with 100-fold excess of the synthetic peptide PEP-001 (Affinity Bioreagents, Inc., Neshanec Station, NJ) used for immunization. The peptide is amino acids 346 to 367 of the human receptor which is included in the amino terminus of region of the receptor.

ER, P450c17 and GR expression were analyzed by Image Analysis on an average of 6 randomly selected areas (157 μm x 130 μm)/slide of 4-8 fetal adrenal sections per animal using an Optiphot-2 microscope attached to a Video-Based Image 1 Analysis System (Universal Imaging Corp, West Chester, PA).

Expression of the width and intensity of labeling of the definitive zone cells positive for immunostaining of the ER was determined by examining 6 randomly selected regions of each adrenal section. The growth fetal zone was quantified by the mean width of P450c17 positive/3βHSDnegative zone of the cortex of on consecutive adrenal sections examined in 6 randomly selected regions of each adrenal section.

Double Immunocytochemistry assay

Sections (4 μm) of paraffin embedded adrenal glands mounted onto Superfrost microscope slides were deparaffinized by heating at 37C overnight and at 60 C for 30 minutes following by three washes in xylene for 30 minutes. After dehydration in 100% ethanol for 15 seconds, slides were incubated with 2% (v/v) H_2O_2 in methanol for 30 to 45 seconds, then washed in 95% ethanol for 20 seconds, followed sequentially by 70% ethanol for 20 seconds, distilled H_20 for 1 minute, and PBS for 5 minutes. High temperature antigen unmasking was performed as described above. Briefly, tissue sections were microwaved for approximately 5.5 minutes in 10mM sodium citrate. After cooling, sections were preblocked with TNK buffer for 30 to 45 minutes.

Staining for the first antigen, estrogen receptor

After 48 hours incubation (4C) with anti-human estrogen receptor monoclonal antibody 6F11 (Novacastra Immunohistochemistry, Novacastra Laboratories Ltd., Burlingame, CA) diluted 1:40 in TNK buffer, sections were washed twice in PBS for 10 minutes and incubated with biotinylated goat anti-mouse (Vector Laboratories, Burlingame CA) diluted 1: 200 in TNK buffer containing 0.5 % of normal baboon serum for one hour. Localization of the primary antibody-biotinylated second antibody complex was then performed using Avidin DH and horseradish peroxidase H (Vectastain Elite Kit, Vector Laboratories, Burlingame CA) for 45 minutes in TNK buffer with diaminobenzadine as the chromogenic substrate until color develops, between 6 to 10 minutes. After two washes in PBS for twenty minutes, tissue sections were prepared to stain with the second antigen.

Staining for the second antigen, 3β-HSD

Tissue sections were preblocked for 15 to 30 minutes with 5% normal goat serum (NGS; , Vector Laboratories, Burlingame CA). After incubation for one hour at room temperature, with polyclonal rabbit antibody to anti-human 3β-HSD enzyme (generously supplied by Dr. Ian Mason) diluted 1:5000 in 5% NGS, sections were washed twice in PBS for 10 minutes and incubated with goat anti-rabbit IgG (Vector Laboratories, Burlingame, CA)) diluted 1:200 in 5% NGS for one hour. After two washes with PBS, tissue sections were incubated for 1 hour with Avidin DH and horseradish peroxidase H (Vectastain Elite Kit, Vector Laboratories, Burlingame CA) for 45 minutes in PBS. Color development was achieved by incubation with a Vector VIP substrate kit for peroxidase (Vector Laboratories, Burlingame CA) and procedures for use supplied by the manufacturer. Sections were lightly counterstained between 2 to 3 minutes with methyl green (Vector) and mounted in Biomount (Fisher) and ER and 3β–HSD expression analyzed by Image Analysis on an average of 6 randomly selected areas (157 μm x 130 μ m)/slide of 4-8 fetal adrenal sections per animal using an Optiphot-2 microscope attached to a Video-Based Image 1 Analysis System (Universal Imaging Corp, West Chester, PA). Expression of the width and intensity of labeling of the definitive zone cells positive for immunostaining of the ER was determined by examining 6 randomly selected regions of each adrenal section and compared with the width of the cell layer immunostaining for 3β–HSD definitive zone cells in the same tissue sections. Specificity of the dual labeling was demonstrated by replacing the anti-ER primary antibody with normal mouse serum.

Fetal pituitary POMC mRNA

Localization and quantification of fetal pituitary POMC mRNA were performed by *in situ* hybridization using our previously published methods (Pepe GJ et al., 1994). Briefly, 0.1 µmol purified POMC antisense (and sense) oligodeoxynucleotide probes complimentary to 30 bases of the human POMC mRNA (Chang ACY et al., 1980) were 3' end-labeled with [^{35}S]dATP (SA > 1000 Ci/mmol; Dupont-New England Nuclear, Boston, MA) via terminal deoxynucleotidyl transferase (20 U; Promega, Madison, WI) to a specific activity of approximately 5000 Ci/mmol. Frozen sections of the fetal pituitary were equilibrated (10 min) to room temperature, and fixed (10 min) in freshly prepared 4% paraformaldehyde in PBS (pH 7.4). Slides were rinse in 2X SSC (2X SCC= 0.30 M NaCl, 0.03 M sodium citrate buffer, pH 7.2) . Sections were dehydrated through an ethanol series, delipidated in chloroform, rehydrated in ethanol and air dried. Sections were then prehybridized for 3 hours (50 C) and incubated overnight (50 C) with approximately 750,000 cpm [^{3}S] labeled antisense or sense probe, washed twice (2X SSC, 50% formamide) at 60 C (approximately 19 C below calculated Tm), rinsed and placed against Kodak X-Omat film in X-ray film holders for 5-7 days. POMC mRNA expression was determined by densitometric analysis using an LKB Bromma Ultroscan XL Enhanced Laser Densitometer (Pharmacia LKB, Piscataway, NY).

Northern analysis of fetal adrenal mRNA

Whole frozen adrenals were homogenized in 4 M guanidine isothiocyanate and RNA extracted with chloroform-isoamyl alcohol (24:1), essentially as described by Chirgwin et al (Chirgwin JM et al., 1979). RNA was isolated by 5.7 M cesium chloride gradient centrifugation

and polyadenylated [poly(A)$^+$] RNA prepared by centrifugation of total RNA over columns of oligo(deoxythymidine)-cellulose (Pharmacia LKB Biotechnology).

Fetal adrenal mRNA levels were determined by Northern blot, using methods implemented in our laboratories (Albrecht ED et al., 1995). Approximately 2.5 μg poly(A)$^+$ RNA were size-fractioned by electrophoresis in a 1.0% agarose gel, transferred onto a nylon membrane (GeneScreen, DuPont-New England Nuclear), and prehybridized in buffer containing 50% formamide, 0.1% polyvinyl pyrrolidone, 0.1% BSA, 0.1% Ficoll, 2.5 x SSPE (0.375 M NaCl, 0.025 M NaH_2P0_4-H_20, and 2.5 mM EDTA–Na_2, pH 7.4), 1.0% SDS, 10% dextran sulfate, and denatured salmon sperm DNA (100 μg/ml) for 18 h at 42 C before the addition of probe. The cDNAs for the baboon ACTH receptor (Albrecht ED et al., 1996) and human 3βHSD (*Eco*RI/*Sst*I insert; provided by Dr. J. Ian Mason; 27), β–actin (No. 65128, American Type Culture Collection, Rockville, MD), P-450scc and P-450_{C17} (generously provided by Dr. Walter Miller, University of California, San Francisco, CA) and the LDL receptor (pLDLR3 No 57004) were each labeled with [α–^{32}P]deoxy-CTP (3000 Ci/mmol; Amersham Corp., Arlington Heights, IL) to a specific activity of approximately 10^9 dpm/μg DNA using the Random-Primed DNA Labeling Kit (Boehringer Mannheim). Hybridization was performed in fresh buffer at 42 C for 23 h with approximately 10^6 cpm/ml ^{32}P-labeled cDNA. After hybridization, the membrane was washed twice in 2 x SSC (0.3 M NaCl, 0.03 M sodium citrate-$2H_20$, pH 7.0), once in 1 x SSC/1% SDS at 65 C, and twice in 0.1 x SSC, then exposed to Kodak X-AR film (Eastman Kodak, Rochester, NY) at -80 C. The intensities of the bands on each Northern blot were analyzed by densitometric autoradiographic scanning using a model 620 Video Densitometer (Bio-Rad, Hercules, CA). Between hybridizations with

different probes, membranes were stripped by washing in 10 mM Tris, pH 8.0, 1 mM Na_2 EDTA, pH 8.0, and 1% SDS, at 100 C for 30 min.

Western Analysis of 3β-HSD and P450c17

Analysis of 3β-HSD and P450c17 peptides in fetal adrenal extracts was performed using procedures developed previously in our laboratories (Pepe GJ et al., 1996). Briefly, adrenals were homogenized on ice in 2.5 ml buffer composed of 1% cholic acid (Sigma), 0.1% SDS, 1mM EDTA in phosphate buffered saline to which had been added 0.1 mg/ml phenylmethylsulfonyl fluoride (PMSF), 10 μg/ml aprotinin and 0.1 mg/ml trypsin inhibitor and centrifuged at 800 x g to remove cell debris. After determination of protein concentrations using the bicinchoninic acid procedure (Sigma), 5x Laemmli buffer (Laemmli UK, 1970) was added to a final concentration of 1x and samples boiled for 2 min, centrifuged (1,000 x g 10 min) and loaded (30 μg protein/lane) onto preformed 10% SDS-polyacrylamide minigels maintained in Bio Rad Mini-Protean II electrophoresis chambers, electrophoresed at 100V, and transferred to Immobilon P (Gibco BRL, Bethesda, MD). After blocking with 3% BSA in 50 mM Tris, pH 7.5 containing 150 mM NaCl and 0.05% Tween 20 (Sigma), samples were incubated (37C, 1h) with polyclonal antibodies to rabbit anti-human placental 3β-HSD or rabbit anti-porcine testicular microsomal P450c17 (generously supplied by Dr. Ian Mason) diluted 1:10,000 or 1:2,000 respectively in 50 mM Tris buffer (pH 7.5) containing 150 mM NaCl, 0.05% Tween 20, 0.05% Nonidet P-40 (Sigma) and 1.5% BSA. Membranes were washed and then incubated with donkey anti-rabbit IgG horseradish peroxidase conjugated second antibody (Amersham) at dilutions recommended by the manufacturer and which contributed no non-specific bands at the concentrations employed. After washing, equal amounts of

enhanced chemiluminescent reagent (ECL; Amersham) were applied to membranes for 1 min, the membranes wrapped in plastic and then placed against Kodak X-Omat film (Kodak) in X-ray film cassettes and exposed for 15-60 sec. Samples were developed and quantified by 1 dimensional densitometry using an LKB Bromma Ultroscan XL Enhanced Laser densitometer.

3β-HSD enzyme activity

The activity of 3β-HSD was determined essentially as described previously (Pepe GJ, Albrecht ED, 1991). Briefly, adrenals were homogenized in 2.5 ml 0.05 M NaH_2PO_4 buffer (pH 7.4) and microsomes (or buffer blank) incubated in duplicate (37C) for 2, 5 and 10 min with 125,000 cpm [7-^{3}H] pregnenolone (SA, 25 Ci/mmol; DuPont NEN), 125 ng radioinert pregnenolone (Sigma) and 2.5 mg NAD^+ (Sigma). Samples were extracted with ethyl acetate, radiolabeled product progesterone isolated by paper chromatography and [^{3}H] concentrations, corrected for procedural losses estimated by recovery of [4-^{14}C] progesterone (SA 35 mCi/mmol; Dupont NEN) added prior to extraction, determined by liquid scintillation spectrometry.

In situ hybridization of ACTH receptor mRNA

Localization of ACTH receptor mRNA was determined as described in recent publication (Aberdeen GW et al., 1997). Briefly, sections of paraffin-embedded fetal adrenal glands were cleared in xylene, rehydrated in graded ethanols, rinsed in PBS and hybridized overnight at 45C with an oligodeoxynucleotide antisense (sense) probe complementary to bases 373-401 of the baboon ACTH receptor (Albrecht ED et al., 1996) and end-labeled with [^{35}S] dATP (Dupont NEN). Slides were washed at 60C, rinsed, dipped in Kodak NTB-2 nuclear track emulsion diluted 1:1 with distilled water,

placed in light tight boxes, exposed for 12-17 days and then developed in Kodak D-19. The cellular distribution of silver grains was determined using an Optiphot 2 microscope attached to a Video Based Image-1 Analysis System (Universal Imaging Corp., West Chester, PA).

Statistical analysis

The data obtained from multiple treatment populations were analyzed by analysis of variance with *post-hoc* comparisons of means by Newman-Keuls multiple comparison test. Comparison of serum steroid, pituitary POMC mRNA and adrenal mRNA levels between untreated and betamethasone-treated fetuses was by Students unpaired *t*-test (Snedecor GW and Cochran WG,1967).

[1]CHAPTER III

EFFECT OF MATERNAL BETAMETHASONE ADMINISTRATION ON BABOON FETAL ADRENAL GLAND DEVELOPMENT AND ACTH RECEPTOR mRNA EXPRESSION AT MIDGESTATION

Abstract

Although ACTH is considered to be important to fetal adrenal growth and steroidogenesis in the second half of primate pregnancy, the role of fetal pituitary ACTH upon adrenal development and function has not been established *in vivo* in the first half of gestation. In the present study, therefore, baboons were treated at midgestation with betamethasone to determine the role of fetal pituitary ACTH upon fetal adrenal growth and development, as well as ACTH receptor and P-450 enzyme messenger ribonucleic acid (mRNA) levels. The administration of betamethasone to baboon mothers on days 60-99 of gestation (term = 184 days) decreased fetal pituitary proopiomelanocortin (POMC) mRNA levels by 54% ($P<0.01$). The decline in POMC expression was associated with a decrease in fetal adrenal weight ($P<0.001$), cortical cell size ($P<0.05$), appearance of apoptosis and cellular disorganization, and a loss of immunocytochemically demonstrable definitive zone-specific Δ^5-3β-hydroxysteroid dehydrogenase (3β-HSD) expression. Moreover, there was approximately a 95% decrease ($P<0.01$) in fetal adrenal expression of the mRNAs for the ACTH receptor, P-450 cholesterol side-chain cleavage (P-450scc) and P-450 17α-hydroxylase, 17/20-lyase ($P\text{-}450_{C17}$) after

[1] Data presented in this chapter has been in part submitted for publication. Aberdeen GW, Leavitt MG, Pepe GJ, Albrecht ED 1997 Effects of maternal betamethasone administration at midgestation on baboon fetal adrenal gland development and ACTH receptor messenger RNA expression. Endocrinology, in revision

betamethasone administration. We conclude that fetal pituitary ACTH is necessary for growth and development of the fetal and definitive cortical zones and the marked coordinated increase in ACTH receptor and P-450scc/P-450_{C17} expression in the baboon fetal adrenal gland in early-mid gestation.

Experimental approach

Hypothesis I. Placental estrogen modulates ACTH-induced growth and maturation of the fetal definitive zone (e.g. 3βHSD, P450c17) while concomitantly acting to modulate ACTH action on fetal cortical cell hyperplasia and /or hypertrophy and synthesis of DHA.

The results of this section will be concerned with three experimental treatments, betamethasone, (synthetic glucocorticoid) administered to the mother (3 mg; n=6) daily between days 60 and 99 of gestation (term=184), simultaneous betamethasone (days 60-99, n=4) and ACTH (25 μg/100μl; days 95-99; n=4); (restoration of ACTH), and ACTH alone (25 μg/100 μl; days 95-99, n=5); (premature elevation of ACTH); and the effect of the three treatments on the adrenal gland growth, cellular remodeling (i.e. apoptosis) and mRNA and/or protein levels/distribution of the enzymes 3βHSD, P450c17 and P450scc and the membrane receptor for ACTH.

Rationale

The human and nonhuman primate fetal adrenal cortex is comprised of an inner fetal zone made up of large eosinophilic cells that are the source of C_{19}-steroid precursors, e.g. dehydroepiandrosterone sulfate (DHAS), utilized for placental estrogen production and an outer definitive zone of smaller basophilic cells which express the Δ^5-3β-hydroxysteroid dehydrogenase (3β-HSD) enzyme required for the synthesis of cortisol (Jaffe RB et al., 1988;

Pepe GJ and Albrecht ED, 1990 for reviews). The fetal zone makes up the majority of the adrenal cortex throughout gestation, while the definitive zone begins to appear and express 3β-HSD at midgestation, but does not undergo extensive maturation until relatively late in gestation. Studies conducted *in vitro* with human fetal adrenal cells (Seron-Ferre M et al., 1978; Carr BR et al.,1980; Simonian MH and Gill GN, 1981; Mason JI et al., 1983; DiBlasio AM et al., 1988) and *in vivo* in the rhesus monkey (Challis JRG et al., 1974; Seron-Ferre M et al.,1978; Walsh SW et al.,1979; Walsh SW et al., 1980; Coulter CL et al., 1996) indicate that ACTH has a major role in regulating cellular proliferation, maturation, and steroidogenesis within the fetal adrenal in the second half of gestation. Although the human fetal adrenal develops normally in anencephalic fetuses through the first trimester, but not thereafter (Benirschke K, 1956; Johannisson E, 1979; Gray ES and Abramovich DR, 1980), the relative importance of fetal pituitary ACTH upon adrenal growth, development, and function in the first half of gestation has not been established *in vivo* in the primate. ACTH is potentially available to the fetal adrenal at this time in development, because human (Siler-Khodr TM et al., 1974; Baker BL and Jaffe RB, 1975) and baboon (Pepe GJ et al., 1994) fetal pituitaries express and/or secrete proopiomelanocortin (POMC) and ACTH by midgestation. Moreover, fetal adrenal ACTH receptor messenger ribonucleic acid (mRNA) levels increased from negligible levels early in baboon gestation to a maximum at midgestation (Albrecht ED et al., 1996), indicating that a mechanism for mediating the action of ACTH exists within the adrenal at this time in development. Although the ACTH receptor is upregulated by ACTH *in vitro* in cultures of human fetal adrenal cells (Lebrethon MC et al., 1994; Mesiano S et al., 1996), the role of ACTH on receptor expression has not been established *in vivo* in the primate.

Therefore, in the present study betamethasone, a synthetic glucocorticoid which readily crosses the placenta and suppresses the fetal pituitary adrenocortical axis (Challis JRG et al., 1974; Walsh SW et al., 1979) was used to investigate the role of pituitary ACTH *in vivo* on fetal adrenal development. Fetal adrenal growth, development of the fetal and definitive zones, and expression of the ACTH receptor and the ACTH-dependent steroidogenic enzymes P-450 cholesterol side-chain cleavage (P-450scc), P-450 17α-hydroxylase-17/20-lyase (P-450$_{C17}$), and 3β-HSD were determined in baboons treated with betamethasone in the latter stage of the first half of gestation.

Materials and Methods

Animal treatment

Pregnant baboons (*Papio anubis*) were housed individually in large aluminum-stainless steel primate cages and received high protein monkey chow (Harlan Teklad, Madison, WI) twice daily, fresh fruit and vitamins daily, and water *ad libitum*, as described previously (Albrecht ED, 1980). Animals were cared for and used strictly in accordance with USDA regulations and the NIH Guide for the Care and Use of Laboratory Animals (Publication 85-23, 1985). The experimental protocol employed in the present study was approved by the Institutional Animal Care and Use Committee of the University of Maryland School of Medicine.

Eight baboons were left untreated and 6 baboons were treated with 3 mg betamethasone (Celestone Soluspan, Schering Corp., Chicago, IL) administered im to the mothers daily after ketamine HCl (10 mg/kg BW, Aveco Co., Ft. Dodge, IA) sedation between days 60 and 99 of gestation (term = 184 days). Maternal saphenous vein blood samples were obtained daily between days 95 and 100 of gestation after ketamine sedation, and serum

estradiol, DHAS and cortisol concentrations determined by solid phase ^{125}I radioimmunoassay (Coat-A-Count, Diagnostic Products Corp, Los Angeles, CA) as described previously (Albrecht ED et al., 1991).

On day 100 of gestation, baboons were sedated with ketamine, anesthetized with halothane, the fetuses delivered by cesarean section, and an umbilical artery blood sample was obtained. The fetal adrenals were weighed and one of the glands was immediately frozen and stored in liquid nitrogen for subsequent Northern analysis of mRNAs. The other gland was fixed in 10% buffered formalin and embedded in paraffin for immunocytochemical analysis of 3β-HSD, quantification of the number of fetal cortical cells, and histological determination of cellular integrity including apoptosis. Fetal pituitaries were placed in cryomolds containing OCT embedding medium (Miles Scientific, Elkhart, IN) and stored at -80 C until analyzed for POMC mRNA.

Preliminary work was also initiated to determine whether the effect of long-term administration of betamethasone on adrenal function could be overcome by concomitant treatment of the fetus with ACTH. Thus, the fetuses of 4 additional betamethasone-treated (3 mg/day, days 60-99) animals and 5 additional untreated baboons were administered 25 μg ACTH (Cortrosyn, Organon Inc., West Orange, NJ) im in 100 μl saline on days 95-99 via maternal transabdominal injection after ketamine sedation and brief anesthetization (halothane, 1.0-1.5%; nitrous oxide, 0.5 liters/min; oxygen, 2.0 liters/min). Fetal adrenals were then obtained on day 100 and processed as described above.

Fetal pituitary POMC mRNA

Localization and quantification of POMC mRNA were performed by *in situ* hybridization using our previously published methods (Pepe GJ et al., 1994). Briefly, 0.1 μmol

purified POMC antisense (and sense) oligodeoxynucleotide probes complimentary to 30 bases of the human POMC mRNA (Chang ACY et al., 1980) were 3′ end-labeled with [^{35}S]dATP (SA > 1000 Ci/mmol; Dupont-New England Nuclear, Boston, MA) via terminal deoxynucleotidyl transferase (20 U; Promega, Madison, WI) to a specific activity of approximately 5000 Ci/mmol. Sections of the fetal pituitary were incubated overnight (50 C) with approximately 750,000 cpm [^{35}S] labeled antisense or sense probe, washed at 60 C (approximately 19 C below calculated Tm), rinsed and placed against Kodak X-Omat film in X-ray film holders for 5-7 days. POMC mRNA expression was determined by densitometric analysis using an LKB Bromma Ultroscan XL Enhanced Laser Densitometer (Pharmacia LKB, Piscataway, NY).

Fetal adrenal morphology and immunocytochemistry

Sections (4 μm) of paraffin embedded fetal adrenal glands were mounted onto Superfrost microscope slides (Fisher Scientific Co., Arlington, VA), heat fixed and endogenous peroxidase blocked with 0.4% H_2O_2 in methanol. After incubation (4 C) overnight with polyclonal antibody to rabbit anti-human 3β-HSD (generously supplied by Dr. Ian Mason, University of Edinburgh, UK), sections were washed and incubated with biotinylated goat anti-rabbit IgG (Boehringer Mannheim, Indianapolis, IN), Avidin DH and horseradish peroxidase H (Vectasin Elite Kit, Vector Laboratories, Burlingame, CA). Sections were lightly counterstained with Gill's hematoxylin (Fisher), coverslipped with Biomount (Fisher) and 3β-HSD expression analyzed on an average of 6 randomly selected areas (157 μm x 130 μm/slide of 4-8 fetal adrenal sections per animal) using an Optiphot-2 microscope attached to a Video-Based Image 1 Analysis System (Universal Imaging Corp., West Chester, PA). The number of fetal zone cells per 0.025 mm^2 was quantified by counting nuclei in 6 randomly selected

sections. The width of the definitive cell layer was quantified by examining those cells which expressed 3β-HSD by immunocytochemistry in 6-randomly selected regions of each adrenal section.

Evaluation of adrenal cells for apoptosis was performed using an Apoptag Plus *in situ* Apoptosis Detection Kit (Oncor, Gaithersburg, MD) and procedures for use with paraffin-embedded tissues supplied by the manufacturer.

Northern analysis of fetal adrenal mRNA

Whole adrenals were homogenized in 4 M guanidine isothiocyanate and RNA extracted with chloroform-isoamyl alcohol (24:1), essentially as described by Chirgwin et al (Chirgwin JM et al., 1979). RNA was isolated by 5.7 M cesium chloride gradient centrifugation and polyadenylated [poly(A)$^+$] RNA prepared by centrifugation of total RNA over columns of oligo(deoxythymidine)-cellulose (Pharmacia LKB Biotechnology).

Fetal adrenal mRNA levels were determined by Northern blot, using methods implemented in our laboratories (Albrecht ED et al., 1995). Approximately 2.5 μg poly(A)$^+$ RNA were size-fractioned by electrophoresis in a 1.0% agarose gel, transferred onto a nylon membrane (GeneScreen, DuPont-New England Nuclear), and prehybridized in buffer containing 50% formamide, 0.1% polyvinyl pyrrolidone, 0.1% BSA, 0.1% Ficoll, 2.5 x SSPE (0.375 M NaCl, 0.025 M NaH_2P0_4-H_20, and 2.5 mM EDTA-Na_2, pH 7.4), 1.0% SDS, 10% dextran sulfate, and denatured salmon sperm DNA (100 μg/ml) for 18 h at 42 C before the addition of probe. The cDNAs for the baboon ACTH receptor (Albrecht ED et al., 1996) and human 3βHSD (*Eco*RI/*Sst*I insert; provided by Dr. J. Ian Mason; 27), β-actin (No. 65128, American Type Culture Collection, Rockville, MD), P-450scc and P-450_{C17} (generously provided by Dr. Walter Miller, University of California, San Francisco, CA) were each labeled

with [α-^{32}P]deoxy-CTP (3000 Ci/mmol; Amersham Corp., Arlington Heights, IL) to a specific activity of approximately 10^9 dpm/μg DNA using the Random-Primed DNA Labeling Kit (Boehringer Mannheim). Hybridization was performed in fresh buffer at 42 C for 23 h with approximately 10^6 cpm/ml ^{32}P-labeled cDNA. After hybridization, the membrane was washed twice in 2 x SSC (0.3 M NaCl, 0.03 M sodium citrate-$2H_20$, pH 7.0), once in 1 x SSC/1% SDS at 65 C, and twice in 0.1 x SSC, then exposed to Kodak X-AR film (Eastman Kodak, Rochester, NY) at -80 C. The intensities of the bands on each Northern blot were analyzed by densitometric autoradiographic scanning using a model 620 Video Densitometer (Bio-Rad, Hercules, CA). Between hybridizations with different probes, membranes were stripped by washing in 10 mM Tris, pH 8.0, 1 mM Na_2 EDTA, pH 8.0, and 1% SDS, at 100 C for 30 min.

Statistical analysis

The data obtained from multiple treatment populations were analyzed by analysis of variance with *post-hoc* comparisons of means by Newman-Keuls multiple comparison test. Comparison of serum steroid, pituitary POMC mRNA and adrenal mRNA levels between untreated and betamethasone-treated fetuses was by Students unpaired t test (Snedecor GW, Cochran WG, 1967).

Results

Pituitary POMC mRNA and serum steroid levels

Fetal pituitary POMC mRNA levels, determined by quantitative *in situ* hybridization, on day 100 of gestation were 54% lower ($P<0.01$) after betamethasone administration than in the untreated controls (Figure3 -1).

The administration of betamethasone to baboon mothers decreased maternal and fetal (i.e. umbilical artery) serum estradiol, DHAS and cortisol concentrations by approximately 90% ($P<0.01$) when compared to the untreated control baboons (Table 3-1).

Fetal adrenal morphology and 3β-HSD immunocytochemistry

Fetal body weights were not significantly altered by betamethasone treatment (Table 3-2). However, fetal adrenal absolute and relative (to body) weights at midgestation were decreased 50-60% ($P<0.001$) by the administration of betamethasone (Figure 3-2, Table 3-2). The decrease in fetal adrenal weight elicited by betamethasone was associated with an approximately 2-fold increase ($P<0.05$) in the number of fetal adrenal cortical cells per unit area (Table 3-2), indicating that cortical cell size was decreased by betamethasone. Although there was no evidence of apoptosis in fetal adrenals of the untreated controls at midgestation (Figure 3-3A), DNA oligonucleosomes indicative of programmed cell death were evident in the fetal adrenal after the administration of betamethasone (Figure 3-3B).

The fetal adrenal cortex of the controls at midgestation was comprised primarily of well defined fetal zone cells that lacked 3β-HSD staining, although a narrow rim of 3β-HSD immunoreactivity indicative of the appearance of definitive zone cells was evident (Figure 3-4A). The administration of betamethasone virtually eliminated the definitive zone (Figure 3-5) with its immunocytochemical expression of definitive zone-specific 3β-HSD (Figure 3-4B), and the fetal zone exhibited cellular disorganization (Figure 3-4B).

Preliminary findings indicated that the daily administration of 25 μg ACTH for 5 days to betamethasone-treated baboon fetuses uniformly restored fetal adrenal absolute and relative weights to values that were not significantly different from those of the untreated controls (Figure 3-2, Table 3-2). Moreover, at this dosage and length of treatment, administration of

ACTH consistently reversed the process of apoptosis induced by long-term administration of betamethasone (Figure 3-3C), uniformly restored cellular integrity of and 3β-HSD expression within the adrenal cortex (Figure 3-4C), and returned the width of the definitive zone to normal (Figure 3-5). ACTH alone increased ($P<0.01$) fetal adrenal weight by 43% (Figure 3-2), but did not change the ratio of adrenal weight to body weight (Table 3-2), and enhanced fetal adrenal definitive zone width to a value that was approximately two-fold greater ($P<0.001$) than normal (Figure 3-5).

mRNAs for ACTH receptor, P-450scc and P-450c17

The baboon ACTH receptor cDNA hybridized with a major 3.4 kilobase (kb) mRNA transcript, two lesser transcripts of 4.0 and 1.8 kb, and very minor transcripts of approximately 7, 10, and 11 kb (data not shown) in the fetal adrenal gland, as we have shown previously (Albrecht et al., 1996). Although the relative changes in expression of each of the ACTH receptor transcripts appeared similar with experimental treatment, only the primary 3.4-kb transcript (Figure 3-6A) was used for statistical analysis of densitometric readings (Figure 3-6C). ACTH receptor mRNA levels in the fetal adrenal gland at midgestation were decreased ($P<0.001$) in all 4 animals by the administration of betamethasone to a mean value that was 4% of the untreated controls (Figure 3-6C). The marked decline in ACTH receptor mRNA levels was also accompanied by a loss ($P<0.001$) in fetal adrenal β-actin mRNA expression (Figure 3-6B).

In the preliminary study, administration of 25 μg ACTH to the fetuses of betamethasone-treated baboons restored the mRNAs for the ACTH receptor and β-actin in 2 of the 4 animals (Figure3-7). However, ACTH administration alone did not alter ACTH receptor expression compared to that in the untreated controls (Figure 3-7).

Expression of the major 2.2 kb and 2.1 kb P-450scc and P-450_{C17} mRNA transcripts, respectively, in the fetal adrenal were uniformly decreased by 97% ($P<0.01$) after betamethasone treatment (Figure 3-8). The administration of ACTH to betamethasone suppressed animals restored P-450scc and P-450_{C17} mRNA levels in 2 of the 4 fetuses, while ACTH alone had no effect.

Discussion

Adrenal morphometry and 3βHSD immunocytochemistry after betamethasone

In the present study, there was a marked decrease in weight and cell size of, and appearance of apoptosis and cellular disorganization in, the adrenal of baboon fetuses in which pituitary POMC was suppressed by betamethasone administration. These effects indicate that fetal pituitary ACTH has a major role in stimulating growth and maintaining structural integrity of the fetal cortical zone at midgestation. The atrophy and decrease in cellular size and integrity of the baboon fetal adrenal after betamethasone treatment at midgestation were also observed in fetal adrenal glands of rhesus monkeys, in which pituitary ACTH was suppressed by either betamethasone administration or fetal decapitation in the second half of gestation (Coulter CL et al., 1996; McNulty WP et al., 1981). The results obtained in the present *in vivo* study are also consistent with the stimulation of growth and cellular proliferation of human fetal adrenal cells by ACTH *in vitro* (DiBlasio AM et al.., 1988; Roos BA, 1974; Kahri AI et al.., 1976) Thus, ACTH appears to be responsible for inducing and maintaining hypertrophy of the fetal zone cells at midgestation. Although we have recently shown that normal cellular maturation and cortical zone development of the primate fetal adrenal gland were not associated with apoptosis (Albrecht ED et al.., 1996), programmed cell death was elicited in the fetal zone by suppressing ACTH in baboons of the present study. These results further

emphasize the importance of ACTH in maintaining the structural integrity of the fetal zone of the primate fetal adrenal cortex. This conclusion is supported by our preliminary findings which indicate that a once daily injection of ACTH to the fetus for 5 days overcame the structural demise elicited by betamethasone suppression of fetal pituitary POMC expression.

Although the definitive zone of the fetal adrenal cortex only begins to emerge and express 3β-HSD at midgestation in the baboon (Pepe GJ and Albrecht ED, 1995) and human (Doody KM et al., 1990; Voutilainen R et al., 1991; Mesiano S et al., 1993), the administration of betamethasone to baboons of the present study virtually eliminated this cellular zone, as demonstrated by the loss of immunocytochemically demonstrable 3β-HSD enzyme. Consequently, it seems that fetal pituitary ACTH, the expression of which we have shown is activated by estrogen-induced changes in transplacental corticosteroid metabolism with advancing baboon pregnancy (Pepe GJ and Albrecht ED, 1995; Pepe GJ et al., 1990), is also responsible for the initial development of the definitive cortical zone and the onset in expression of enzymes critical to steroidogenesis. Indeed, the administration of ACTH to betamethasone-suppressed fetuses restored the definitive zone and its expression of 3β-HSD.

Effects of betamethasone on mRNAs for ACTH receptor, P-450scc and P-450c17

The decline in fetal adrenal ACTH receptor mRNA expression in baboons by betamethasone administration is consistent with a role for pituitary ACTH in generating the marked 13-fold rise in receptor expression that occurs between early and midgestation (Albrecht ED et al., 1996). The present *in vivo* observations are also consistent with recent *in vitro* studies, which showed an ACTH-induced increase in ACTH receptor mRNA levels (Lebrethon MC et al., 1994; Mesiano S et al.,1996) and ACTH binding capacity (Rainey WE et al., 1991) in cultures of human fetal adrenal cells. The corresponding decline in ACTH-

depleted baboons of mRNA expression for P-450scc and P-450_{C17}, enzymes expressed in the fetal zone (Mesiano S et al.., 1993) and stimulated *in vitro* by ACTH (Mesiano S et al.,1996; Mesiano S et al., 1993; DiBlasio AM et al.., 1987; Voutilainen R and Miller WL, 1988; Ilvesmaki V and Voutilainen R , 1991), support the concept of a role for the receptor-mediated action of ACTH *in vivo* in regulating those enzymes critical to fetal adrenal C_{19}-steroid biosynthesis and thus estrogen production at midgestation. Indeed, acute administration of ACTH to baboon fetuses at midgestation enhanced fetal adrenal DHA secretion (Pepe GJ et al., 1989). Prior observations of a decrease in fetal adrenal P-450scc and P-450_{C17} mRNA levels in rhesus monkeys treated with betamethasone (Coulter CL et al.., 1996) and a decrease and subsequent increase in fetal plasma DHAS, estradiol, and cortisol in acutely betamethasone suppressed/ACTH-stimulated rhesus monkey fetuses in the second half of pregnancy (Walsh SW et al., 1979; Walsh SW et al., 1980) are also consistent with the findings in baboons of the present study. Moreover, Mesiano et al (Mesiano S et al., 1996) have recently shown that the ACTH receptor and P-450scc are coordinately expressed in human fetal adrenal cells in culture and they have suggested that the ACTH receptor belongs to a cohort of ACTH responsive genes required to maintain fetal adrenal differentiation and responsiveness to ACTH. The results of the present study demonstrate that a similar ACTH-dependent coordinated regulation of the ACTH receptor and P-450scc and P-450_{C17} enzymes exists *in vivo* within the baboon fetal adrenal at midgestation.

The mRNA levels for the ACTH receptor were not corrected for that of β-actin in the fetal adrenals of the present study, because β-actin which is typically used as a constitutively expressed gene marker was also suppressed by betamethasone. It is unlikely that the striking decrease in mRNA levels for the ACTH receptor and other factors reflected methodological

problems, e.g. gel loading, because the mRNAs for the ACTH receptor and β-actin were relatively uniformly expressed in the untreated control and ACTH-treated baboons of the present study. Rather, we suggest that the decrease in expression of β-actin in ACTH-suppressed fetuses further points to the absolute requirement of ACTH for the structural and functional integrity of the primate fetal adrenal gland at midgestation.

Although fetal adrenal weight/growth, cellular integrity and 3β-HSD expression were uniformly restored to normal by the administration of ACTH to betamethasone-suppressed baboons, the mRNA levels for the ACTH receptor, P-450scc, and P-450_{C17}, were only restored in 2 of the 4 fetuses injected with the particular dose of ACTH used in the preliminary study. There is no obvious explanation for this inconsistent response in ACTH receptor mRNA expression to ACTH. Because the loss in adrenal weight and structural organization was completely reversed in all cases, it does not seem that the inconsistent effects on mRNA expression resulted from a procedural problem associated with maternal transabdominal injection of ACTH to the fetus. It is possible that the level of ACTH required to consistently induce the ACTH receptor and the P-450 genes essential to steroidogenesis is greater than that needed to stimulate growth and differentiation of the fetal adrenal. Clearly, further study with additional doses of ACTH is needed to more precisely define the absolute level of ACTH required *in vivo* to fully achieve all aspects of normal fetal adrenal function at midgestation.

In addition to ACTH, placental corticotropin releasing hormone (CRH) produced in high levels during human and baboon pregnancy (Goland RS et al., 1992), may have a role in regulating adrenal steroidogenesis (Jones CT and Edwards AV, 1992) and extrahypothalamic pituitary ACTH release (Challis JRG and Brooks AN, 1989). The potential interaction of

ACTH and CRH in regulating development of the primate fetal adrenal., however, has not been investigated.

Summary

Suppression of fetal pituitary POMC expression in baboons by betamethasone administration at midgestation resulted in apoptosis, cellular disorganization, loss of 3β-HSD expression, and a decline in ACTH receptor and P-450 steroidogenic enzyme mRNA levels in the fetal adrenal gland. It is concluded that ACTH is necessary for the growth and development of the fetal and definitive zones of, as well as a coordinated increase in expression of the ACTH receptor and P-450 enzyme components critical to C_{19}-steroid estrogen precursor production within, the primate fetal adrenal cortex at midgestation.

Table 3-1. Effect of betamethasone administration on maternal and fetal serum estradiol, DHAS, and cortisol concentrations in baboons at midgestation

	Maternal vein			Umbilical artery		
Treatment[a]	Estradiol (ng/ml)	DHAS (μg/dl)	Cortisol (μg/dl)	Estradiol (ng/ml)	DHAS (μg/dl)	Cortisol (μg/dl)
Control (n=7)	1.67±0.16	20.1±3.8	39.8±3.9	0.52±0.05	77.5±8.8	12.4±0.6
Betamethasone (n=4)	0.17±0.04[b]	2.7±1.5[b]	0.6±0.1[b]	0.11±0.03[b]	5.6±3.4[b]	0.7±0.1[b]

[a]Betamethasone (3 mg daily via maternal im injection) was administered to baboons on days 60-99 of gestation (term = 184 days). Serum steroid levels represent the means (±SE) on samples obtained daily on days 95-100 from the maternal vein and on day 100 only from the umbilical artery. The number of animals studied are in parentheses.

[b]Significantly different at $P<0.01$ from untreated controls (Student's t test).

Table 3-2. Effect of betamethasone and ACTH administration on baboon fetal adrenal cortical cell number and relative weight at midgestation

Treatment	Body Weight (g)	Fetal adrenal cells/0.025 mm^2	Adrenal wt / Body wt
Control (8)	174 ± 7^a	66.8 ± 3.7^a	0.80 ± 0.01^a
Betamethasone (6)	140 ± 13^a	120.9 ± 15.6^b	0.35 ± 0.05^b
Betamethasone + ACTH (4)	133 ± 15^a	95.1 ± 13.7^b	0.72 ± 0.17^a
ACTH (5)	189 ± 20^a	50.1 ± 6.1^a	0.81 ± 0.12^a

Betamethasone (3 mg/day) was administered on days 60-99 to the mother and ACTH (25 μg/day) on days 95-99 to the baboon fetus. Values (means ±SE) with different letter superscripts differ from each other at $P<0.01$ (analysis of variance and Newman-Keuls multiple comparison test).

Figure 3-1. Fetal pituitary POMC mRNA levels (means ±SE) determined by quantitative *in situ* hybridization on day 100 of gestation in untreated control baboons (n = 3) and in animals treated maternally with 3 mg betamethasone on days 60 - 99 (n = 4, term = 184 days).

*Significantly different ($P<0.01$) from the untreated controls (Students t test).

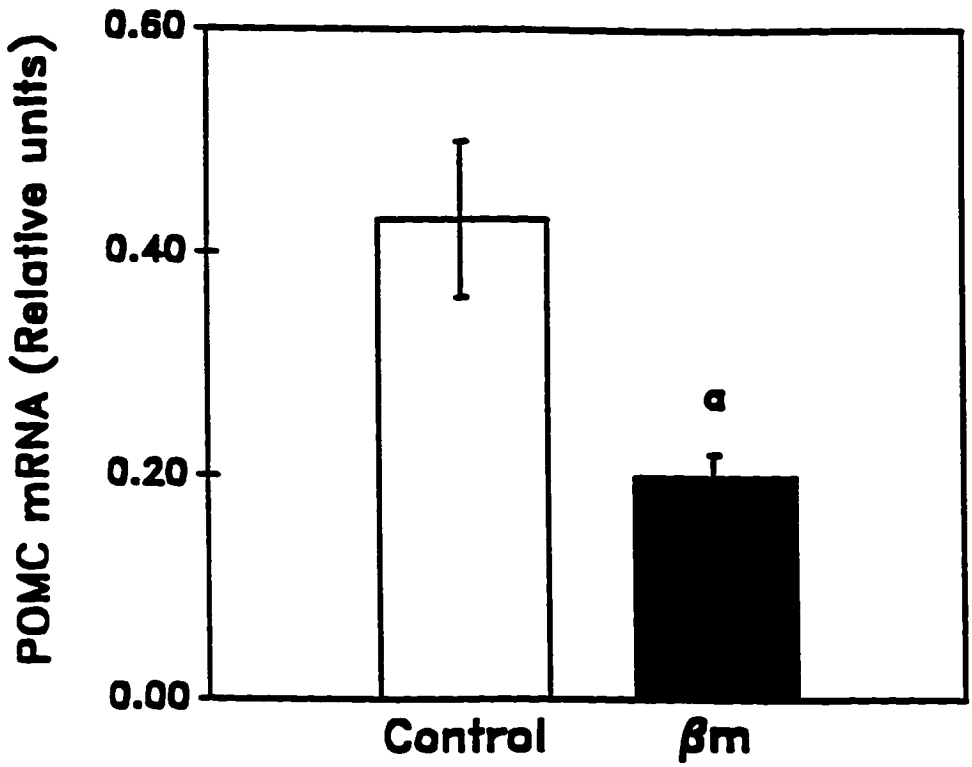


Figure 3-2. Adrenal weights (means ±SE) of baboon fetuses delivered on day 100 of gestation after no treatment (n = 8); after maternal administration of betamethasone on days 60-99 (n = 6); after maternal administration of betamethasone on days 60 - 99 and fetal administration of ACTH on days 95-99 (n = 4); and after fetal administration of ACTH only on days 95-99 (n = 5). See footnotes of Tables 1 and 2 for additional details. Values designated with different letter superscripts are different from each other at $P<0.01$ (analysis of variance and Newman-Keuls multiple comparison test).

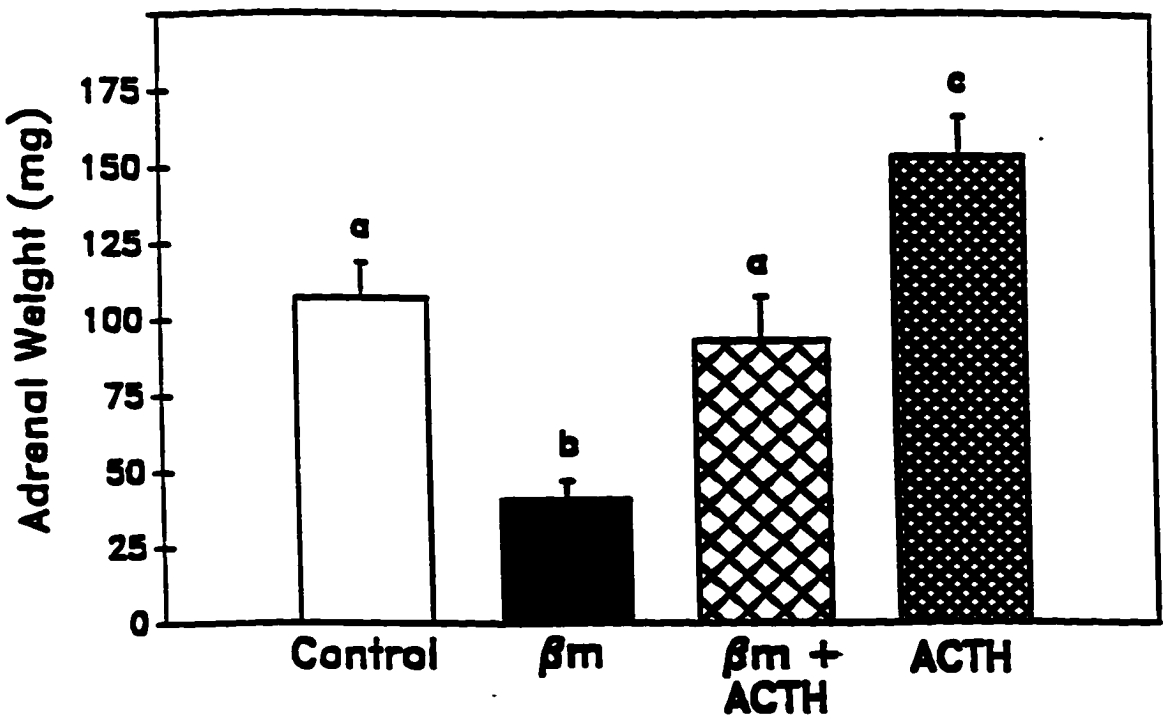


Figure 3-3. Immunocytochemical analysis of apoptosis in fetal adrenal glands obtained on day 100 of gestation from baboons that: (A) were untreated; (B) received betamethasone; or (C) received betamethasone and ACTH as detailed in the footnotes of Tables 3-1 and 3-2. Magnification of photomicrographs is approximately 100 x..

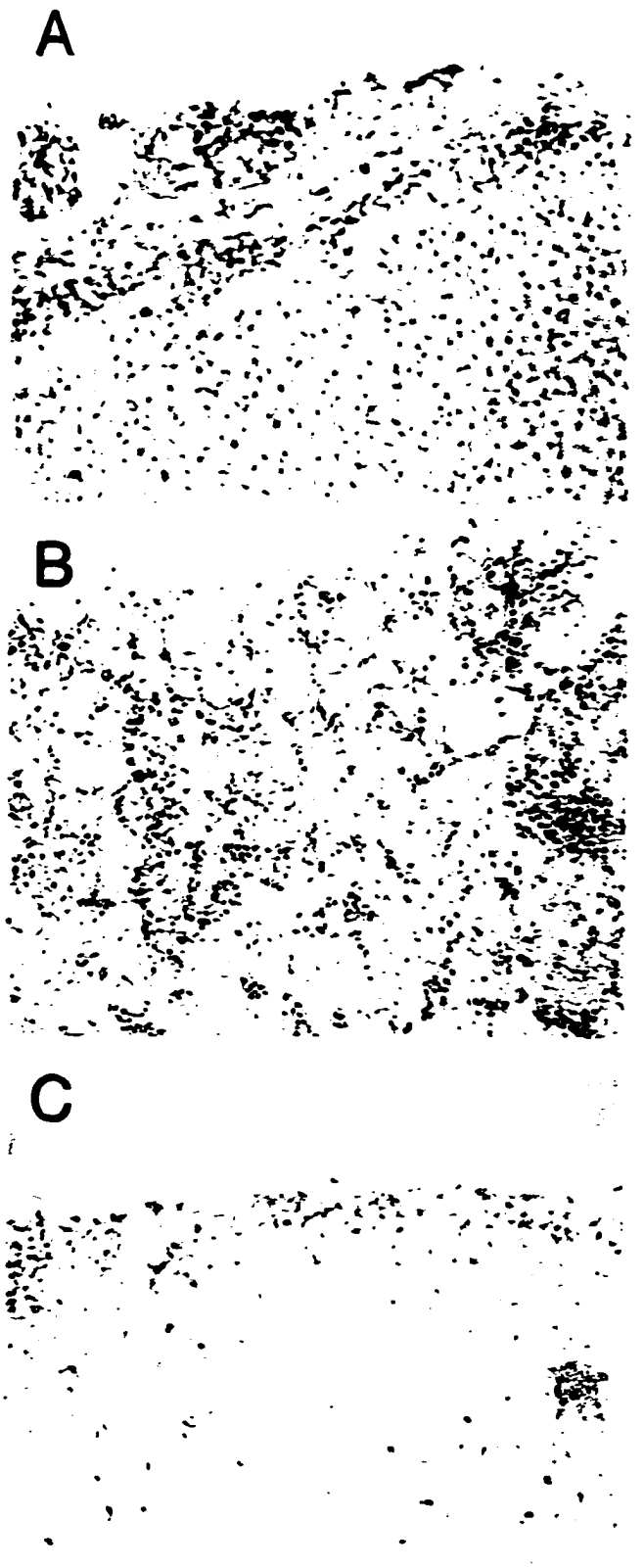


Figure 3-4. Photomicrographs of 3β-HSD immunocytochemistry and hematoxylin staining of fetal adrenal glands obtained on day 100 of gestation from baboons after no treatment (A); after maternal administration of betamethasone (B); and after maternal administration of betamethasone and fetal administration of ACTH (C). The arrow indicates 3β-HSD staining in the definitive zone. Panel D shows an adrenal of an untreated control in which the primary 3β-HSD antibody was deleted. Magnification of photomicrographs A, B, C is approximately 100 x and D is approximately 200 x.

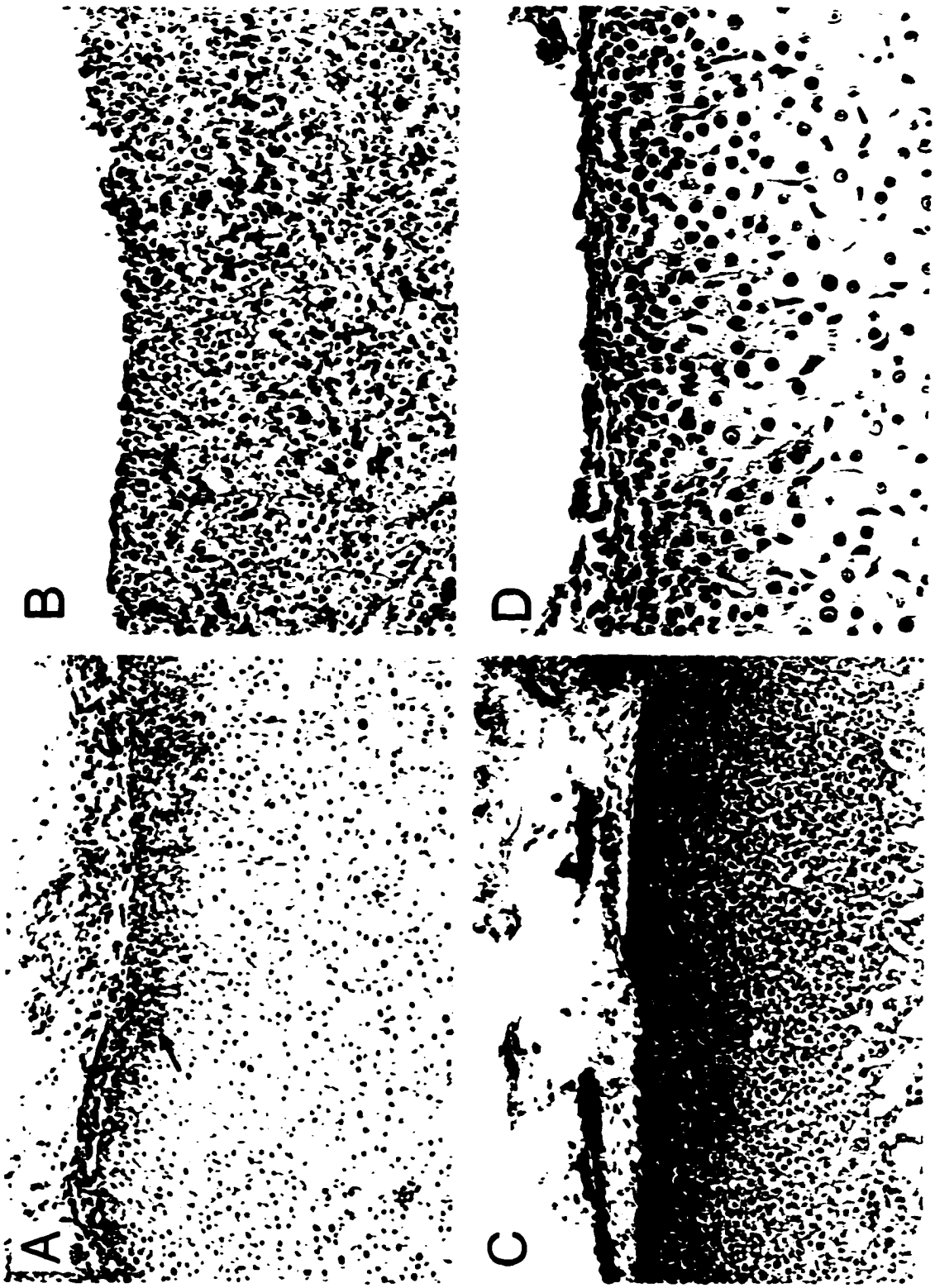


Figure 3-5. Width (μm) of the definitive zone of the fetal adrenal in baboons that were untreated (n = 8); treated with betamethasone (n = 6); betamethasone and ACTH (n = 4); or ACTH alone (n = 5). The width of the definitive zone was quantified by image analysis of definitive zone-specific immunocytochemical expression of 3β-HSD. Values (means ±SE) with different letter superscripts differ at $P<0.01$ (analysis of variance and Newman-Keuls multiple comparison test).

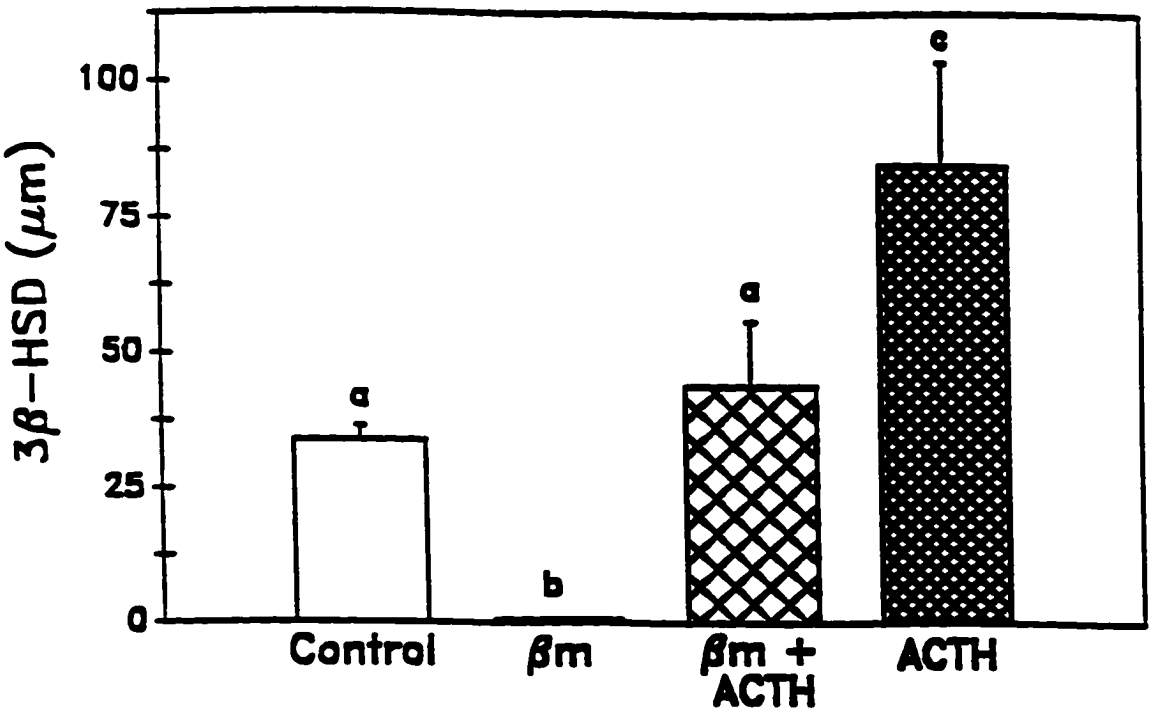


Figure 3-6. (A) Expression of the ACTH receptor 3.4-kb mRNA transcript determined by Northern analysis in fetal adrenal glands obtained on day 100 of gestation from baboons after no treatment (Control, n=4) and after maternal betamethasone administration on days 60-99 (βm, n = 4). Approximately 2.5 μg poly (A^+) RNA was size-fractioned in a 1.0% agarose gel, transferred onto nylon membrane, hybridized with 10^6 cpm/ml ^{32}P-labeled baboon ACTH receptor cDNA, and exposed to autoradiogram film. (B) β-Actin mRNA expression determined in the same RNA samples depicted in panel A. After hybridization with ACTH receptor cDNA, the membrane was washed in Tris, EDTA, 1% SDS, reprobed with the β-actin cDNA, and exposed to film for 2 h. (C) Mean ±SE ACTH mRNA levels (arbitrary units) determined by autoradiographic densitometry of the samples shown in panel A. *Value significantly different at $P<0.01$ (Students t test).

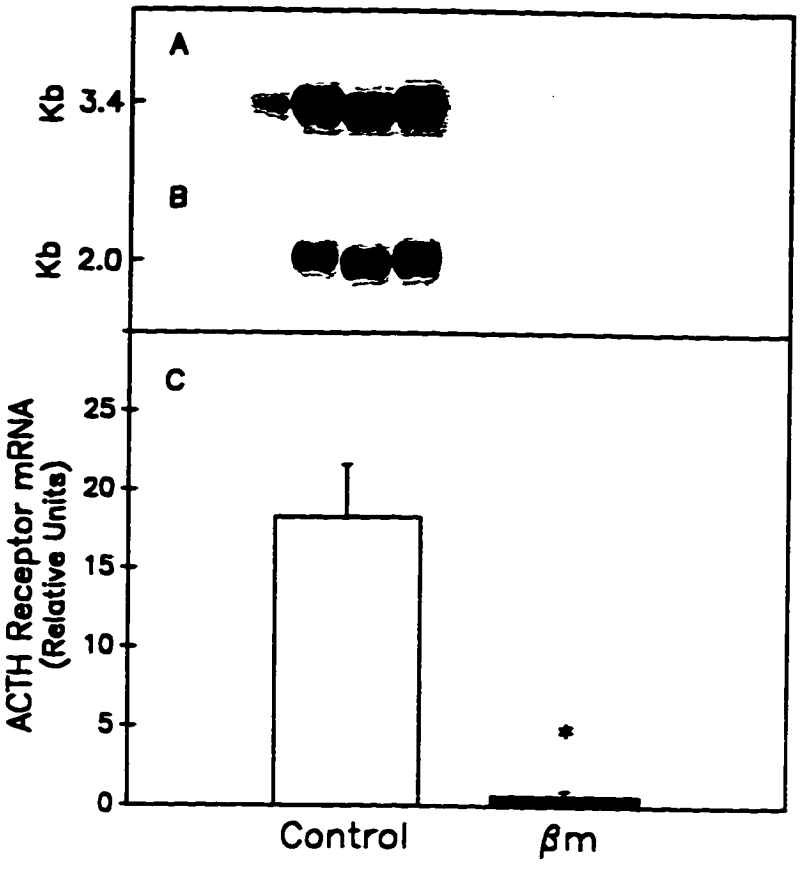


Figure 3-7. Northern blot of baboon fetal adrenal ACTH receptor and β-actin mRNA expression after no treatment (Control, n=4); after fetal ACTH administration (ACTH, n=4); after maternal administration of betamethesone (βm, n=4); and after maternal administration of betamethasone and fetal administration of ACTH (βm + ACTH, n=4).

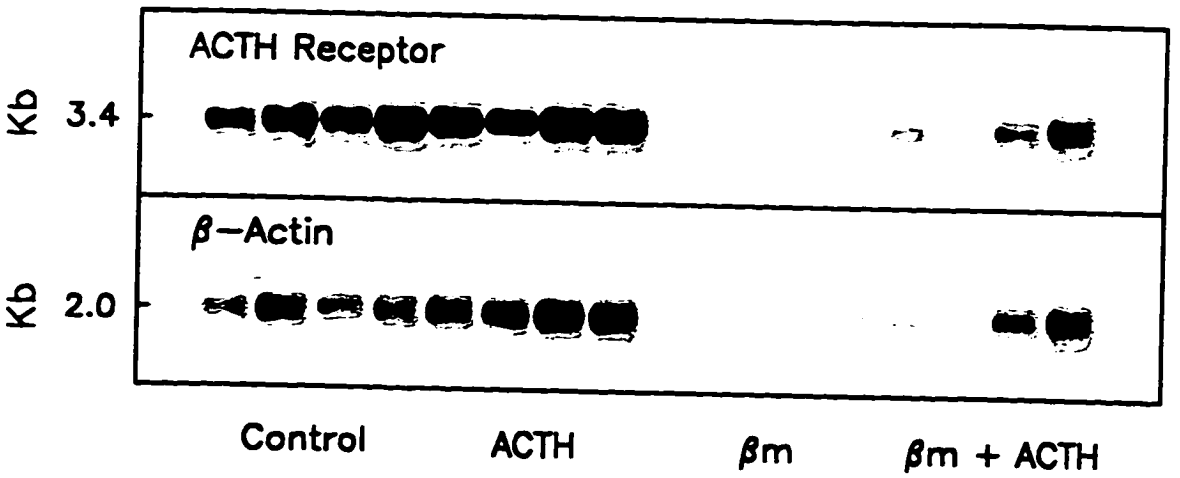
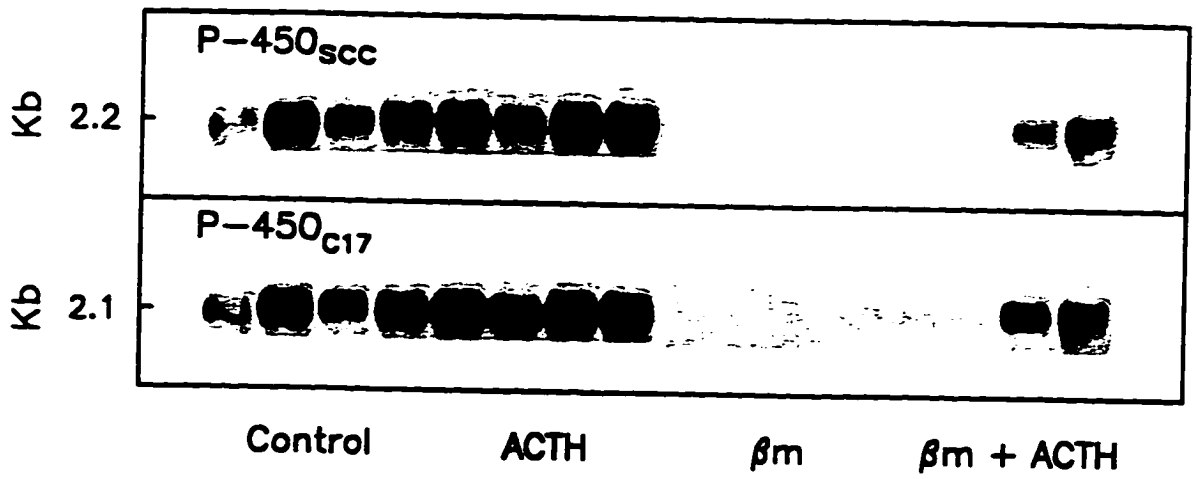


Figure 3-8. Northern blot of P-450scc and P-450_{C17} mRNA expression in the fetal adrenal glands of the baboons shown in Fig. 3-7.



[1]CHAPTER IV

INHIBITION OF FETAL ADRENAL ACTH mRNA EXPRESSION BY BETAMETHASONE ADMINISTRATION TO THE BABOON FETUS IN LATE GESTATION

Abstract

Throughout the majority of intrauterine development, the primate fetal adrenal gland is comprised primarily of fetal zone cells and only late in gestation do definitive zone cells, which express the enzyme Δ^5-3β-hydroxysteroid dehydrogenase/isomerase (3β-HSD) emerge to produce cortisol. The present study was designed to determine whether the induction of definitive zone ACTH receptor mRNA levels and components of the steroidogenic pathway known to be expressed specifically in the definitive zone, e.g. the 3β-HSD enzyme, are dependent upon fetal pituitary ACTH. Fetal pituitaries and adrenal glands were obtained on day 165 (term = day 184) from untreated controls (n=7) and from baboons in which betamethasone was administered im to the fetus (0.6 mg/100μl; n=4) or to the fetus (0.6 mg) and mother (6 mg/ml; n=4) every other day between days 150 and 164 of gestation. Although fetal pituitary weight was not altered by betamethasone, proopiomelanocortin (POMC) mRNA levels determined by *in situ* hybridization were lower ($P<0.05$) in betamethasone-treated (0.34 ± 0.07 arbitrary densitometric units) than in untreated controls (0.63 ± 0.04). Associated with this decline in pituitary POMC, levels of the major 3.4 kb mRNA transcript for the ACTH receptor expressed as a ratio of β-actin were approximately 80% lower ($P<0.05$) in fetal adrenals of betamethasone-treated baboons (0.12 ± 0.02) than in untreated controls (0.84 ± 0.05). *In situ* hybridization indicated that ACTH receptor mRNA expression in the definitive

[1]Data presented in this chapter has been in part submitted for publication. Leavitt MG, Aberdeen GW, Burch MG , Albrecht ED, Pepe GJ 1997 Inhibition of fetal ACTH receptor messenger ribonucleic acid expression by betamethasone administration to the baboon fetus late in gestation. Endocrynology, In press

zone exceeded that in the fetal zone and was reduced by betamethasone. Associated with the decrease in ACTH receptor expression, fetal adrenal weight was suppressed ($P<0.05$) by 50% and reflected a marked reduction ($P<0.05$) in the size of the cells of the definitive and fetal zones. Betamethasone treatment also induced a decrease ($P<0.05$) in the width (μm) of the definitive zone (183 ± 14 vs 128 ± 7; determined by immunohistochemical expression of 3β-HSD), as well as the levels of the mRNA and protein for 3β-HSD. Levels of the mRNA for the LDL-receptor and the enzymes 17α-hydroxylase-$C_{17,20}$ lyase and P450 cholesterol side chain cleavage were also suppressed in adrenals of betamethasone-treated baboons. These findings indicate that treatment of the baboon fetus with betamethasone in late gestation suppressed fetal pituitary POMC mRNA expression and ACTH receptor mRNA levels in the fetal adrenal gland, as well as the hypertrophy and ACTH receptor mRNA and 3β-HSD mRNA/protein levels in the cells comprising the newly emerging definitive zone. We conclude that ACTH is necessary for the up-regulation of the mRNAs for the ACTH receptor and steroidogenic enzymes in the definitive zone of the primate fetal adrenal gland in late gestation.

Experimental approach

Hypothesis I. Placental estrogen modulates ACTH-induced growth and maturation of the fetal definitive zone (e.g. 3βHSD, P450c17) while concomitantly acting to modulate ACTH action on fetal cortical cell hyperplasia and /or hypertrophy and synthesis of DHA.

The results of this section will be concerned with two experimental treatments, betamethasone, (synthetic glucocorticoid) administered to the fetus (0.6 mg/100μl; n=4) or to the fetus (0.6 mg/100μl; n=4) and the mother (6mg/ml; n=4) every other day between days 150 and 164 of gestation (term=184); and the effect of the two treatments on the adrenal gland mRNA and/or protein levels/distribution of the enzymes 3βHSD, P450c17 and P450scc and the membrane receptors for LDL and ACTH.

Rationale

Albrecht ED et al (Albrecht ED et al., 1996) have previously demonstrated that messenger ribonucleic acid (mRNA) levels for the ACTH receptor in the baboon fetal adrenal at midgestation, a time when the gland is composed almost exclusively of fetal zone cells (Jaffe RB et al., 1988; Pepe GJ and Albrecht ED, 1990), are greater than respective values in adrenals of late gestation, a time during which there is development of definitive zone cells (Pepe GJ et al., 1977; Mesiano S et al., 1993; Pepe GJ and Albrecht ED, 1991). The increase in ACTH receptor mRNA expression in the fetal adrenal at midgestation would provide a mechanism to mediate ACTH-dependent dehydroepiandrosterone (DHA) production (Walker ML et al., 1988; Pepe GJ and Albrecht ED, 1995). Albrecht ED et al (Albrecht ED et al., 1996) have proposed, however, that the decrease in ACTH receptor mRNA with advancing gestation reflected a decline in receptor in the fetal zone cells and its appearance in the developing definitive zone. In support of this hypothesis, Aberdeen GW et al (Aberdeen GW et al., 1997) have recently shown using *in situ* hybridization that there was a decrease in ACTH receptor mRNA concentrations in the fetal zone between mid and late gestation. Moreover, expression of ACTH receptor mRNA in definitive zone cells late in gestation exceeded respective values in fetal zone cells. Our present studies are aimed at elucidating the mechanisms regulating this biphasic developmental pattern of ACTH receptor mRNA expression in zone-specific cells of the fetal adrenal cortex.

Under *in vitro* conditions, ACTH up-regulates ACTH binding (Rainey WE et al., 1989; Penhoat A et al., 1989) or ACTH receptor mRNA expression in human and bovine adult (Lebrethon MC et al., 1994; Mountjoy KG et al., 1994; Penhoat A et al 1994) and human fetal (Lebrethon MC et al., 1994; Mesiano S et al., 1996) adrenocortical cells. Recently, Aberdeen GW et al (Aberdeen GW et al., 1997) demonstrated that treatment of baboon fetuses at midgestation with ACTH enhanced ACTH receptor mRNA levels in fetal adrenal glands obtained from baboons in which ACTH receptor expression was depleted by betamethasone. These findings indicate that ACTH up-regulates its own

receptor in fetal zone cells *in vivo*. However, because ACTH receptor mRNA levels in the fetal zone decline with advancing gestation in the baboon, it is not known whether the concomitant induction of ACTH receptor mRNA in the adrenal definitive zone cells of near term baboon fetuses is regulated by ACTH. Therefore, the present study was designed to determine whether the induction of definitive zone ACTH receptor mRNA levels and components of the steroidogenic pathway known to be expressed specifically in the definitive zone, e.g. the Δ^5-3β-hydroxysteroid dehydrogenase-isomerase (3β-HSD) enzyme, are dependent upon fetal pituitary ACTH.

Materials and methods

Animals

Female baboons (*Papio anubis*) weighing 10-15 kg were housed individually in stainless steel cages in air-conditioned quarters and fed Purina monkey chow (Ralston Purina, St. Louis, MO) and fresh fruit and/or carrots daily and water ad libitum. Females were paired with males for 5 days at the anticipated time of ovulation and pregnancy confirmed 30 days post ovulation. Baboons were cared for and used strictly in accordance with USDA regulations and the NIH Guide for the Care and Use of Laboratory Animals (Publication 85-23, 1985). The experimental protocol employed in this study was approved by the Institutional Animal Care and Use Committee of the Eastern Virginia Medical School.

Experimental protocol

Fetal adrenal glands and pituitaries were collected on day 165 from untreated controls (n=7) and from baboons in which betamethasone (Celestone Soluspan, Schering Corp., Chicago IL) was administered to the fetus (0.6 mg/100 μl; n=4) or to the fetus (0.6 mg/100 μl) and the mother (6.0 mg/ml, im; n=4) every other day between days 150 and 164 of gestation (term = day 184). Baboons were sedated with ketamine-HCl (10 mg/kg BW; Parke-Davis, Detroit, MI), anesthetized with halothane:nitrous oxide, and betamethasone administered im to the fetus via a 25 gauge needle and maternal

transabdominal injection under ultrasound. At 1-2 day intervals between days 140 and 165 of gestation, all baboons were sedated with ketamine-HCl and a maternal saphenous vein blood sample (4-7 ml) collected. Two of the 4 baboons in which betamethasone was administered only to the fetus delivered spontaneously on days 160 and 164 or approximately 96 and 1 h before their last injection of steroid. Since labor (vaginal bleeding) was initiated between 1 and 2 pm, neonates were recovered from ketamine-sedated mothers during or 30 min after delivery. The neonates were sedated with ketamine and after collecting a blood sample, a lethal dose of Beuthanasia (Butler Corp., Fredericksburg, VA) was administered and tissues obtained within 60 min. In all other animals, umbilical venous blood samples were obtained at the time of cesarean section and samples stored at -20C until assayed for estradiol and cortisol by solid phase ^{125}I RIAs (Coat-A-Count, Diagnostic Products Corp., Los Angeles, CA) as described previously (Pepe GJ et al., 1988; Pepe GJ et al., 1990).

Adrenals were weighed and representative sections frozen in liquid nitrogen for subsequent analysis of the mRNAs for ACTH receptor, LDL receptor, 17α-hydroxylase-$C_{17,20}$ lyase (P450c17), P450 cholesterol side chain cleavage (P450scc) and 3β-HSD and peptide levels of P450c17 and 3β-HSD. In some instances, fetal adrenal sections were stored at -80C for subsequent analysis of 3β-HSD enzyme activity. Fetal pituitaries of 4 control baboons and of 4 baboons in which betamethasone was administered to the fetus (n=3) or to the fetus and mother (n=1) were placed in sterile cryomolds containing OCT embedding medium (Miles Scientific, Elkhart, IN) and stored at -80C until analyzed for POMC mRNA.

Adrenal morphometry and immunocytochemistry

Sections (4 μm) of paraffin embedded adrenal glands were mounted onto Superfrost microscope slides (Fischer Scientific Co., Arlington, VA), heat fixed and endogenous peroxidase blocked with 0.4% H_2O_2 in methanol. After incubation (4C) overnight with anti-PCNA/cyclin monoclonal antibody PC-10 diluted 1:200 in 5% normal

goat serum (NGS; Boehringer Mannheim, Indianapolis, IN) or with polyclonal antibody to rabbit anti-human 3β-HSD (generously supplied by Dr. Ian Mason) diluted 1:5000 in 5% NGS, sections were washed and incubated with biotinylated goat anti-mouse or goat anti-rabbit IgG (Boehringer Mannheim), Avidin DH and horseradish peroxidase H (Vectastain Elite Kit, Vector Laboratories, Burlingame CA). Sections were lightly counterstained with Gill's hematoxylin (Fisher) and mounted in Biomount (Fisher) and PCNA and 3β-HSD expression analyzed by Image Analysis on an average of 6 randomly selected areas (157 μ m x 130 μm)/slide of 4-8 fetal adrenal sections per animal using an Optiphot-2 microscope attached to a Video-Based Image 1 Analysis System (Universal Imaging Corp, West Chester, PA). The number of definitive and fetal cortical cells per 0.025 mm^2 was quantified by counting nuclei in 6 randomly selected sections and results compared with the number of cells in which nuclear expression of PCNA was 5-fold greater than background. The growth of the definitive zone cells was quantified as the width of the cell layer immunostaining for 3β-HSD and determined by examining 6 randomly selected regions of each adrenal section.

mRNA for 3β-HSD, P450c17, P450scc and the ACTH and LDL receptors

These were determined by Northern blot essentially as described previously (Albrecht ED et al., 1995). Briefly, approximately 10 μg of fetal adrenal poly(A+) RNA was denatured and size-fractionated by electrophoresis in 1.0% agarose gel containing 0.66M formaldehyde and 20 mM MOPS. RNA was transferred overnight by capillary action onto nylon membrane (GeneScreen, DuPont-New England Nuclear, Boston, MA), UV cross-linked, baked in a vacuum oven (80C for 2h) and prehybridized in buffer containing 50% formamide, 0.1% polyvinyl pyrrolidine, 0.1% BSA, 0.1% Ficoll, 2.5 x SSPE (0.375 M NaCl, 0.025M NaH_2PO4-H_2O and 2.5 mM EDTA-Na_2, pH 7.4), 1.0% SDS, 10% dextran sulfate, and denatured salmon sperm DNA (100 μg/ml) for 24h at 42C before addition of labeled probe. The cDNAs for the baboon ACTH receptor prepared by us (Albrecht ED et al., 1996), the 3β-HSD (provided by Dr. Ian Mason), the P450c17,

P450scc (provided by Dr. Walter Miller), and the LDL receptor (pLDLR3 No 57004) and β-actin (No. 65128) both obtained from the American Type Culture Collection (Rockville, MD) were labeled with approximately 50 μCi [α-^{32}P]deoxy-CTP (3000 Ci/mmol; Amersham Corp., Arlington Hts. IL) to a specific activity of approximately 10^9 dpm/μg DNA using the Random-Primed DNA labeling kit (Boehringer-Mannheim) according to the methods of Feinberg and Vogelstein (Feinberg AP, Vogelstein B, 1983). Hybridization was performed in fresh buffer at 42C for 23 h with ^{32}P-labeled cDNA. After washing under stringent conditions, the membranes were exposed to Kodak X-AR film (Eastman Kodak, Rochester, NY) at -80C and the intensities of the bands on each Northern blot analyzed by densitometric autoradiographic scanning using a model 620 Video Densitometer (Bio-Rad, Hercules, CA).

Western Analysis of 3β-HSD and P450c17

Analysis of 3β-HSD and P450c17 peptides in fetal adrenal extracts was performed using procedures developed previously in our laboratories (Pepe GJ et al., 1996). Briefly, adrenals were homogenized on ice in 2.5 ml buffer composed of 1% cholic acid (Sigma), 0.1% SDS, 1mM EDTA in phosphate buffered saline to which had been added 0.1 mg/ml phenylmethylsulfonyl fluoride (PMSF), 10 μg/ml aprotinin and 0.1 mg/ml trypsin inhibitor and centrifuged at 800 x g to remove cell debris. After determination of protein concentrations using the bicinchoninic acid procedure (Sigma), 5x Laemmli buffer (Laemmli UK, 1970) was added to a final concentration of 1x and samples boiled for 2 min, centrifuged (1,000 x g 10 min) and loaded (30 μg protein/lane) onto preformed 10% SDS-polyacrylamide minigels maintained in Bio Rad Mini-Protean II electrophoresis chambers, electrophoresed at 100V, and transferred to Immobilon P (Gibco BRL, Bethesda, MD). After blocking with 3% BSA in 50 mM Tris, pH 7.5 containing 150 mM NaCl and 0.05% Tween 20 (Sigma), samples were incubated (37C, 1h) with polyclonal antibodies to rabbit anti-human placental 3β-HSD or rabbit anti-porcine testicular microsomal P450c17 (generously supplied by Dr. Ian Mason) diluted 1:10,000 or 1:2,000

respectively in 50 mM Tris buffer (pH 7.5) containing 150 mM NaCl, 0.05% Tween 20, 0.05% Nonidet P-40 (Sigma) and 1.5% BSA. Membranes were washed and then incubated with donkey anti-rabbit IgG horseradish peroxidase conjugated second antibody (Amersham) at dilutions recommended by the manufacturer and which contributed no non-specific bands at the concentrations employed. After washing, equal amounts of enhanced chemiluminescent reagent (ECL; Amersham) were applied to membranes for 1 min, the membranes wrapped in plastic and then placed against Kodak X-Omat film (Kodak) in X-ray film cassettes and exposed for 15-60 sec. Samples were developed and quantified by 1 dimensional densitometry using an LKB Bromma Ultroscan XL Enhanced Laser densitometer.

3β-HSD enzyme activity

The activity of 3β-HSD was determined essentially as described previously (Pepe GJ, Albrecht ED, 1991). Briefly, adrenals were homogenized in 2.5 ml 0.05 M NaH_2PO_4 buffer (pH 7.4) and microsomes (or buffer blank) incubated in duplicate (37C) for 2, 5 and 10 min with 125,000 cpm [7-^{3}H] pregnenolone (SA, 25 Ci/mmol; DuPont NEN), 125 ng radioinert pregnenolone (Sigma) and 2.5 mg NAD^+ (Sigma). Samples were extracted with ethyl acetate, radiolabeled product progesterone isolated by paper chromatography and [^{3}H] concentrations, corrected for procedural losses estimated by recovery of [4-^{14}C] progesterone (SA 35 mCi/mmol; Dupont NEN) added prior to extraction, determined by liquid scintillation spectrometry.

In situ hybridization histochemistry of POMC and ACTH receptor mRNAs

In situ hybridization detection and quantification of POMC mRNA expression were performed using our previously published methods (Pepe GJ et al., 1994). Briefly, 0.1μmol purified POMC antisense (and sense) oligodeoxynucleotide probes were 3' end-labeled with [^{35}S]dATP (SA > 1000 Ci/mmol; NEN) and terminal deoxynucleotidyl transferase (20 U; Promega, Madison, WI) to a specific activity of approximately 5000 Ci/mmol. Sections of the fetal pituitary were selected, incubated overnight (50C) with 40μ

l labeled antisense or sense probe and then washed at 60C, rinsed and placed against Kodak X-Omat film in X-ray film holders and exposed for 5-7 days. POMC mRNA expression was determined by densitometric analysis using an LKB Bromma Ultroscan XL Enhanced Laser Densitometer (Pharmacia LKB, Piscataway, NY).

Localization of ACTH receptor mRNA was determined using our previously published procedures (Aberdeen GW et al., 1997). Briefly, sections of paraffin-embedded fetal adrenal glands were cleared in xylene, rehydrated in graded ethanols, rinsed in PBS and hybridized overnight at 45C with an oligodeoxynucleotide antisense (sense) probe complementary to bases 373-401 of the baboon ACTH receptor (Albrecht ED et al., 1996) and end-labeled with [^{35}S] dATP (Dupont NEN). Slides were washed at 60C, rinsed, dipped in Kodak NTB-2 nuclear track emulsion diluted 1:1 with distilled water, placed in light tight boxes, exposed for 12-17 days and then developed in Kodak D-19. The cellular distribution of silver grains was determined using an Optiphot 2 microscope attached to a Video Based Image-1 Analysis System (Universal Imaging Corp., West Chester, PA).

Statistics

Because fetal adrenal weight and fetal pituitary POMC mRNA expression was not different following maternal and/or fetal betamethasone, values for the various parameters examined in these two treatment groups were combined and expressed as an overall mean for the effects of betamethasone. Data were analyzed by analysis of variance with *post hoc* comparison of the means by the Student Newman Keuls statistic or were compared by Students' *t* tests for independent or dependent observations.

Results

Blood samples

Maternal serum cortisol and estradiol concentrations in untreated control baboons were not significantly changed during the study period (Fig 4-1, Panels A and B). In contrast, betamethasone administration to the mother and fetus resulted in a rapid and

sustained decrease in maternal serum estradiol and cortisol concentrations (Fig 4-1, Panels E and F). However, in baboons in which betamethasone was administered only to the fetus, reductions in maternal serum estradiol and cortisol concentrations, although significant, were much less marked (Fig 4-1, Panels C and D). Moreover, umbilical venous serum concentrations (mean ± SE) of cortisol and estradiol obtained at the time of delivery were decreased ($P<0.05$) when betamethasone was administered to the fetus and further reduced in animals in which both the mother and fetus received betamethasone (Table 4-1) although the latter was not confirmed statistically due to heterogeneity of variance between the groups.

POMC and ACTH mRNA levels

Although fetal pituitary and body weights were not altered by betamethasone (Table 5-1), POMC mRNA levels (Fig 4-2) were lower ($P<0.05$) in betamethasone-treated (0.34 ± 0.07 arbitrary densitometric units) than in untreated control (0.63 ± 0.04) baboons. Associated with the decline in pituitary POMC mRNA, levels of the major 3.4 kb mRNA transcript for the ACTH receptor (Fig 4-3) expressed as a ratio of β-actin, were approximately 80% lower ($P<0.05$) in the fetal adrenal of baboons in which betamethasone was administered to the fetus and/or to the mother and fetus (0.12 ± 0.02) than in untreated controls (0.84 ± 0.05). However, in the two animals in which the fetus was delivered spontaneously, ACTH receptor mRNA levels (1.06 and 0.63) were similar to those of the untreated controls. *In situ* hybridization indicated that in control baboons ACTH receptor mRNA levels, expressed as the number of silver grains/0.025 mm^2, were greater ($P<0.01$) in the definitive zone than in the corresponding fetal zone and that the reduction of ACTH receptor mRNA expression elicited by betamethasone occurred primarily in the definitive zone (Fig 4-4). Associated with the decrease in ACTH receptor expression, fetal adrenal weight was reduced ($P<0.05$) approximately 50% by the administration of betamethasone to the fetus (Table 4-1), but was not further suppressed by administration of betamethasone to both the mother and fetus. The decrease in adrenal

weight was associated with an increase ($P<0.05$) in the number of fetal and definitive zone cells per unit area (Table 4-2), indicating that the size of these cells was reduced by betamethasone administration. The number of fetal adrenal cells expressing PCNA, however, was similar in control and betamethasone-treated animals (Table 4-2).

Protein and mRNA levels of 3βHSD

Betamethasone treatment also induced a significant decrease ($P<0.05$) in the width (μm) of the definitive zone (183 ± 14 vs. 128 ± 7), as determined by immunohistochemical expression of 3β-HSD (Fig 4-5, panel A). The decrease in definitive zone width was associated with a reduction ($P<0.05$) in expression of the single 2.0 kb 3β-HSD mRNA transcript (Fig 4-5B), the 36 kDa 3β-HSD peptide (Fig 4-5C), and 3β-HSD enzyme activity (2.2 pmol/mg; Fig 5D), which was 20-fold lower than that previously measured by us in adrenals of untreated baboons (41 ± 11 pmol/mg) at the same time in gestation (Pepe GJ and Albrecht ED, 1991).

Protein and/or mRNA levels of the enzymes 3β-HSD, P450c17 and P450scc and the membrane receptor LDL

Associated with the reduction in ACTH receptor mRNA, the levels of the 6.2 kb mRNA for the LDL receptor (Fig 4-6A), the 2.2 kb mRNA for P450scc (Fig 4-6B) and the 2.1 kb mRNA for P450c17 (Fig 4-6C) in adrenals of term controls (1.23 ± 0.20; 1.35 ± 0.07 and 1.61 ± 0.09 respectively) were also decreased ($P<0.05$) by approximately 80-90% by betamethasone in cesarean (0.09 ± 0.02; 0.25 ± 0.05; and 0.30 ± 0.06; respectively), but not spontaneously delivered baboons. The levels of the 55 kDa P450c17 protein in adrenals of term controls (3.15 ± 0.19) were also decreased ($P<0.05$) by betamethasone in cesarean delivered (0.88 ± 0.18) but not spontaneously delivered (2.1) baboons (data not shown).

Disscusion

Reduced POMC and ACTH mRNA levels

The results of the present study indicate that fetal adrenal ACTH receptor mRNA levels were markedly suppressed in baboons in which fetal pituitary POMC mRNA expression was reduced by treatment with betamethasone during late gestation. Pepe GJ and colleagues (Pepe GJ et al., 1994) have previously shown that the increase in fetal pituitary POMC mRNA levels with advancing baboon gestation is associated with a concomitant increase in the number of pituitary corticotrophes expressing and presumably secreting ACTH peptide. Therefore, we suggest that ACTH of fetal pituitary origin regulates fetal adrenal ACTH receptor mRNA expression in the baboon fetus late in gestation. Previous studies in rhesus monkeys have also shown that maternal or fetal administration of dexamethasone/betamethasone (Challis JRG et al., 1974; Coulter CL et al., 1996) markedly suppressed fetal adrenal function/maturation late in gestation. However, the results of the present study are the first to show that these glucocorticoid-induced changes in late gestation are due to suppression of fetal pituitary POMC mRNA/ACTH and inhibition of fetal adrenal ACTH receptor mRNA expression. Although additional studies in which ACTH is infused into the late gestation fetus remain to be performed, the results of the present study are consistent with *in vitro* observations demonstrating that ACTH receptor mRNA levels were stimulated by ACTH in cultures of human fetal adrenal cells (Lebrethon MC et al., 1994; Mountjoy Kget al.,1994; Penhoat A et al., 1994; Lebrethon MC et al., 1994; Mesiano S et al., 1996) and with our recent *in vivo* studies showing that ACTH partially reversed the decline in ACTH receptor mRNA elicited by betamethasone at midgestation (Aberdeen GW et al., 1997). Mesiano et al (Mesiano et al., 1996) have also demonstrated that ACTH receptor mRNA expression was maximally enhanced 12-16 h after treatment of cultured human fetal adrenal cells with ACTH in a dose-dependent manner. This apparent relatively rapid increase in ACTH receptor mRNA induction as measured *in vitro* may also occur *in vivo*. Thus, ACTH

receptor mRNA levels were up-regulated in two animals of the present study that labored and delivered spontaneously and in which ACTH levels were probably elevated (Challis JRG and Lye SJ, 1994; Coulter CL et al., 1996).

The present results also indicate that the ACTH receptor was primarily expressed in cells of the newly emerging definitive zone of the fetal adrenal and exceeded that in cells of the fetal zone. Mesiano et al (Mesiano S et al., 1996) noted a similar pattern of ACTH receptor mRNA expression in the human fetal adrenal. Aberdeen GW et al (Aberdeen GW et al., 1997) have previously shown that ACTH receptor mRNA levels declined in the fetal zone with advancing baboon gestation and have suggested that the concomitant developmental increase in ACTH receptor expression in the definitive zone indicates that the definitive cells are more sensitive to ACTH than cells of the fetal zone late in pregnancy. The results of the present study are consistent with this suggestion, since ACTH receptor mRNA levels as assessed by *in situ* hybridization and expressed per adrenal cellular area were decreased in the definitive zone, but not the fetal zone, in baboons in which fetal pituitary POMC mRNA was suppressed. Moreover, we (Berghon KA et al., 1995) have previously shown that basal as well as ACTH-dependent DHA production by the baboon fetal adrenal *in vitro* is lower at term than at midgestation, whereas basal and ACTH stimulable cortisol synthesis at term exceeds that at midgestation. Because fetal pituitary POMC mRNA expression and presumably secretion of ACTH increases with advancing gestation in the baboon (Pepe GJ et al., 1994) and perhaps the human (Siler-Khodr TM et al., 1974), it appears that factors in addition to ACTH play a role in modulating ACTH receptor expression in the fetal zone of the primate fetal adrenal gland. Although additional studies utilizing *in situ* hybridization and molecular approaches are required to address this possibility, we have recently shown that estrogen acts directly on the fetal adrenal gland to modulate ACTH-dependent DHA production (Albrecht ED and Pepe GJ, 1987; Albrecht ED and Pepe GJ, 1987). However, whether estrogen acts to modulate ACTH receptor mRNA also remains to be determined.

Modulation of protein and/or mRNA levels of the enzymes 3β-HSD, P450c17 and P450scc and the membrane receptor LDL

The present study also indicates that the mRNA and/or protein levels of the enzymes 3β-HSD, P450c17 and P450scc and the membrane receptor for LDL were decreased in adrenals of betamethasone-treated animals near term. The activities and/or mRNAs for the 3β-HSD and the P450scc/P450c17 enzymes were regulated by ACTH in cultures of human adrenal cells (Simpson ER et al., 1988; Diblasio AM et al., 1987; McAllister JM et al., 1988) and *in vivo* in the sheep fetus (Tangalakis K et al., 1990). Moreover, expression of P450c17 mRNA is very sensitive to ACTH, because the mRNA for this enzyme rapidly declines under culture conditions in the absence of ACTH (DiBlasio AM et al., 1987). Similarly, LDL receptor binding is lower in adrenals of anencephalic fetuses and increased following treatment of fetal adrenal cells *in vitro* with ACTH (Ohashi M et al., 1981). As with ACTH receptor expression, the mRNA and protein levels of P450c17 and mRNA levels for P450scc and the LDL receptor were similar in adrenals of control cesarean delivered and betamethasone-treated spontaneously delivered baboons. Collectively, these observations indicate that the ACTH receptor mRNA is regulated coordinately with other ACTH-dependent genes, *in utero* in the primate fetus as recently suggested by Mesiano et al (Mesiano S et al., 1996) based on studies of human adrenal cells in culture.

Adrenal morphometry and immunocytochemistry

Our findings also indicate that following suppression of ACTH receptor mRNA there was a reduction in cell size of both the fetal and definitive zones. Thus, the decrease in width of the definitive zone, as assessed by immunohistochemical expression of zone-specific 3β-HSD (Doody KM et al., 1990), apparently reflected the smaller size of these cells. Therefore, one of the actions of ACTH may be to stimulate hypertrophy of the fetal and definitive zone cells as previously demonstrated in the fetal rhesus monkey (McNulty WP et al., 1981; Coulter CL et al.; 1996). ACTH also stimulated proliferation of

adrenocortical cells in the rat, but this effect occurred only after stimulation of cellular hypertrophy (Malendowicz LK et al., 1992). Although it remains to be determined whether the latter sequence also occurs in the fetal adrenal, in baboons of the present study the percentage of fetal and definitive zone cells expressing PCNA was not altered by betamethasone. Therefore, it is possible that the capacity of these cells to undergo replication if appropriately stimulated was not compromised. Indeed, Jaffe and colleagues have shown that fetal adrenal cells in culture proliferate in the continuous presence of physiologic levels of ACTH (DiBlasio AM et al., 1990).

In animals of the present study in which the fetal pituitary-adrenal axis was maximally suppressed by fetal administration of betamethasone, estradiol levels were only reduced by 50%. This suggests that the maternal adrenal is also a contributor of adrenal C-19 steroid precursors for placental estrogen production in nonhuman primate pregnancy as in humans (Albrecht ED and Pepe GJ; 1990). Thus, only when the maternal pituitary-adrenal axis was blocked was there a more marked suppression of estrogen synthesis.

Summary

The present study shows that treatment of baboon fetuses with betamethasone in late gestation suppressed fetal pituitary POMC mRNA expression and ACTH receptor mRNA levels and 3β-HSD expression in the definitive zone of the fetal adrenal gland. Moreover, in association with these changes in ACTH receptor expression, there was a significant decrease in fetal adrenal size and growth of the definitive zone. It is concluded that ACTH is necessary for the up-regulation of the mRNAs for the ACTH receptor and steroidogenic enzymes in the definitive zone of the primate fetal adrenal in late gestation.

Table 4-1. Effects of administration of betamethasone during baboon pregnancy on umbilical vein serum levels of cortisol and estradiol and on fetal adrenal, pituitary and body weights.

	Umbilical		Weight		
	Estradiol (ng/ml)	Cortisol (μg/dl)	Adrenal (mg)	Pituitary (mg)	Fetus (g)
Control (n=7)	2.2 ± 0.5^a	33 ± 4^a	378 ± 21^a	24 ± 2	794 ± 68
Fetal betamethasone (n=4)	0.6 ± 0.1^b	14 ± 5^b	183 ± 20^b	21 ± 3	754 ± 88
Fetal/maternal betamethasone (n=4)	0.1 ± 0.1^b	1 ± 1^b	183 ± 9^b	25 ± 2	733 ± 85

Betamethasone was injected into the fetus (0.6 mg) or the mother (6 mg) and fetus (0.6 mg) every other day between days 150 and 164 of gestation (term = day 184).

Values (mean ± SE) with different letter superscripts differ from each other at $P<0.05$ (Analysis of variance and Student Newman Keuls multiple comparison test).

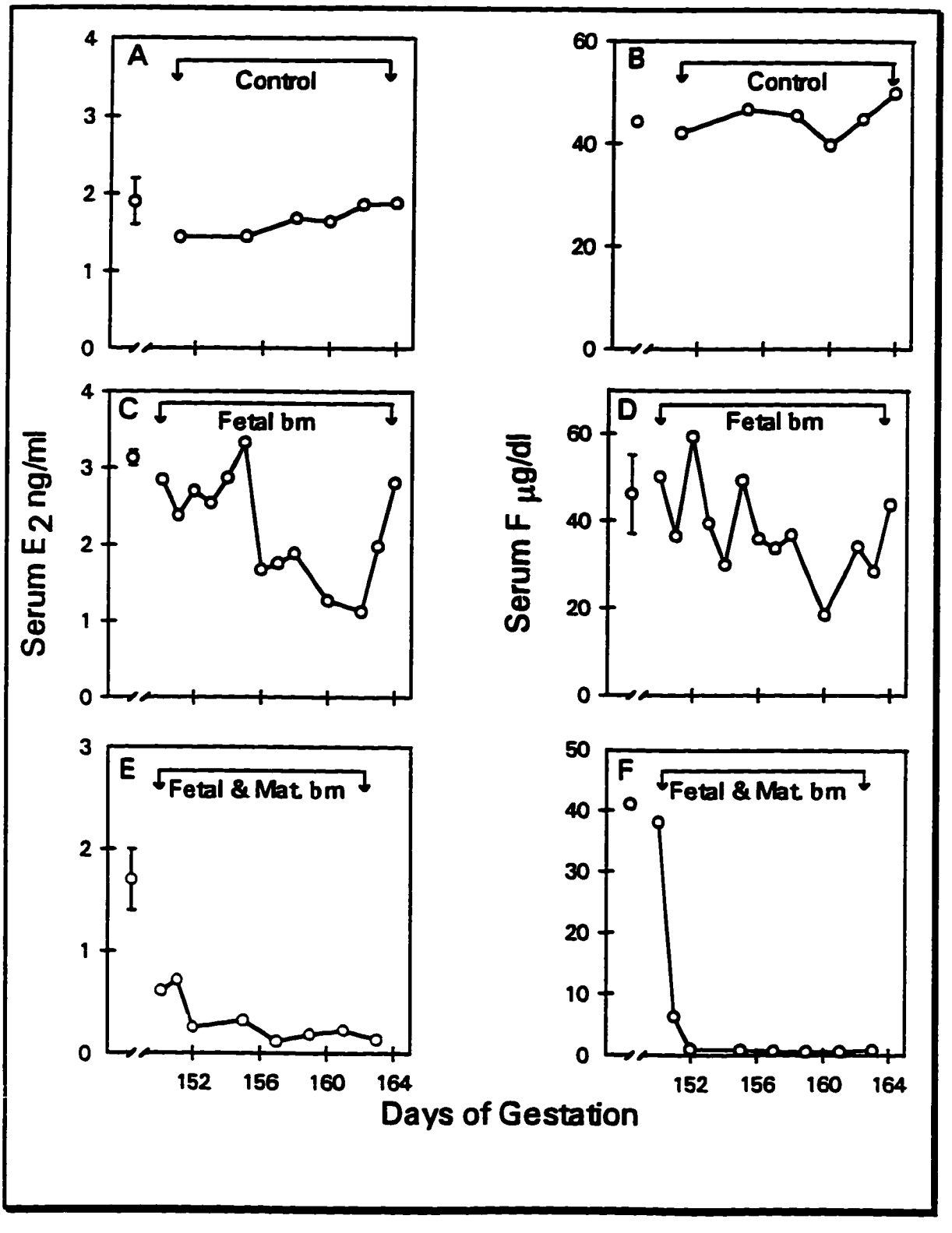
Table 4-2. Effect of administration of betamethasone during baboon pregnancy on fetal and definitive zone cell number and expression of proliferating cell nuclear antigen (PCNA).

	Fetal zone		Definitive zone	
	Cells/0.025 mm^2	% PCNA+	Cells/0.025 mm^2	% PCNA+
Control (n=7)	81 ± 8	55 ± 3	174 ± 12	51 ± 8
Betamethasone (n=3)	182 ± 11*	65 ± 8	232 ± 14*	77 ± 8

Betamethasone was injected into the fetus and the mother between days 150 and 164 of gestation. See legend of Table 1 for details. Adrenal sections stained with hematoxylin/eosin and number of cells/unit area calculated.

* Asterisk indicates mean (± SE) value is different ($P<0.05$) from that in the control (*t* test).

Figure 4-1. Serum estradiol (E_2) and cortisol (F) concentrations in maternal saphenous vein blood samples obtained between days 140 and 164 of gestation (term = day 184) from representative baboons untreated (Panels A and B) or treated with betamethasone administered to the fetus (Panels C and D) or to the mother and fetus (Panels E and F) every other day between days 150 and 164 of gestation. See footnote of Table 4 -1 for details.



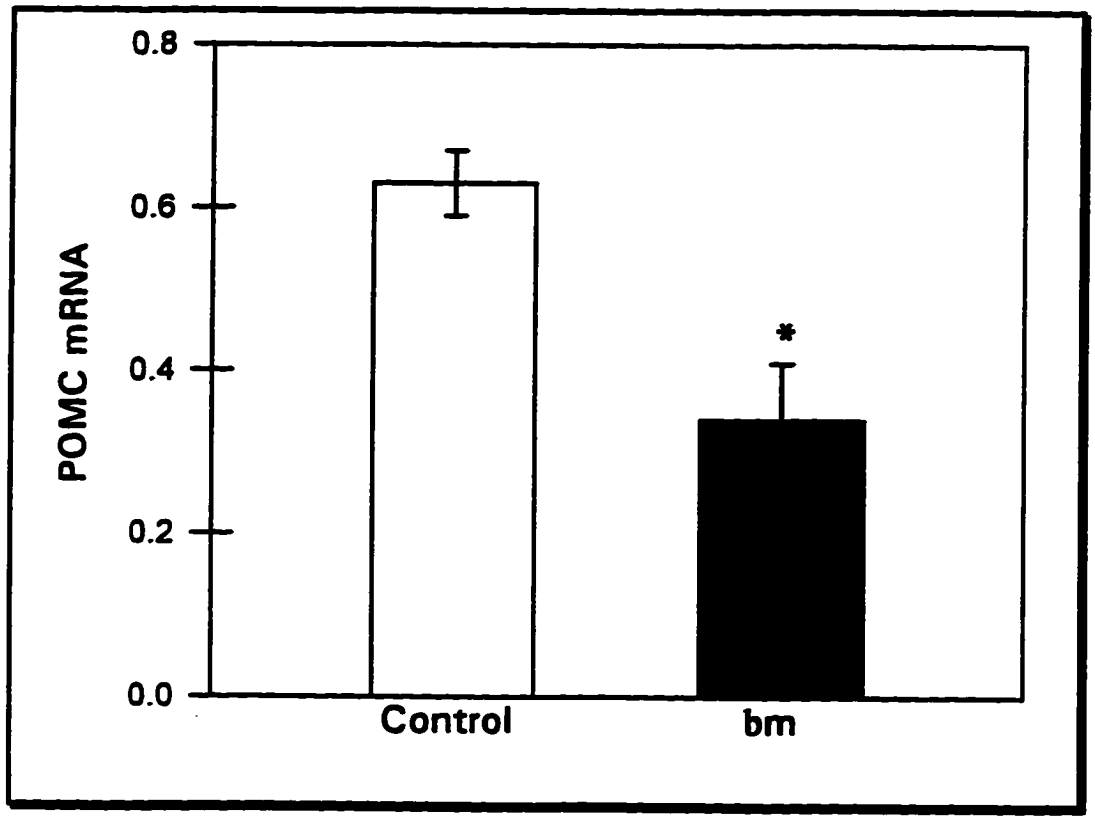


Figure 4-2. Effect of administration of betamethasone during baboon pregnancy on fetal pituitary proopiomelanocortin (POMC) mRNA levels (mean ± SE) determined by *in situ* hybridization. Pituitary glands were obtained on day 165 of gestation (term = day 184) from 3 baboons in which the fetus alone was injected with betamethasone and from one animal in which the fetus and mother were treated with betamethasone between days 150 and 164 of gestation. POMC mRNA in the fetal pituitary of the one baboon in which both the mother and fetus received betamethasone (0.32) was similar to the mean value (0.36) in the 3 animals in which betamethasone was administered only to the fetus. Asterisk indicates mean value differs from that in the control (n=5) at $P<0.05$ (Students' *t* test).

Figure 4-3. Representative Northern blot of the mRNA for ACTH receptor (A) and β-actin (B) in fetal adrenal glands obtained on day 165 of gestation by cesarean section from untreated control baboons (lanes 1-3) and from animals in which the mother and/or fetus were administered betamethasone (βm) between days 150 and 164 of gestation and the fetus delivered by cesarean section (CS; lanes 5,6,8,9) or spontaneously (SD; lanes 4 and 7) 96 or 1 h prior to day 165. (C) Mean (±SE) levels of the 3.4 kb mRNA transcript for the ACTH receptor determined by Northern analysis in fetal adrenal glands of control (n=5) and betamethasone-treated baboons delivered by cesarean section (n=4) or spontaneously (n=2). Asterisk indicates value differs from that in the control at $P<0.05$ (Students' *t* test).

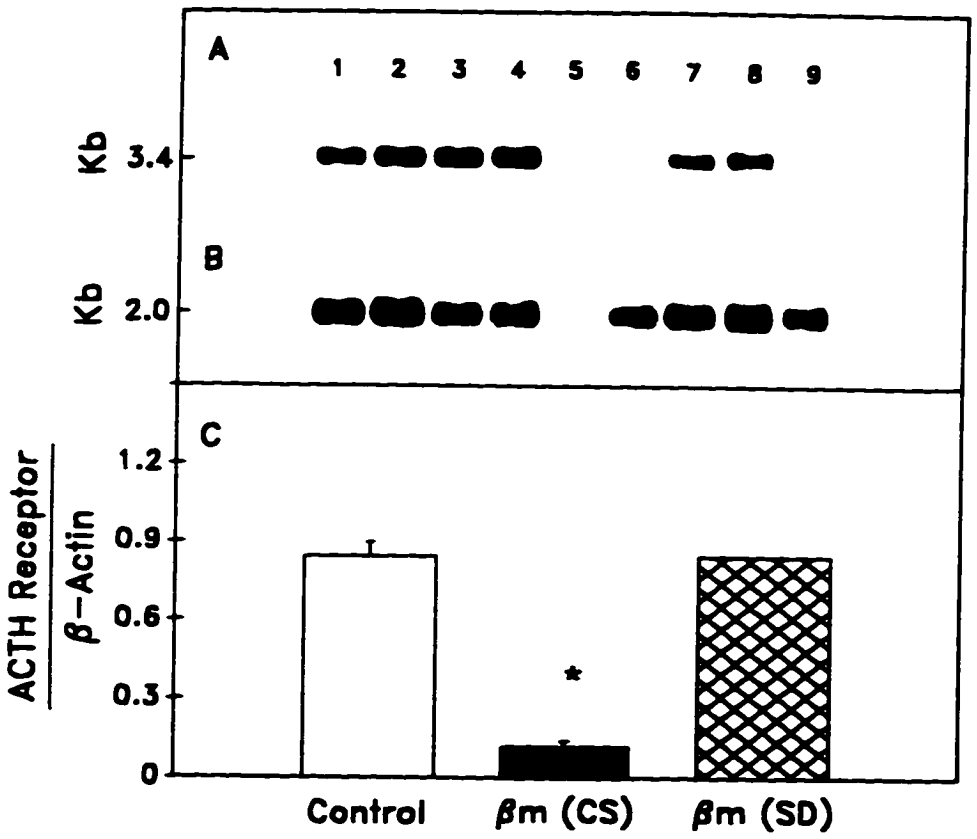


Figure 4-4. Representative darkfield photomicrographs of sections of baboon fetal adrenal glands hybridized with ^{35}S-labeled ACTH receptor antisense (Panels A and B) or sense (Panel C) oligonucleotide probes (magnification = x100; magnification bar = 100 μm). Adrenal glands obtained on day 165 of gestation from untreated (Panels A and C) baboons and from animals in which the mother and fetus were treated with betamethasone between days 150 and 164 of gestation (Panel B). The arrow head denotes the demarcation of the definitive (top) and fetal cortical zones. Insert in Panel A depicts a brightfield photograph (magnification = x 1000) of representative cells of the definitive cortex of sections of near term fetal adrenal from untreated baboons hybridized with ^{35}S-labeled ACTH receptor antisense probe. Panel D depicts ACTH receptor mRNA levels determined by *in situ* hybridization expressed as the mean (± SE) number of silver grains/0.025 mm^2 of definitive zone and fetal zone of fetal adrenals from untreated (n=4) and betamethasone-treated (βm) baboons (n=3) of late gestation.

* - Different from values in control ($P<0.01$; *t* test for dependent observations); † - Different from respective control value ($P<0.02$; *t* test for independent observations).

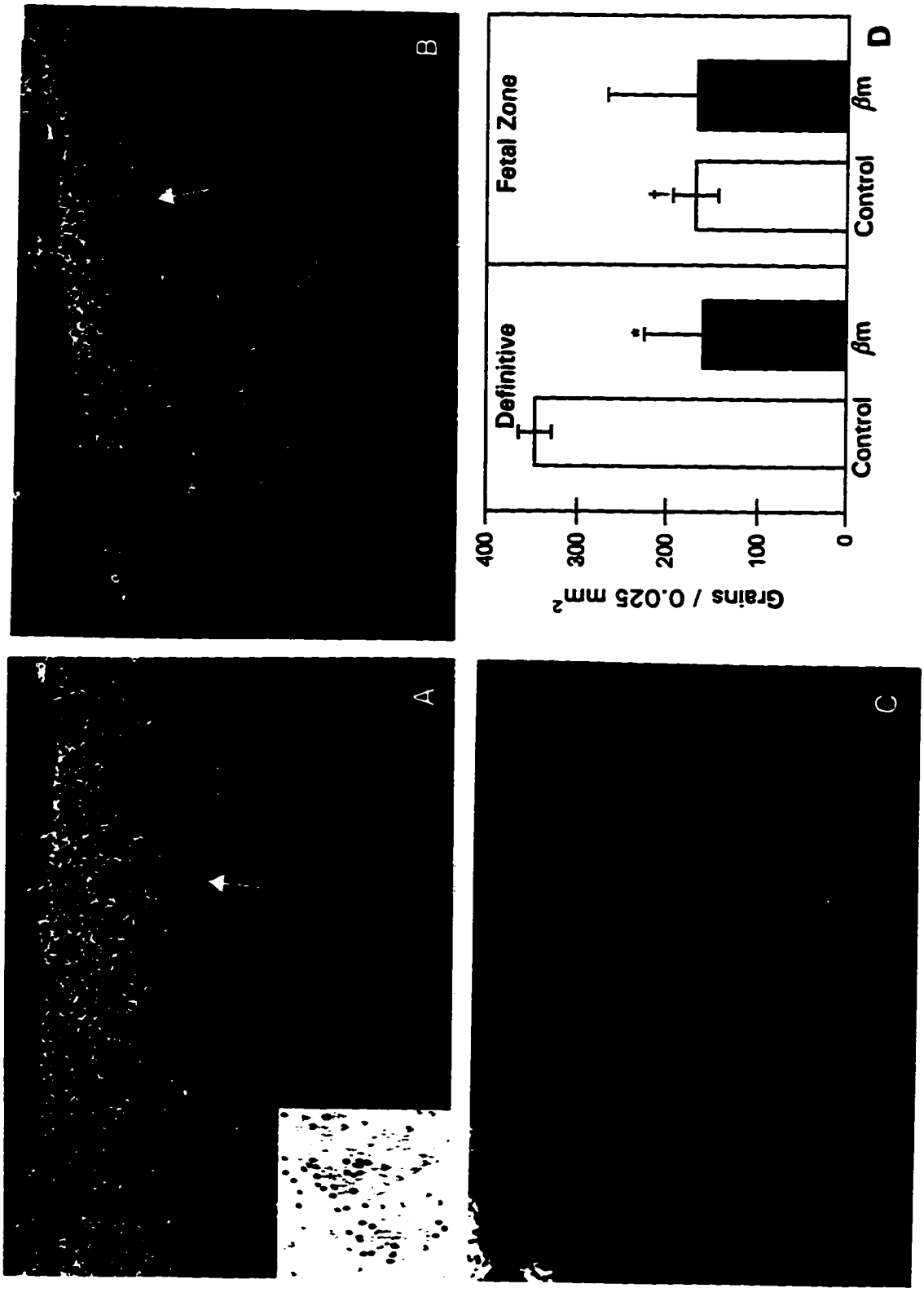


Figure 4-5. Effect of betamethasone on baboon fetal adrenal definitive zone: (A) width in microns (determined by 3β-HSD immunocytochemical expression), (B) mRNA levels (ratio to β-actin), (C) protein levels (arbitrary units), and (D) enzyme activity (pmol pregnenolone converted to progesterone/min/mg tissue). Adrenals obtained on day 165 of gestation from untreated controls and from baboons in which the mother and/or fetus were treated with betamethasone every other day between days 150 and 164 of gestation. For each parameter, the number of adrenals analyzed ranged from 4-7 for the controls and 3-5 for the betamethasone-treated group. Because fetal adrenal weight was not different following maternal and/or fetal betamethasone (See Table 4 - 1), 3β-HSD in this figure represents analyses from both treatment groups, pooled and presented as the overall effects of betamethasone. Asterisk indicates value (mean ± SE) differs from the control at $P<0.05$ (Students' *t* test).

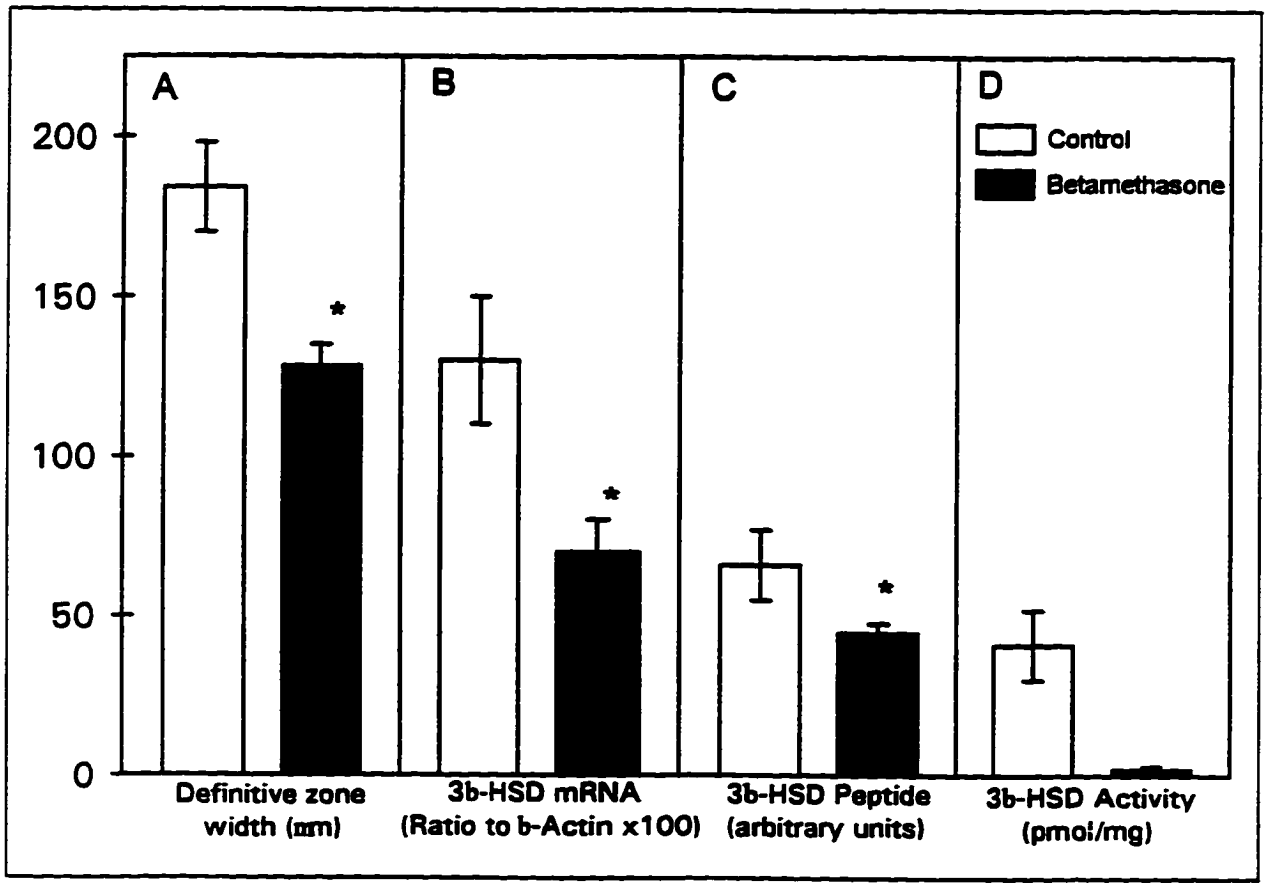
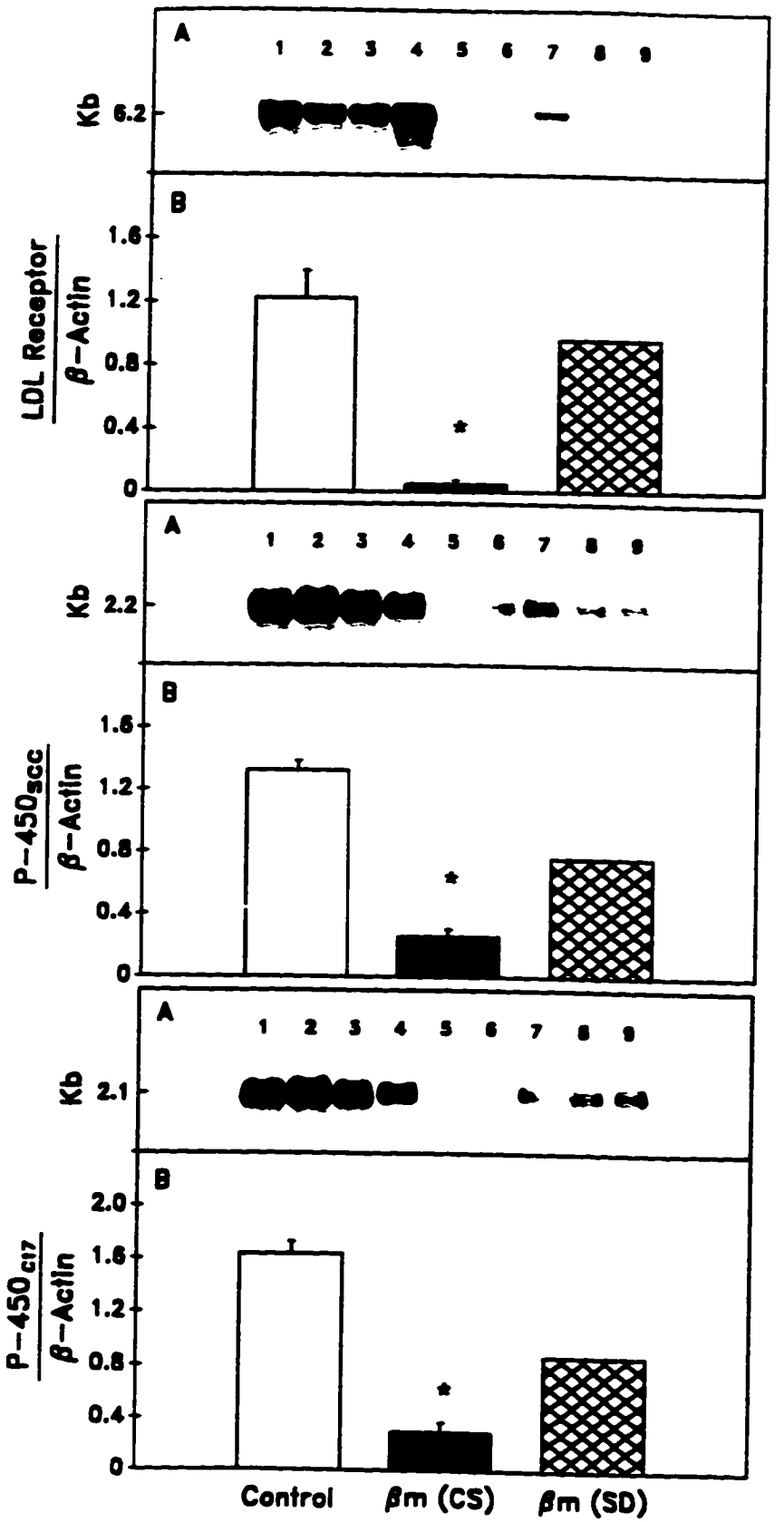


Figure 4-6. Mean (± SE) levels of the 6.2 kb mRNA for the LDL receptor (Panel A), the 2.2 kb mRNA for P450scc (Panel B) and the 2.1 kb mRNA for P450c17 (Panel C) determined by Northern analysis in fetal adrenals obtained by cesarean section from untreated controls baboons (n=4-7/analysis) and from animals in which the mother and/or fetus were administered betamethasone (βm) between days 150 and 164 of gestation and the fetus delivered by cesarean section (CS; n=3-5/analysis) or spontaneously (SD) 96 or 1 h prior to day 165 (n=2). Asterisk indicates value differs from that in the control at $P<0.05$ (Student's *t* tests). Representative examples of the size and relative intensities of the mRNA for each parameter are indicated above the histogram. Lanes 1-3 = controls; lanes 5,6,8,9 = βm - CS; lanes 4 and 7 = βm - SD.



CHAPTER V

ESTROGEN RECEPTOR AND THE BABOON FETAL ADRENAL

Abstract

To test the hypothesis that estrogen modulates the ACTH effects on both the fetal adrenal definitive and fetal zones, the aromatase inhibitor CGS 20267 was administered (125μg/kg body weight/day; sc) to baboon mothers between days 100 and 170 of gestation (n=3) to suppress maternal and fetal peripheral serum estradiol to less than 2% of control levels. Specifity of CGS effects was demonstrated by simultaneous administration of CGS and estradiol between days 100 and 164 of gestation (n=3).

CGS treatment enhanced fetal adrenal weight. Associated with this increase in weight, cortical cells size was decreased. CGS and estradiol reversed the weight increase to values similar to untreated control. CGS treatment also reduced the total width of the definitive zone but enhanced the intensity and immunoexpresion of P450c17 to groups of cells just underneath the adrenal capsule. Thus, CGS appeared to retard the ontogenic development of the fetal adrenal. Simultaneous administration of CGS and estrogen restored all aspects of fetal adrenal development.

To determine whether the estrogen-mediated modulation of ACTH action reflected a direct action of estrogen on the adrenal, we examined fetal adrenal glands for the presence of the estrogen receptor (ER) in control as well as animals treated with CGS or with betamethasone at mid and late gestation as discussed in chapter III and IV. At midgestation, ER expression was not detectable in any treatment group. By term gestation, nuclear ER was observed in the cells of the continuous narrow definitive zone

adjacent to the capsule of untreated adrenals but was not observed in either the transitional or fetal zone cells. CGS or CGS and estrogen had no effect on the expression of the estrogen receptor. Betamethasone treatment, however, induced an increase in both the intensity and width of the zone of expression of the ER at term. The betamethasone effect may be mediated by a glucocorticoid receptor (GR) since the GR was observed in both the definitive and fetal zones at mid and term gestation.

The results of the present study demonstrated that a reduction in placental estrogen production enhance ACTH-induced growth of the adrenal fetal zone and prevented the formation of the definitive zone, but have no significant effect on ER expression in the definitive zone. Thus, estrogen appears to be essential in repressing ACTH-modulation of P450c17 immunoexpression in the definitive zone contributing to its differentiation late in gestation. We hypothesize that increased endogenous levels of cortisol acting in a paracrine/autocrine fashion via a GR-mediated mechanism increase estrogen receptor expression in the definitive zone late in gestation. Thus, full maturation and differentiation of adrenal definitive zone cells is linked to estrogen modulation of ACTH action most likely through an ER mediated-pathway.

Experimental approach

Hypothesis II. Estrogen exerts its action(s) in the fetal adrenal gland via a receptor-mediated mechanism.

The studies of this section will first be concerned with determining whether and which cells of the baboon fetal adrenal cortical cells express the nuclear estrogen receptor. Adrenals from three *in vivo* experimental treatments will be examined, i.e. betamethasone, (synthetic glucocorticoid), CGS (inhibition of androgen to estrogen

conversion; i.e. aromatase inhibitor) and, simultaneous CGS and E_2 (restoration of estrogen).

Rationale

The rapid growth of the primate fetal adrenal from mid to term gestation is regulated by ACTH secreted by the fetal pituitary (ACTH cannot cross the placenta). Thus, glucocorticoid treatment is commonly employed in investigations dealing with function and morphology of the adrenal cortex. The administration of synthetic glucocorticoids, which can cross the placenta in their biologically active form, (e.g. dexamethasone, betamethasone) to pregnant human (NIH consensus conference, 1995; Albrecht ED and Pepe GJ, 1995) and nonhuman primates (Challis et al., 1974) results in inhibition of the hypothalamic-pituitary-adrenal axis (HPA) and decreasing levels of placental estrogens and endogenous adrenal glucocorticoids (Challis JG et al., 1974; NIH Consensus Conference, 1994; Chapters III and IV). Similar inhibitory effects of HPA were observed in the rat where single doses of dexamethasone resulted in reduction of adrenal maturation and function (Hristic M et al., 1992-3, Malendowicz LK et al., 1992). Moreover in primates, glucocorticoids appear to inhibit expression of pituitary POMC mRNA and ACTH peptide the levels of which increase normally between midgestation and term (Pepe GJ et al., 1994; Davies WA et al., 1996, Nathanielsz et al., 1995).

Reduced development of the transitional zone at term after betamethasone treatment has also been described *in vivo* in rhesus monkeys, where accelerated maturation and formation of the transitional zone at midgestation was observed after levels of fetal pituitary ACTH secretion were prematurely increased by metyrapone (Coulter et al., 1996).

We have previously demonstrated the inhibitory effect of betamethasone on P450c17 and 3βHSD immunoexpression, fetal adrenal weight, and ACTH receptor mRNA expression both in the fetal zone at midgestation and in the definitive zone late in gestation (see chapters 3 and 4).

To determine the effect of ACTH on induction and distribution of ER in the adrenal glands as well as immunolocalization of ACTH-dependent steroidogenic enzymes P450c17 and 3βHSD, betamethasone was administered to the baboon fetuses (0.6 mg/100μl) at term gestation (days 150 - 164) and to the fetus (0.6 mg) and mother (6 mg/ml) (every other day, days 150 - 164; term=day 184). Adrenals were also obtained on day 100 from animals in which the mother was treated either with betamethasone alone (days 60-99) or betamethasone in conjunction with fetal ACTH injections (days 95-99) to restore levels of ACTH or from fetuses treated with transabdominal injections of only ACTH (days 95-99) to study the induction of premature adrenal growth and maturation.

In the baboon, the placenta has been shown to be the major site of E_2 production by day 30 of pregnancy (Castracane VD et al., 1986). However, due to the absence of the enzyme 17α-20 desmolase and 17α-hydroxylase (Sholl SA et al., 1981), *de novo* estrogen synthesis is limited and the placenta utilizes androgen precursors of fetal and maternal origin (Jaffe RB et al., 1981; Pepe GJ and Albrecht ED, 1985; Albrecht et al., 1980; Albrecht ED and Pepe JG 1995). Fetal and maternal adrenal androgens are aromatized by the placenta with the subsequent formation of estrogens. With advancing gestation fetal adrenal androgen production (Townsley JD et al., 1977) and placental utilization (Schint HAJ et al., 1978) for estrogen synthesis has been shown to increase. As a result, peripheral serum E_2 levels rises in the second half of pregnancy (Dawood MY et al.,

1980).

Pepe and Albrecht (1988) recently demonstrated that placental estrogens reduced the responsivity of the baboon fetal adrenal gland to ACTH with respect to the formation of DHA. Moreover, *in vivo* regulation of maternal and fetal C19-steroid production by placental estrogen was further confirmed when maternal treatment with estradiol after fetectomy reduced levels of ACTH-induced maternal adrenal DHA and DHAS secretion, while cortisol level were not altered. Collectively, these results indicate a negative feedback system *in utero* whereby placental estrogen regulates maternal and fetal adrenal C19-steroid production (Albrecht ED and Pepe GJ, 1995). To determine the effects of the progressive increase in endogenous estrogen production and secretion into the fetus observed in the second half of pregnancy on fetal adrenal function, the aromatase inhibitor CGS 20267 was administered (125μg/kg body weight/day; sc) to baboon mothers between days 100 and 170 of gestation to suppress maternal and fetal peripheral serum estradiol. That placental estrogen production and delivery to the fetus was reduced was confirmed by the marked decrease in serum estradiol concentrations in the uterine and umbilical veins as measured on day 170 (Table 5-1).

Thus, protocols were utilized to experimentally suppress maternal and fetal pituitary ACTH production and formation of placental estrogens to determine the respective roles of ACTH and placental estrogen on the induction and regulation of ER with advancing gestation.

Immunocytochemistry

The availability of the mouse monoclonal 6F11 anti-ER antibody and rabbit polyclonal anti-3βHSD and anti P450c17 antibodies renders feasible immunocytochemical

studies of the receptor and enzymes, respectively. It resolves the problem of limited quantities of cell material as well as allowing determination of the intracellular localization and adrenal distribution of these proteins, gland zonation and ACTH and/or steroid regulation. Additionally, modulation of the intensity of immunolabeling and extent of tissue distribution by endocrine manipulations can be monitored.

The fetal adrenal outer definitive zone synthesizes aldosterone, but not cortisol nor androgens, so contains 3βHSD but not P450c17 (Figure 1). The fetal zone synthesizes androgens, but not cortisol, and contains P450c17 but not 3βHSD. The inner definitive zone or transitional zone synthesizes cortisol so contains both 3βHSD and P450c17 . Thus, expression of 3 βHSD is particularly relevant for the *de novo* synthesis of mineralocorticoids and glucocorticoids, and expression of P450c17 is essential for C_{19} steroid synthesis (See introduction for steroid biosynthetic pathway and associated enzymes).

Immunolocalization of 3βHSD and P450c17 steroidogenic enzymes was performed on consecutive adrenal sections and used to define the outer definitive, the fetal zone and the inner definitive or transitional zone. The mean width of each zone was calibrated using an Optiphot 2 microscope attached to a Video Based Image-1 Analysis System. The anatomical localization and steroidogenic enzymes expression of transitional zone cells has been described by Mesiano et al (1993). The transitional zone cells express P450scc, P450c17 and 3βHSD whereas fetal zone cells only express P450scc and P450c17. Recent studies have differentiated transitional zone cells as a subclass of the definitive zone based on their morphological appearance (Coulter CL et al., 1996; Coulter LC et al., 1996). Characterization of this zone based solely on morphometric analysis,

however, may lead to equivocal results. Thus, McNulty et al., (1981) infused a continuous dose of ACTH into the fetus which caused a pronounced increase in fetal adrenal cell size and conversion of definitive zone into cells morphologically similar to those in the fetal zone. Moreover in a recent study, effects of metyrapone treatment, to increase fetal pituitary ACTH, resulted in hypertrophy of definitive, transitional and fetal zones (Coulter LC et al., 1996), indicating that morphometric analysis of these zones may not be sufficient to define the functional differentiation. In the present study we observed a marked reduction in fetal adrenal weight consistent with a reduction in cell size of definitive and fetal zone cells in betamethasone/ACTH suppressed -baboon fetuses in the second half of pregnancy. We considered the need to define the transitional zone by immunolocalization of the steroidogenic enzymes P450c17 and 3βHSD, since fetal adrenal morphology and zonation were significantly altered after betamethasone treatment.

In the present study, to define with more precision the cell type of the transitional zone and its physiological significance, the mean width of the P450c17 negative/3βHSD positive zone under the capsule was subtracted from the total mean width of the fetal 3βHSD positive zone on consecutive adrenal sections (Figure 1). The difference in width between the two zones represented cells expressing both P450c17 and 3βHSD proteins and thus is the immunocytochemical definition of transitional zone; the cells of which have the steroidogenic potential to synthesize cortisol.

Results

Effects on weight and cell number

As demonstrated in Chapter IV, betamethasone treatment reduced adrenal weight (Table 5-1) by reducing the size of the cells of the fetal and definitive zone (Table 5-2).

Interestingly, there was a significant increase in the cross-sectional area of the adrenal medulla in relation to control groups. There was no significant difference in the number of fetal adrenal cells expressing PCNA in control and betamethasone-treated animals (Table 5-2).

CGS treatment increased the number of fetal and definitive zone cells per unit area compared to control (Table 5-2), indicating that increased weight of the gland was likely due to hyperplasia. However, the percentage of fetal and definitive zone cells per unit area expressing PCNA was reduced in both fetal and definitive zones after CGS treatment compared to control group (Table 5-2).

Simultaneous treatment with CGS and estradiol restored adrenal weight to values similar to the control, indicating that estrogen reversed the growth of the fetal adrenal gland induced by CGS. There was no apparent decrease in the number of either fetal or definitive zone cells per unit area after CGS and E_2 treatment compared to CGS group, indicating that the restoration of reduced weight after CGS plus E_2 treatment was not due to a reduction in the number of fetal and definitive zone cells per unit area (Table 5-2). Moreover, the percentage of fetal and definitive zone cells expressing PCNA was not significantly different in either fetal or definitive zones after CGS and E_2 compared to CGS group (Table 5-2).

Expression of P450c17

In the mid control adrenal gland, P450c17 protein was detected in all adrenal cortical cells except for the 2 to 3-cell layer (30μm) adjacent to the capsule; these latter cells are 3βHSD positive. Thus, at midgestation, adrenal cortical cells expressed either 3βHSD or P450c17, but not both simultaneously. The P450c17 positive cells comprise the

fetal zone. In adrenals of untreated baboons near term, expression of P450c17 protein was not detected in the densely packed cells under the capsule, but was present in the fetal zone. The intensity of the signal decreased toward the more central areas of the fetal zone; however, individual cell groups adjacent to medullary cells labeled intensely (Figure 2).

Betamethasone treatment increased the width (μm) of the P450c17 negative zone under the capsule (130 ± 13) compared to control animals (100 ± 6; Table 5-3). Betamethasone also decreased the overall intensity of the signal. The abundance of P450c17 was again highest in the outer zone decreasing toward the central area. However, a dense band with high levels of P450c17 label was observed in the innermost fetal zone adjacent to the medulla. P450c17 expression was absent from adrenal medullary cells.

Maternal administration of the aromatase inhibitor CGS 20267 throughout the second half of gestation enhanced the intensity and expression of P450c17 to groups of cells just underneath the adrenal capsule (Figure 2). Simultaneous treatment of CGS and E_2 in the second half of pregnancy restored the width of P450c17 negative zone under the capsule to values similar to those of the control group (Table 5-3).

Expression of 3βHSD

At midgestation, 3βHSD expression was localized exclusively to cells (e.g. definitive zone) under the capsule. As determined by image analysis, the mean width of the definitive zone was increased from 34 ± 2μm on day 100 of gestation to 208 ± 14μm on day 165 of gestation (Figure 3). Betamethasone treatment in late gestation decreased ($p<0.05$) the width of the definitive zone (Figure 2, Table 5-3). Moreover, the zone of cells positive for both P450c17 and 3βHSD (transitional zone) was virtually eliminated

(Figure 2, Table 5-3), suggesting that the reduction in fetal pituitary POMC mRNA by betamethasone treatment prevented development of the transitional zone.

In baboons in which maternal peripheral serum estradiol concentrations were reduced to 2% of normal during the second half of gestation by treatment with CGS 20267, fetal adrenal expression of 3βHSD was also reduced (Figure 2, Table 5-3). Therefore, CGS treatment resulted in reduction of the total width of cell positive for 3βHSD. As discussed earlier, CGS enhanced the intensity and signal of P450c17 protein to the outermost zone of the adrenal, to groups of cells underneath the capsule. Thus, in CGS treated baboons the width of cell positive for both 3βHSD and P450c17 was similar to that in the control (Table 5-3), despite the fact that total width of the zone of cells positive only for 3βHSD was reduced (Table 5-3). Thus, CGS treatment appears to retard the development of the definitive zone but not the transitional zone. Occasionally expression of 3βHSD was observed in clusters of cells in fetal cortical tissue near the medulla in CGS 20267 treated baboons but not in other treatments.

Simultaneous treatment of CGS and E_2 resulted in restored the width of cells positive for 3βHSD and the zone positive for both 3βHSD and P450c17 (Table 5-3).

Expression of Estrogen Receptor

At midgestation, ER was not detectable by immunohistochemistry in either the fetal or the definitive zone of untreated baboon adrenals. Administration of ACTH to the fetus or betamethasone in conjunction with ACTH to prematurely elevate circulating fetal ACTH or to restore ACTH levels after betamethasone suppression, also apparently failed to induce nuclear expression of ER. Occasionally, groups of nuclei adjacent to the capsule were observed which labeled very weakly for ER. By term gestation, nuclear ER

expression was detected in the continuous narrow definitive zone adjacent to the capsule but not the fetal or transitional zones (Figure 4). The immunostaining varied from cell to cell in positivity and intensity. In adrenals of untreated baboons, the overall intensity of the labeling ranged from moderate to minimal (Table 5-3). When the estrogen receptor antibody, ER-6F11, was preabsorbed with 15-fold excess of the whole recombinant ER molecule, the nuclear staining was completely abolished indicating specificity of the ER monoclonal antibody (Figure 4).

Betamethasone treatment induced a significant increase in both the intensity and width (μm) of the zone of cells expressing the ER (Figure 3, Table 5-3). CGS 20267-treatment did not alter the intensity or pattern of ER adrenal immunolabeling suggesting that inhibition of placental estrogen synthesis does not influence ER expression in the baboon fetal adrenals (Figure 4). Moreover, simultaneous administration of CGS and E_2 also did not change the pattern nor intensity of expression of the ER further indicating that estrogen does not regulate ER expression by the fetal adrenal.

Colocalization of ER and 3βHSD

To better understand the interrelationship of the expression of nuclear ER and its cellular localization in relation to 3βHSD, an enzyme marker of the fetal definitive zone, we developed a double primary antibody immunolabeling technique. Immunocolocalization of nuclear ER with the mouse monoclonal antibody and cytoplasmic 3βHSD with the rabbit polyclonal antibody was observed in single cells in the outermost definitive zone extending toward the fetal zone of the near-term fetal adrenal. Intensity of nuclear staining varied throughout the definitive zone but was relatively homogenous for 3βHSD (Figure 5). Interestingly, not all 3βHSD positive cells expressed

nuclear ER labeling. Thus, the abundance of ER protein decreased toward the fetal zone in 3βHSD positive cells. Betamethasone induced a significant increase in intensity and width of the definitive zone expressing nuclear ER label; by contrast the width for cytoplasmic 3βHSD immunostaining zone was reduced (Table 5-3). Thus, in the betamethasone-treated animals the entire width of 3βHSD positive cells (e.g. definitive zone) was now also positive for the presence of ER. Specificity of the dual labeling was demonstrated by replacing the anti-ER primary antibody with normal mouse serum (Figure 5, C and D).

Expression of Glucocorticoid Receptor

Because betamethasone treatment induced a significant increase in the width and intensity of the zone of cells expressing the ER, it was important to determine whether the ER containing cells also express the glucocorticoid receptor and ascertain whether the effect of betamethasone was directly on the cells of the definitive zone. To test this possibility, adrenal sections were analyzed using the polyclonal (rabbit) anti-human glucocorticoid receptor (GR) antibody PA1-511. At midgestation, nuclear GR was detected throughout the fetal zone (Figure 6) with stronger staining in the inner fetal zone. Very weak staining was observed in the definitive zone cells adjacent to the capsule whereas medullary cells labeled with the strongest intensity. At term, nuclear GR expression was also observed throughout both the fetal and definitive zone with an overall intensity of labeling which appeared stronger than at midgestation (Figure 6). Intensity of the labeling was relatively weak in the definitive zone, and increased toward the center of the gland with medullary cells also expressing the GR. Betamethasone treatment induced an apparent increase in the intensity of the nuclear signal in both the inner zone of the fetal zone and in medullary cells. However, GR expression in cells of the definitive zone under

the capsule was still weak. Distinguishing the nuclear signal in the definitive zone was often rendered difficult by unspecific cytoplasmic labeling as determined by preabsorbing the GR antibody with 100-fold excess of the synthetic peptide PEP-001; the nuclear staining was essentially abolished but some cytoplasmic staining was still often observed (Figure 6, D).

Discussion

Adrenal growth and differentiation

Maturation and activation of the fetal adrenal gland is crucial to fetal organ development in preparation for extrauterine life. In the baboon, we have demonstrated that ACTH is a major trophic factor for adrenal growth and steroidogenesis *in vivo* from midgestation to term. Changes in maternal cortisol secretion to the fetus by term result in activation of the fetal pituitary leading to increased production of ACTH and steroidogenic maturation of the adrenal gland (Pepe GJ and Albrecht ED, 1990; 1995 for review). Thus, administration of the cortisol agonist betamethasone (3mg/day) to baboons at midgestation and at term to the fetus or the fetus and mother to inhibit fetal pituitary ACTH secretion, reduced maternal estrogen, cortisol and DHA levels by 80-95%. Inhibition of pituitary ACTH synthesis by betamethasone administration was confirmed by the suppressed fetal pituitary mRNA levels for the ACTH precursor proopiomelanocortin (POMC) (Pepe GJ et al., 1994; Davis WA et al., 1996). Associated with the decline in pituitary POMC, fetal adrenal weight was markedly suppressed at midgestation (62%) and term (50%) which reflected a marked reduction in both the size and number of fetal adrenocortical cells. Betamethasone treatment also elicited a decline in fetal adrenal expression of the mRNAs for the ACTH receptor at midgestation (95%) and term (80%)

(Aberdeen GN et al., 1997) which was probably a consequence of reduced fetal pituitary ACTH secretion; ACTH has been demonstrated to increase adrenal ACTH mRNA levels in both the adult (Kolanowski J et al., 1977) and the fetus (Aberdeen GN et al., 1997).

Levels of the mRNA for steroidogenic enzymes P450scc, P450c17 and 3βHSD mRNA/protein were also reduced by betamethasone. Thus in the fetal, as in the adult (Pepe GJ and Albrecht ED, 1990; 1995 for review) adrenal, ACTH is necessary for the up-regulation and/or maintenance of steroidogenic enzymes of the definitive and fetal cortical zones.

While regulation of the growth and differentiation of the fetal definitive zone have been more defined, the mechanism(s) regulating fetal adrenal androgen production are less clear. In contrast to the developmental increases in growth and expression of steroidogenic enzymes, the output per cell of fetal-zone specific DHA, determined by perfusion, declined between mid and late baboon gestation (Berghorn KA et al., 1995). Although the secretion of total DHAS increased with advancing gestation, possibly reflecting contribution from the definitive zone (Doody KM et al., 1990) and more likely the increase in the size of the adrenal gland (Seron-Ferre M et al., 1983), the absolute levels of DHA and DHAS were lower than those of cortisol near term (Berghorn KA et al., 1995). Moreover, the rate of growth of the fetal zone appeared to be much lower than that of the definitive zone in the latter third of gestation. These observations are consistent with studies in rhesus monkey in which fetal serum cortisol concentrations at term were suppressed by 90% by dexamethasone while DHAS secretion was only suppressed by 60% after 48 hr treatment (Jaffe RB et al., 1981; Challis JRG et al., 1974).

Studies of the signaling pathways of activation of the definitive and fetal zones steroidogenesis also suggest differences in the regulatory mechanisms and patterns of these two zones. Adrenal steroidogenesis in the adult is modulated by interaction of ACTH with cell-surface ACTH receptors which in turn results in the stimulation of the production of intracellular adenylate cyclase and a rise in cAMP. Cyclic AMP then modulates different protein kinases function which leads to an increase steroidogenesis. ACTH upregulation of steroidogenesis in the fetal adrenal is apparently also mediated in the human (Lebrethon et al., 1994; Mesiano et al., 1996) and baboon (Davies WA et al., 1993) adrenals by a PKA mechanism. In support of these data, Mason et al., (1995) demonstrated enhanced production of C19-steroid and induction of P450c17 in the adrenocortical tumor cell line H295 by forskolin and dibutryl cAMP via a protein kinase A pathway. Though regulation of steroidogenesis occurs predominately via a PKA pathway, modulation of P450 c17 expression and activity in the fetal zone may also occur by other mechanisms. For example, an increase in the mRNA and activity of P450c17 were stimulated by angiotensin II and K+ which was mediated by an intracellular calcium mechanism (Mason JI et al., 1995). Moreover, angiotensin II was described to induce steroidogenesis in cultured human fetal adrenocortical cells by activating phospholipase C and presumably PKC (Rainey WE et al., 1992). Thus, the diversity of pathways to regulate P450c17 and therefore C19-steroid secretion indicate that ACTH regulation of the fetal zone may differ from that of the definitive zone. Factors other than ACTH may contribute to the regulation of the fetal zone and consequently androgen secretion with advancing gestation.

There is considerable evidence that various factors have the capacity to mediate

the action of ACTH, i.e. estrogen, prolactin, insulin-like growth factors, and epidermal growth factor (Jaffe RB et al, 1988; Crickard K and Jaffe RB, 1981; Coulter et al, 1996; Pepe GJ and Albrecht ED, 1985; Albrecht ED and Pepe GJ, 1987; Albrecht ED and Pepe GJ, 1995; Voutilainen R and Miller WL,, 1988; Mesiano S and al., 1993). In the fetus these growth factors seem to modulate preferentially *in utero* growth of the fetal zone. In support of the latter concept, estrogen administration at midgestation suppressed the responsivity of the baboon fetal adrenal to ACTH with respect to the secretion of fetal zone-specific DHA, but not of definitive zone-specific cortisol, as determined *in vitro* (Albrecht et al., 1987; Albrecht et al., 1990) and *in vivo* (Pepe GJ et al., 1989). Based on these results Albrecht et al., (1996) proposed that estrogen normally feeds back to restrain ACTH-regulated growth and steroidogenesis in the fetal zone, while simultaneously in an indirect way (Pepe et al., 1988), via placental 11βHSD, enhances ACTH-secretion and thus development and function of the definitive zone. The mechanism for estrogen action in the fetal zone is as yet unclear since neither Hirst and colleagues (Hirst JJ et al., 1992) nor we in the present study have observed the presence of ER in these cells (see below).

With advancing gestation the developmental increase in placental estrogen secretion may lead to inhibitory effects of estrogen on Δ^5C_{19}-steroid production while enhancing cortisol synthesis by inducing expression of 3βHSD in the definitive zone cells. Therefore, the purpose of study 3 was: (1) to test whether or not the ER is present in the baboon fetal adrenal; (2) if so, to study its distribution among the fetal adrenal zones; (3) to study its developmental pattern; and (4) finally, to study the regulation of the content and distribution of the fetal adrenal ER and to relate these to the physiological effects of

estrogen on fetal adrenal development and function.

ER in fetal adrenals

To assess the role of ACTH in the developmental expression of ER we used immunohistochemical techniques and sections of baboon fetal adrenal glands. No ER immunolabeling was detected in early (d60) or midgestation (d100) adrenocortical cells. However, near term (d164) expression of this nuclear protein appears in the cells of the outer zone of the definitive zone adjacent to the capsule; ER immunolabeling was absent, however, in the transition and fetal zones; the latter which still comprises the major portion of the near-term fetal adrenal gland. Our results were similar to those of Hirst JJ et al. (1992), who observed expression of ER in only the definitive zone cells of the fetal adrenal at term in rhesus monkey. Thus, using gradient shift assays and immunocytochemistry they detected low levels of ER that localized exclusively to the narrow definitive zone (Hirst JJ et al., 1992). In our studies, the observed immunostaining was heterogeneous, i.e. not all definitive zone cells were stained and staining varied in intensity from cell to cell. Such staining heterogeneity, even in cells of the same tissue and same type, had been previously reported in ER immunohistochemical studies of normal breast tissue (Malet CA et al., 1991) and uterus (Tseng L et al., 1977; Bayardcortisolet al., 1978) which suggests different degree of maturation and function of different cells within one tissue.

Specificity for ER labeling with the monoclonal antibody 6F11 was demonstrated by preabsorbing the antibody with a 15-fold excess of the whole recombinant ER molecule which completely abolished the nuclear labeling. Specificity of the anti-ER 6F11 monoclonal antibody was further assessed by using it in established estrogen target baboon

tissues such as the uterus. ER labeling was detected in adult baboon endometrium and ovary; tissues previously demonstrated to be positive for the presence of the estrogen receptor by the use of different monoclonal antibodies (Billiar et al., 1992). Additionally, immunoblots of electrophoresed proteins from baboon reproductive tracts with anti ER-6F11 monoclonal antibody showed a single band at approximately 67,000 mol wt (data not shown), which is the predicted weight for the size of the ER (Greco TL et al., 1991;). Preliminary data in our laboratory has also identified using RT-PCR the presence of the ER mRNA at midgestation and term and demonstrated that ER mRNA levels were much higher at term than at midgestation gestation in the baboon fetal adrenals (Pepe GJ and Albrecht ED, in progress). Thus, immunocytochemical localization of ER in fetal adrenals is supported by preabsortion of the antibody with estrogen receptor, by Western immunoblot, and by polymerase chain amplification analyses of the ER mRNA levels.

The absence of immunodetection of ER in fetal zone was somewhat unexpected and may indicate that estrogen modulation of fetal zone growth and steroidogenesis requires lower concentration of ER in the fetal zone cells which are not detected by the sensitivity of our method or the estrogen modulation of the fetal zone is mediated by other growth factors or perhaps by a different subclass of ER. Recently, the discovery of another estrogen receptor, estrogen receptor-beta (Kuiper GG et al., 1996), has been reported. This novel estrogen receptor (ERβ) has a high degree of conservation of the DNA-binding domain and of the ligand binding domain of the classical ER (ERα). If a different isoform of the ER were present in adrenocortical cells, the monoclonal antibody 6F11 most likely would not detect it. Also it is possible that the growth of the fetal zone is modulated indirectly through the ER in the outer definitive zone. Indeed, Cunha et al.,

(1983) has provided evidence that the estrogenic effects observed in mouse epithelial cells are mediated in a paracrine manner via ER-regulated growth factors from the adjacent stroma cells. Thus, further studies are necessary to determine the mechanism(s) of estrogenic effects on growth and differentiation of the fetal zone of adrenocortical cells from mid to late gestation.

Effects of *in vivo* betamethasone on adrenal ER expression

ER is present in the definitive zone late in pregnancy at a time when 3βHSD expression is also induced in the transitional zone (Table 5-3). Since the hypothalamic-pituitary axis plays a major role in regulation of the fetal adrenal gland, the ontogenic increase in fetal pituitary ACTH secretion may also be an important factor responsible for the induction of ER expression as for adrenal growth and regulation of 3βHSD and P450c17. To asses the role of ACTH in the induction of ER in the fetal adrenal *in vivo*, we administered betamethasone to the fetus or the fetus and mother at term to decrease fetal pituitary ACTH secretion. We observed that betamethasone treatment decreased significantly the cortical width but surprisingly, increased both the intensity and distribution of ER immunolabeling. In contrast betamethasone prevented expression of P450c17 in the transitional zone and decreased the extent of 3βHSD expression; both reflective of decreased presence of ACTH levels. Thus, the presence of ER in the term adrenal is apparently not due to a direct effect of ACTH.

The stimulatory effect of betamethasone on ER immunoexpression in the outer definitive zone at term may be explained in either of two ways: Indirectly by a mechanism involving decreased placental estrogen production due to lower fetal androgen production and thus lower fetal estrogen concentrations; or directly on the adrenal gland itself. In the

uterus low concentrations of estrogen increase the ER but higher concentrations decrease estrogen receptor content. The levels of estrogen in the primate fetus are high at term (in the absence of betamethasone treatment). Betamethasone through a negative feedback on fetal pituitary ACTH secretion, could reduce ACTH-dependent C19-steroid synthesis in the fetal adrenal and therefore reduce the androgen precursor concentration for placental estrogen synthesis. Thus, decreased placental estrogen secretion might upregulate adrenal ER if estrogen acts in the adrenal as it does in the uterus on the ER expression.

We investigated the possibility that suppression of fetal estrogen resulted in the increased adrenal ER content by selecting an alternative method to lower fetal estrogen levels, namely the administration of the aromatase inhibitor CGS 20267. It was administered daily to baboon mothers between days 100 and 170 to suppress the formation of placental estrogen and to severely decrease the level of circulating fetal estrogen concentration in the second half of the pregnancy without increasing fetal glucocorticoid levels (Table 5-1). CGS treatment reduced maternal peripheral serum estradiol concentrations to <50 pg/ml or 2% of normal for this time in gestation. Interestingly, the weight of the fetal adrenal glands were significantly increased after CGS treatment (Table 5-1), which was reflected in an apparent marked enhancement of the fetal zone. However, there was no increase in the intensity nor the cortical width of the nuclear ER expression compared to the control group. Consistent with lack of induction of adrenal ER at term after CGS treatment, restoration of placental estrogen by simultaneous administration of estradiol with the CGS throughout the second half of gestation also did not alter the intensity nor cortical width of the nuclear ER. Thus, reduced placental estrogens appear to enhance preferentially ACTH induced growth of adrenal fetal zone,

while having no significant effect on ER expression in the definitive zone. The absence of ER in fetal zone may indicate that modulation of fetal zone growth and steroidogenesis by estrogen does not appear to involve a direct receptor mediated pathway or that our method is not sufficiently sensitive to detect minimal levels of ER in these cells. These results also suggest that glucocorticoids may act directly on the fetal adrenal to increase ER, (as discussed below).

Tissue specific regulation of ER mRNA and protein have been previously described in different species. For example, in the rat the mRNAs encoding for ER in uterus, liver and pituitary were regulated in different directions by high levels of estrogen. In uterus, ER mRNA increases after ovariectomy, returning to basal levels after E_2 replacement. In contrast, liver ER mRNA declines after ovariectomy, returning to normal levels after E_2 replacement. Pituitary ER mRNA decreased after ovariectomy, increasing after E_2 replacement to return subsequently to basal levels (Shupnik MA et al., 1989). Thus, in the rat estrogen at high concentrations appears to reduce expression of ER in uterus while up regulating expression in liver and pituitary. In the monkey uterus, progesterone down-regulates ER and ER mRNA in glandular epithelial cells of the functionalis but not in epithelial cells of the basalis (Koji T and Brenner RM, 1993). In another study, it was observed that changes in ER protein in the hypothalamus were not in phase with those of the uterus during the rat estrous cycle, further suggesting that the regulation of ER protein is tissue-specific (Zhou Y et al., 1995). In normal human breast epithelial and fibroblast cells cultured separately, E_2 increased the intensity of ER protein in epithelial cells but had no effect on fibroblast (Malet CA et al., 1991). Collectively, these studies suggest that the tissue-specific pattern of ER protein and mRNA levels are

regulated differently by circulating levels of estrogen and/or other hormones. Thus, expression of ER appears to be controlled by different mechanisms specific to the cell type. How this tissue-specific expression pattern of ER is controlled is not completely understood. Some recent studies have proposed tissue specific differential promoter utilization of the human estrogen receptor gene (Grandien K et al, 1995;), ligand-independent activation of the ER by tyrosine phosphorylation (Arnold SF et al., 1995; Auricchio F et al., 1995) or protein-protein interaction (Skipper JK et al., 1993; Landel CC et al., 1994).

There is little information concerning the regulation of the ER in the adrenal gland either in the fetus or in the adult. In contrast with numerous studies of localization and regulation of ER by E_2 in rhesus monkey endometrium (Slayden OD et al. 1993; Koji T and Brenner RM, 1993; McClellan MC et al, 1984) and oviducts (Brenner RM et al., 1974;), only one study examined the concentration and distribution of ER in primate fetal and adult adrenals (Hirst et al., 1992). In agreement with the study of Hirst, ER staining expression was only in the narrow definitive zone adjacent to the capsule in the term adrenal and was absent in fetal zone in both mid and late gestation. Moreover, our data demonstrate that in the fetal adrenal gland ER immunoexpression was not present in the day 60 or midterm adrenal. Hirst et al.,(1992) only studied term fetuses.

Our findings also indicate that following administration of CGS there was a reduction in cell size of both the fetal and definitive zone (Table 5-2). Thus, the enhancement of the fetal zone, as assessed by immunohistochemical expression of P450c17, reflected cellular hyperplasia. Therefore, one of the actions of estrogen may be to stimulate cellular hypertrophy of adrenocortical cells by term gestation. This effect

contrasts with a role for estrogen as a mitogenic agent. Thus, an increase in DNA synthesis and proliferation after administration of diethylstilbestrol (DES), a nonsteroidal estrogen, was observed in mouse uterine cells (Yamashita S et al., 1989). Likewise, activation of ER through tyrosine phosphorylation, induces cell proliferation in estradiol-responsive MCF-7 cells (Auricchiocortisolet al., 1995). It is possible that estrogenic effects vary depending on the tissue affected. We can hypothesize that E_2 modulates ACTH-induced hypertrophy and differentiation of the fetal adrenal rather than hyperplasia, thereby contributing to the maturity of the definitive zone cells at term.

That the percentage of fetal and definitive zone cells expressing PCNA, the proliferating cell nuclear antigen, was significantly reduced following CGS administration in relation to control groups was unexpected. However, ACTH-induced adrenal growth *in vivo* in sheep (Boshier DP and Holloway H, 1989; Dallman MF, 1984/85) and rats (Malendowicz et al., 1992) is primarily mediated by adrenal hypertrophy which is subsequently followed by hyperplasia. Our data appear to support this sequence of cell growth and cell division. Thus, there was evidence for prior CGS induced hyperplasia, which may have been completed by the time of our sampling, accompanied, or possibly followed, by inhibition of cell differentiation and/or hypertrophy. Though simultaneous administration of estradiol with the CGS reversed the increased weight and enhancement of the fetal zone induced by CGS alone, the total number of cells and percentage of PCNA positive cells of both the fetal and definitive zone were not altered, indicating that estrogenic effects were not sufficient to restore hyperplasia induced by CGS.

Adrenal ER expression and glucocorticoid regulation

In the present study we found that betamethasone treatment increased both the intensity and distribution of ER immunolabeling in relation to control groups. Recent studies in sheep point to a role for glucocorticoids in the regulation of uterine ER levels during the last third of pregnancy and labor. Thus, Wu WX et al, (1995) demonstrated that ER mRNA was dramatically increased in the myometrium both during cortisol-induced premature labor and term spontaneous labor. The same authors (Wu WX et al., 1996) concluded that an increase in the population of immunoreactive ER-positive cells was associated with the increment of ER mRNA during glucocorticoid-induced labor. This increase for ER protein and ER mRNA was observed in smooth muscle cells and the endothelial cells of the blood vessels of the myometrium. In agreement with these results, dexamethasone was also responsible for a direct induction of the ER mRNA of Xenopus liver cells (Ulisse S and Tata JR, 1994). Collectively, these studies suggest that, at least in sheep, glucocorticoids can increase ER mRNA and protein content, presumably acting through the glucocorticoid receptor (GR) present in the pregnant sheep myometrium. These studies are consistent with a role for sex steroids hormones and glucocorticoids influencing ER levels in the nonpregnant uterus (Bergman MD et al., 1992), in a breast cancer cell line (Read LD et al., 1989; Bianchini RP et al., 1988) and in rat hepatocytes (Freyschuss BO et al., 1993).

Cortisol is responsible for maturation of several fetal organs in preparation for extrauterine life (Liggins GC, 1994). Liggins first demonstrated that the administration of synthetic glucocorticoids to fetal lambs resulted in accelerated lung maturation and the onset of labor (Liggins GC, 1969). Subsequently, others demonstrated that

glucocorticoids enhanced surfactant levels in lungs of fetal rabbits (Kotas RV et al., 1973). A number of *in vitro* and *in vivo* studies in the rat (Rooney SA et al., 1986; Hundertmark S et al., 1994), rabbit (Rooney SA et al., 1979; Chu AJ and Rooney SA, 1985), and human (Sharma A et al., 1993), have demonstrated that glucocorticoids have very complex actions in fetal lungs, involving not only stimulation of surfactant synthesis and secretion but also connective tissue maturation, alveolar epithelial differentiation, including enhanced glycogenolysis and the induction of antioxidant enzymes (Pepe ED and Albrecht GJ, 1995; Liggins GC, 1994). The maturation effects of cortisol alone, and in conjunction with other hormones, e.g. estrogen, androgens, PRL, peptide growth factors and insulin, have been demonstrated in lung and other fetal organ systems (Pepe GJ and Albrecht ED, 1995; Liggins GC, 1994). For example, glucocorticoids in conjunction with thyroid hormone stimulate synthesis of dipalmitoyl phosphatidylcholine (DPPC), the major component of surfactant, and its precursor phosphatidylcholine (PC). The actions of glucocorticoids and thyroid hormone in lung are probably receptor mediated, and receptors for these hormones have been characterized in fetal lung (Giannopoulos G, 1973; Gross et al., 1983; Gonzales LW and Ballard PL, 1981). Glucocorticoids have also dose-dependent, biphasic effects on the synthesis of the largest and most abundant surfactant protein, SP-A. Thus, dexamethasone at concentrations of 10^{-10}-10^{-9} M stimulated SP-A mRNA levels in human fetal explants, while at concentrations of greater than 10^{-8} M, SP-A expression was inhibited (Boggaram V and Mendelson CR, 1988; Boggaram V et al., 1989). Thus, fetal tissues have glucocorticoid receptors and can respond physiologically to glucocorticoids.

As discussed above, betamethasone regulation of fetal adrenal steroidogenesis

through a negative feedback mechanism in pituitary ACTH secretion has been extensively documented. Thus, *in vivo* dexamethasone or glucocorticoids inhibit ACTH-stimulation of adrenal DNA synthesis (Saez JM et al., 1977) and decrease expression of 3βHSD protein (Coulter CL et al., 1996). However, several *in vitro* and *in vivo* studies have also suggested the possibility of direct actions of betamethasone on the fetal adrenal itself. Thus, *in vitro* betamethasone suppressed adrenal cortical cell proliferation and steroidogenesis (Saito et al., 1979; Loose DS et al., 1980). Also an *in vivo* direct action of cortisol in sheep fetal adrenal gland has been postulated by Boshier et al., (1981). Thus in hypophysectomized sheep fetuses, the definitive zone has been shown to be approximately one-third as thick as that in controls consistent with suppressive effects of reduced ACTH. However, in hypophysectomized animals treated with cortisol, the number of mitochondria and the amount of smooth endoplasmic reticulum in the inner cortical cells exceeded that in control adrenals suggesting that cortisol may have a direct effect on the maturation of the inner cortical cells and/or the zona fasciculata. Though levels of cyto-differentiation exceeded that in control adrenals, fetal plasma cortisol did not reach the levels expected of the volume of inner cortex present, indicating that a additional factor(s) could be necessary for the complete maturation of the fetal sheep adrenal cortex. Further, Liggins et al., (1977) reported that the output of cortisol by the sheep fetal adrenal gland in response to an ACTH challenge *in vivo* was enhanced if the fetuses had been pretreated for 48 hr with dexamethasone. Although the effects of cortisol on its own production have not clearly been defined, it appears that cortisol, presumably acting through a glucocorticoid receptor (GR) present in adrenals, inhibits 18-hydroxylation of the steroid nucleus, but not 11β-hydroxylation or 17α-hydroxylation, thereby facilitating its own

production (Kahri et al., 1979). Supporting these data, modulation by cortisol of ACTH-induced activation of adrenal function in fetal sheep and 17α-hydroxylation appears to be at the level of cAMP (Challis JRG et al., 1985).

The mechanism(s) responsible for the increased activity of cortisol in the fetus most likely involves a receptor-mediated pathway. Indeed, GR has been characterized in sheep (Berdusco et al., 1993) and human (Sun M et al., 1996) fetal tissue. In sheep, northern blots of GR mRNA demonstrated that the relative abundance of GR mRNA was similar in fetal liver, lung, kidney and adrenal throughout fetal development, and a significant decrease was only observed in the kidneys of newborn lambs and not in the other tissues. In humans, GR are present at term and preterm labor in fetal membranes (Sun M et al., 1996).

Although cortisol may act trough a receptor mediated mechanism in fetal adrenals the distribution of the GR has not been evaluated yet. Recently Wu WX et al., (1997) demonstrated increased levels of glucocorticoid mRNA receptor in the fetal sheep adrenal late in gestation following betamethasone treatment. These studies indicated that, at least in fetal sheep, an increase in glucocorticoid biosynthesis late in gestation may be implicated in the direct activation of fetal adrenal function *in utero* through an adrenal GR-mediated mechanism.

In the light of the previous observations, the significant increase in the mean width of the ER positive cells under the capsule as well as the increase in the amount of ER staining by betamethasone in relation to control groups, could reflect a direct effect of cortisol on the development of the baboon fetal adrenal gland development. Indeed our results, characterizing the expression and distribution of GR in mid and late fetal adrenals,

are consistent with a receptor mediated mechanism for a direct effect of cortisol. Betamethasone appears to prevent the development of the transitional zone (Table 5-3), as determined immunohistochemically by absence of cells positive for both 3βHSD and P450c17, presumably by inhibiting fetal pituitary ACTH secretion. In earlier studies (Gray ES and Abramovich DR, 1980; Benirschke K, 1956), the decrease in ACTH in anencephalic fetuses was associated with a reduced adrenal weight, including inhibition of the fetal zone. We not observed only a reduction of the fetal zone (data not shown) but also a virtual elimination of the transitional zone. Our approach to assess adrenal cortical zonation by determining which steroidogenic enzymes are present in each zone, provides a more precise understanding of ACTH-regulation of fetal adrenal cortex functional differentiation. Thus, betamethasone treatment appears to have an indirect effect to restrain growth and adrenal steroidogenesis (Coulter CL et al., 1996), while simultaneously having a direct effect (i.e. replacing locally synthesized F) to increase intensity and expression of ER in previously induced (i.e. before day 150), definitive cortical zone cells. Therefore, induction of ER by ACTH is suggested to be mediated by an increased endogenous fetal cortisol secretion in ACTH-induced and estrogen-modulated differentiated definitive cells. Therefore, full maturation and differentiation of these cells is being linked to estrogen modulation of ACTH action.

Recently, Aberdeen et al., have shown that ACTH receptor mRNA levels declined in the fetal zone with advancing gestation even though secretion of pituitary ACTH is increasing during this time period (Pepe GJ et al., 1994; Graham WA et al., 1997). Although it is not known if ACTH protein levels parallel the mRNA patterns, as discussed above, basal as well as ACTH-stimulated DHA production by the baboon fetal adrenal *in*

vitro is lower per cell at term than at midgestation; whereas basal and ACTH stimulated cortisol synthesis and ACTH receptor mRNA levels in the definitive zone at term were higher than that at midgestation (Berhorn KA et al., 1995; Leavitt et al., 1997). Serum cortisol concentrations in instrumented rhesus monkey fetuses were suppressed 90% by dexamethasone while DHAS secretion was only suppressed by 60% (Jaffe RB et al., 1981). Moreover, we previously demonstrated that betamethasone treatment late in gestation elicited a reduction in the mRNA for ACTH receptor in the definitive zone but had no significant effect in the fetal zone, suggesting reduced ACTH-regulation of the fetal zone as pregnancy advanced (Aberdeen GW et al., 1997). This is paralleled by the reduced rate of growth of the fetal zone compared to that of the definitive zone in the second half of baboon pregnancy (Figure 7). These results indicate that the definitive zone cells are more sensitive to ACTH than cells of the fetal zone late in pregnancy. Thus, we propose that secretion of cortisol late in gestation may play an important paracrine/ autocrine role in the ACTH-induced maturation of the definitive and transitional zones late in primate pregnancy and that part of this cortisol effect is mediated by its regulation of the estrogen receptor in the definitive zone. Thus, by term elevated concentrations of placental estrogen acting through a receptor mediated pathway in the definitive zone could be responsible for the increased sensitivity of definitive zone to ACTH-mediated growth and steroidogenesis.

Effects of gestational age on adrenal ER expression: in-vivo ACTH and ACTH and betamethasone administration at midgestation

We could not detect ER in the fetal adrenal at midgestation but could detect it in the definitive zone cells near term. As discussed above, fetal ACTH secretion is a

necessary component of the formation of the definitive zone. To assess the role of ACTH in the induction of ER in the fetal adrenal *in vivo* and the role of ontogeny in this process, baboon fetuses were either treated with ACTH alone at midgestation to study if ER could be induced prematurely, or betamethasone followed by ACTH to study the combined stimulatory effects of both treatments. No ER was detected in any adrenal from animals treated with ACTH alone though occasionally, groups of nuclei adjacent to the capsule were observed staining very weakly for ER. Also, no ER expression was observed when combining stimulatory effects of both ACTH and glucocorticoids at midgestation.

In contrast with the lack of induction of ER in the definitive zone cells by ACTH treatment at midgestation, ACTH enhanced fetal adrenal definitive zone width to a value that was approximately two-fold greater than normal. However, ACTH induction of 3βHSD immunoreactivity was still 4-fold less than term control groups (Figure 3). ACTH did not have a significant effect on P450scc nor P450c17 mRNA levels as discussed earlier (Chapter 2). Administration of ACTH to betamethasone treated baboons did not induce ER but restored the width of the definitive zone (as assessed by 3βHSD expression) to normal size for a midterm fetus (Data not shown). Therefore, despite levels of fetal ACTH which induced an increase in the 3βHSD zonation following ACTH administration, betamethasone could not induce the ER at midgestation. That ACTH or betamethasone followed by ACTH had no direct effect at this stage of gestation is surprising since the midterm control baboon adrenal expresses the glucocorticoid receptor (Figure 7). Presumably, sufficient ACTH treatment, duration and/or concentration, was not presented to the midterm adrenal for induction of the definitive/transitional zones or other ontogenic factors limit the fetal adrenal response to ACTH at this stage.

Our present data indicates that the sensitivity of the definitive zone to ACTH at midgestation is different for the induction of ER and 3βHSD proteins. Thus, exogenous ACTH can induce prematurely 3βHSD protein. In contrast, induction of ER requires additional ontogenic development of the adrenal and the presence of glucocorticoid and the GR are not sufficient to induce expression of the ER since there is abundant GR present in the midgestation fetal adrenal cortex.

Summary

At midgestation ER was not detectable by immunohistochemistry in the fetal or definitive zone of untreated baboon adrenals. Administration of ACTH or betamethasone followed by ACTH to the fetus either to prematurely elevate fetal ACTH concentration or restore ACTH levels after betamethasone suppression, respectively, failed to induce nuclear expression of ER. However, by term nuclear ER was observed by immunohistochemistry in the continuous narrow definitive zone adjacent to the capsule of untreated adrenals but was not detectable in the fetal zone. At term, betamethasone treatment reduced adrenal weight and immunohistochemical expression of both 3βHSD and P450c17 proteins, preventing development of the transitional zone (the zone of cells positive for both P450c17 and 3βHSD). However, betamethasone treatment induced a significant increase in the width and intensity of the zone of cells expressing the ER. We investigated the possibility that suppression of placental estrogen by decreased secretion of fetal adrenal androgen precursors in betamethasone treated animals resulted in the increased adrenal ER content by administration of the aromatase inhibitor CGS 20267 in the second half of the pregnancy. CGS treatment reduced maternal peripheral serum estradiol concentrations to 2% of normal for this time in gestation. Interestingly, the

weight of the fetal adrenal glands was significantly increased after CGS treatment, which was reflected in a marked enhancement of the fetal zone. However, there was no increase in the intensity nor the cortical width of the nuclear ER expression compared to the control groups. Consistent with lack of ER induction of adrenal ER at term after CGS treatment, restoration of placental estrogen by simultaneous administration of E_2 with the CGS throughout the second half of gestation did not increase the intensity nor cortical width of the nuclear ER. Thus, reduced placental estrogen appears to enhance preferentially ACTH induced growth of the fetal zone, retarding maturation of the definitive zone while having no significant effect on ER expression. Collectively, these results suggest that glucocorticoids may act directly on the fetal adrenal to increase ER. Characterization of the GR in mid and term adrenals throughout both the fetal and definitive zone appears to indicate that the cortisol effect in the fetus most likely involves a receptor-mediated pathway. Thus, betamethasone treatment appears to have an indirect effect to restrain growth and differentiation of the definitive zone (i.e. reduced 3βHSD expression), while simultaneously having a direct effect (i.e. replacing locally synthesized cortisol) to increase the intensity and expression of ER in previously induced, i.e. before day 150, definitive cortical zone cells. Therefore, induction of ER by ACTH is suggested to be mediated by an increase in endogenous fetal cortisol secretion in ACTH-induced and estrogen-modulated differentiated definitive zone cells; full maturation and differentiation of these cells is linked to estrogen modulation of ACTH action through ER mediation.

The absence of ER in fetal zone may indicate that modulation of fetal zone growth and steroidogenesis by estrogen does not appear to involve a direct action of estrogen on

these cells.

Localization of GR at midgestation suggests that its presence alone is not sufficient to induce expression of the ER. Ontogenic development and ACTH-induced maturation and differentiation of the definitive zone cells appears to be necessary for glucocorticoid enhancement of ACTH-induced ER expression.

Table 5-1. Effects of administration of betamethasone, CGS, and CGS and E_2 during baboon pregnancy on umbilical vein serum levels of cortisol and estradiol and on fetal adrenal, pituitary and body weights.

	Umbilical		Weight		
	Estradiol (ng/ml)	Cortisol (μg/dl)	Adrenal (mg)	Pituitary (mg)	Fetus (g)
Control (n=7)	2.2 ± 0.5[a]	33 ± 4[a]	378 ± 21[a]	24 ± 2	794 ± 68
Fetal betamethasone (n=4), days 150-164	0.6 ± 0.1[b]	14 ± 5[b]	183 ± 20[b]	21 ± 3	754 ± 88
Fetal/maternal betamethasone (n=4), days 150-164	0.1 ± 0.1[b]	1 ± 1[b]	183 ± 9[b]	25 ± 2	733 ± 85
CGS (n=2), days 100-170	0.07	17.4	553	16.1	740
CGS and E_2 (n=2), days 100-170	0.15	20.4	366	19.3	646

Values (mean ± SE) with different letter superscripts differ from each other at $P<0.05$

Table 5-2. Effect of administration of betamethasone, CGS, and CGS and E_2 during baboon pregnancy on fetal and definitive zone cell number and expression of proliferating cell nuclear antigen (PCNA).

	Fetal zone		Definitive zone	
	Cells/0.025 mm^2	% PCNA+	Cells/0.025 mm^2	% PCNA+
Control (n = 7)	81 ± 8	55 ± 3	174 ± 12	51 ± 8
Betamethasone (n = 3)	182 ± 11*	48 ± 20	232 ± 14*	59 ± 18
CGS (n = 2)	133	1	296	29
CGS and E_2 (n = 2)	108	4	232	13

Asterisk indicates mean (± SE) value is different ($P<0.05$) from that in the control.

Betamethasone was administered between days 150-164

CGS and GCS + E_2 between days 100-164

All adrenal glands were harvested on G.A. day 164

Table 5-3. Effect of administration of betamethasone, CGS and CGS and E_2 at term on fetal adrenal zonation and expression of estrogen receptor (ER).

Treatment	Mg Wet WT	Total 3βHSD DZ + TZ μm	3βHSD not P450c17 DZ μm	+ 3βHSD/ + P450c17 TZ μm	ER μm DZ μm	ER intensity of label
Control (n = 5)	368 ± 29	187 ± 23	100 ± 13	87	83 ± 5	-, + or + +
Betamethasone (n = 3)	178 ± 9	126 ± 9	130 ± 6	0	126 ± 11	+ + / + + +
CGS 60 days (n = 2)	553	149	(82)*	(66)*	68	-, + or + +
CGS and E_2 (n = 2)	366	176	75	101	94	+ or +/-

* Expression of P450c17 reached to the outermost zone of the adrenal, to groups of cells under the capsule and thus uniform measurements through the course of the DZ cannot be made and directly compared with the measurements of the three other treatment groups.

Figure 5-1. To distinguish inner definitive or transitional zone cells located between the outer definitive and fetal zone cells by term gestation, immunolocalization of 3βHSD and P450c17 steroidogenic enzymes was used in consecutive adrenal sections. To define the cell type of the transitional zone, the mean width of the zone under the capsule negative for P450c17 was subtracted from the mean width of the 3βHSD positive zone on consecutive adrenal sections. The difference in width measurement between the two zones represented cells expressing both P450c17 and 3βHSD proteins or transitional zone.

Steroidogenic enzyme expression of the transitional zone at term

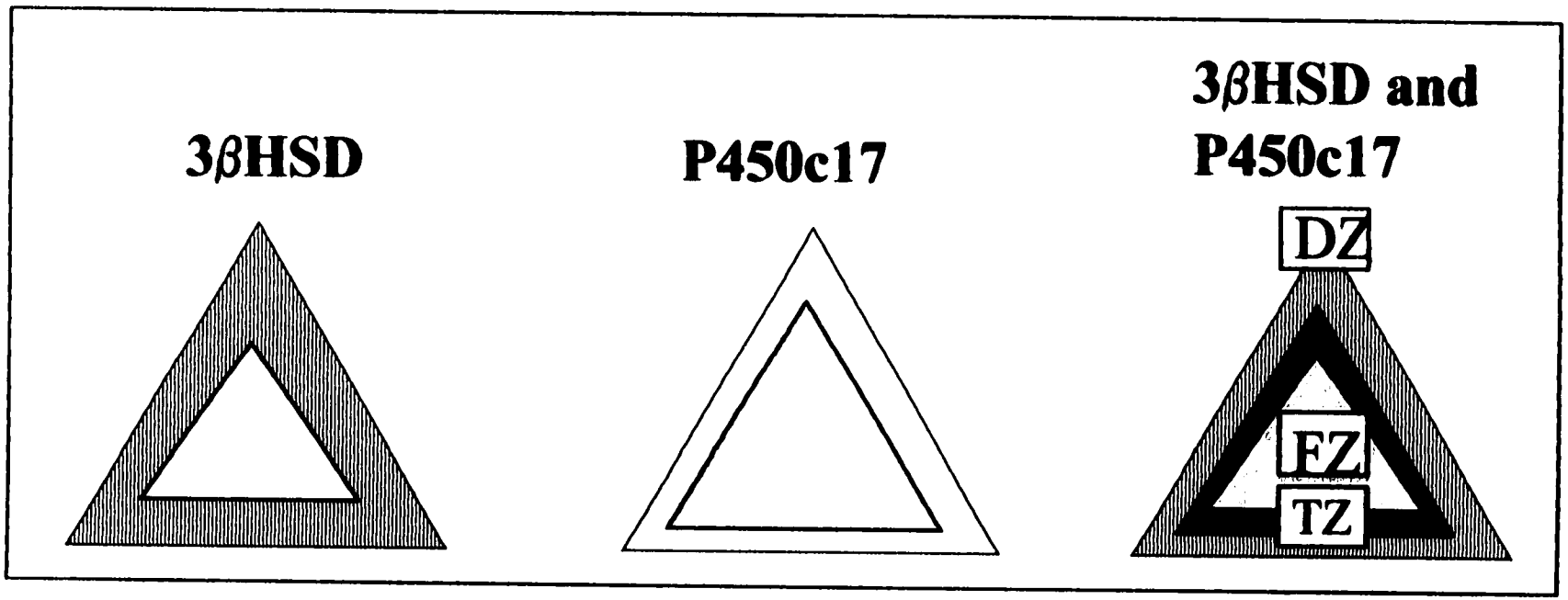


Figure 5-2. Immunohistochemistry of sections of term adrenal glands from control, betamethasone and CGS-treated fetal baboons. Localization of 3βHSD immunostaining in adrenal gland sections from representative fetuses of untreated (A), or treated from days 150 to 164 of gestation either with betamethasone (B) or CGS (C). Localization of P450c17 immunostaining in adrenal gland sections from representative baboon fetuses of untreated (D), or treated from days 150 to 164 of gestation either with betamethasone (E) or CGS (F). The capsule/definitive zone interface is marked in each figure by a bar (❙❙). DZ, definitive zone; FZ, fetal zone; M, medulla (original magnification=X100).

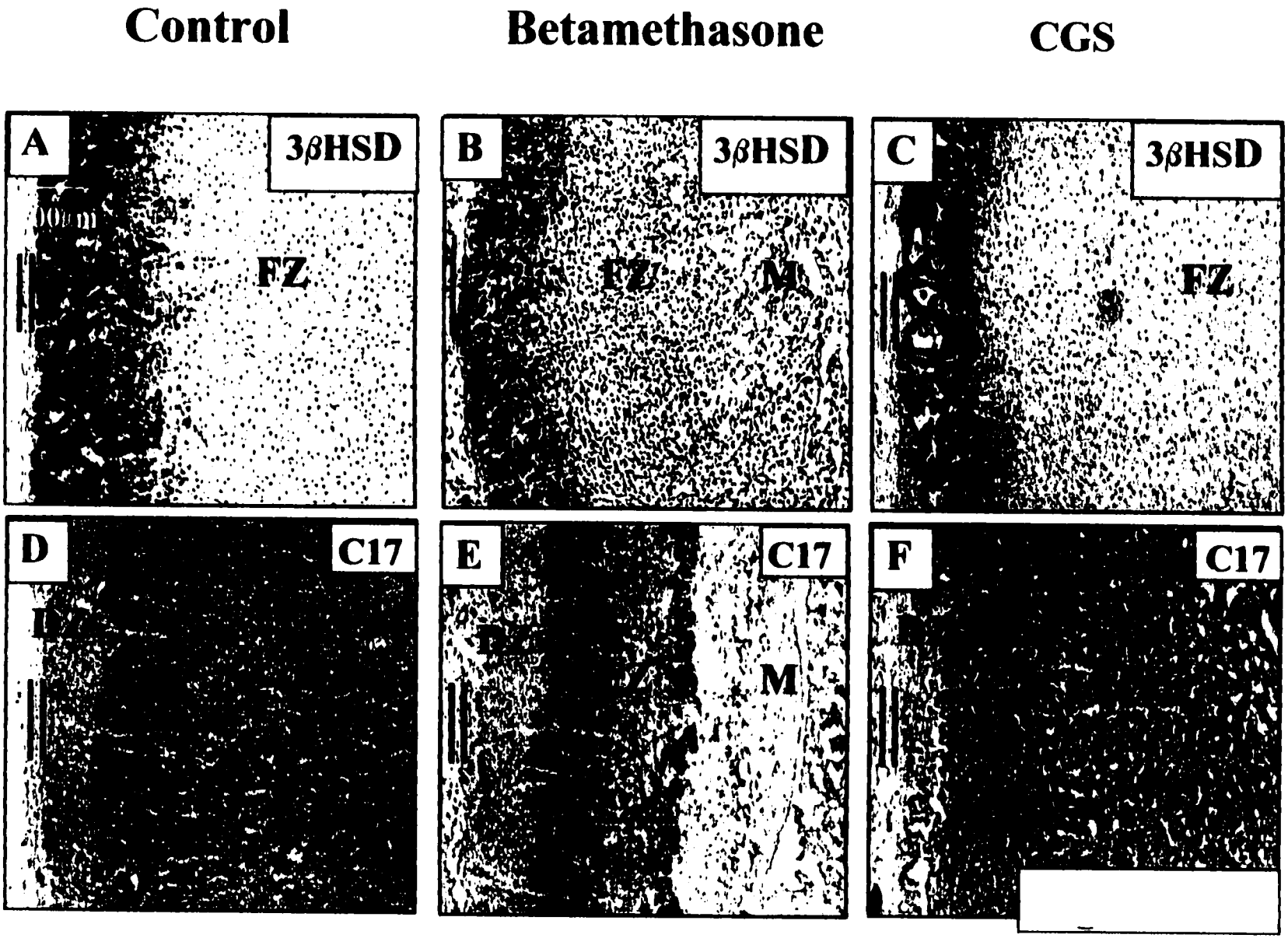
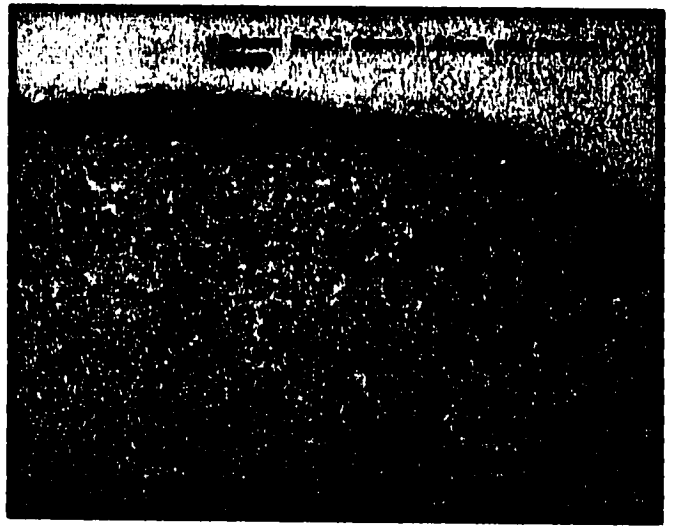
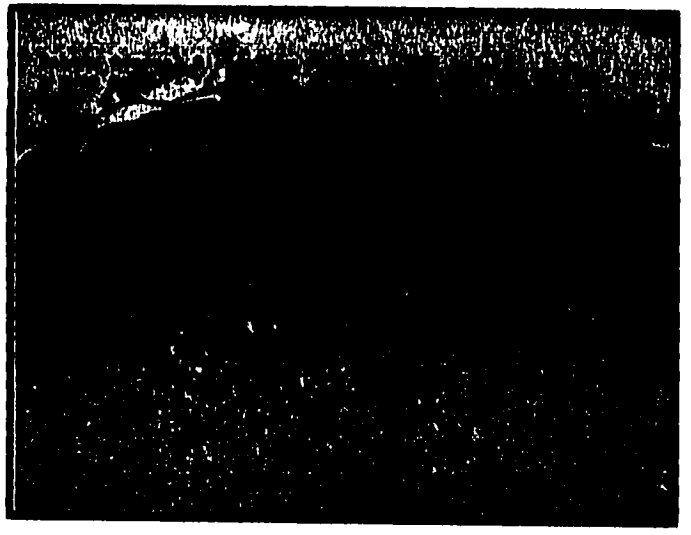


Figure 5-3. Photomicrographs of sections of adrenal glands from mid and term untreated fetal baboons. Localization of 3βHSD immunostaining in adrenal gland sections from representative untreated fetuses at mid (A) and term gestation (B) (original magnification=X100).

Immunolocalization of Adrenal 3βHSD



Control Mid



Control Term

Figure 5-4. Immunohistochemistry of ER in sections from term adrenal glands from control, betamethasone and CGS-treated fetal baboons. Localization of nuclear ER immunostaining in adrenal gland sections from representative fetuses of untreated (A), or treated form days 150 to 164 of gestation either with CGS (B) or betamethasone (C). The specificity of ER staining was demonstrated by lack of immunoreactivity in adrenal sections (D, day 164) treated with ER antibody that has been preabsorbed with 15-fold excess of whole ER recombinant molecule (c = capsule; original magnification=X100).

Immunolocalization of Adrenal Estrogen Receptor

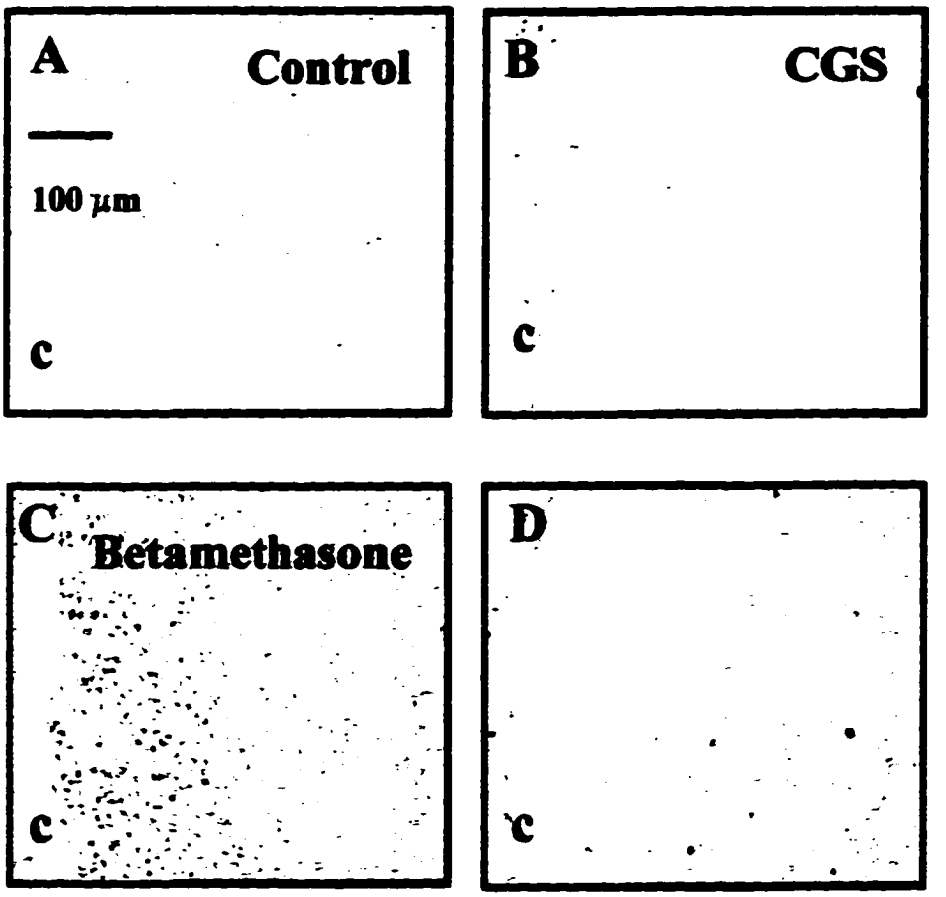


Figure 5-5. Photomicrographs of sections of adrenal glands from term control and betamethasone-treated fetal baboons. Colocalization of 3βHSD and ER expression in representative adrenal gland sections of untreated (A,E) or betamethasone-treated from days 150 to 164 of gestation (B,F). A,B magnification X100; C-F magnification X200. A 164-days section of fetal baboon adrenal of untreated (C), or betamethasone-treated (D) where ER antibody was replaced with normal mouse serum. Nuclei stained with methyl green (original magnification=X200).

Control **Betamethasone**

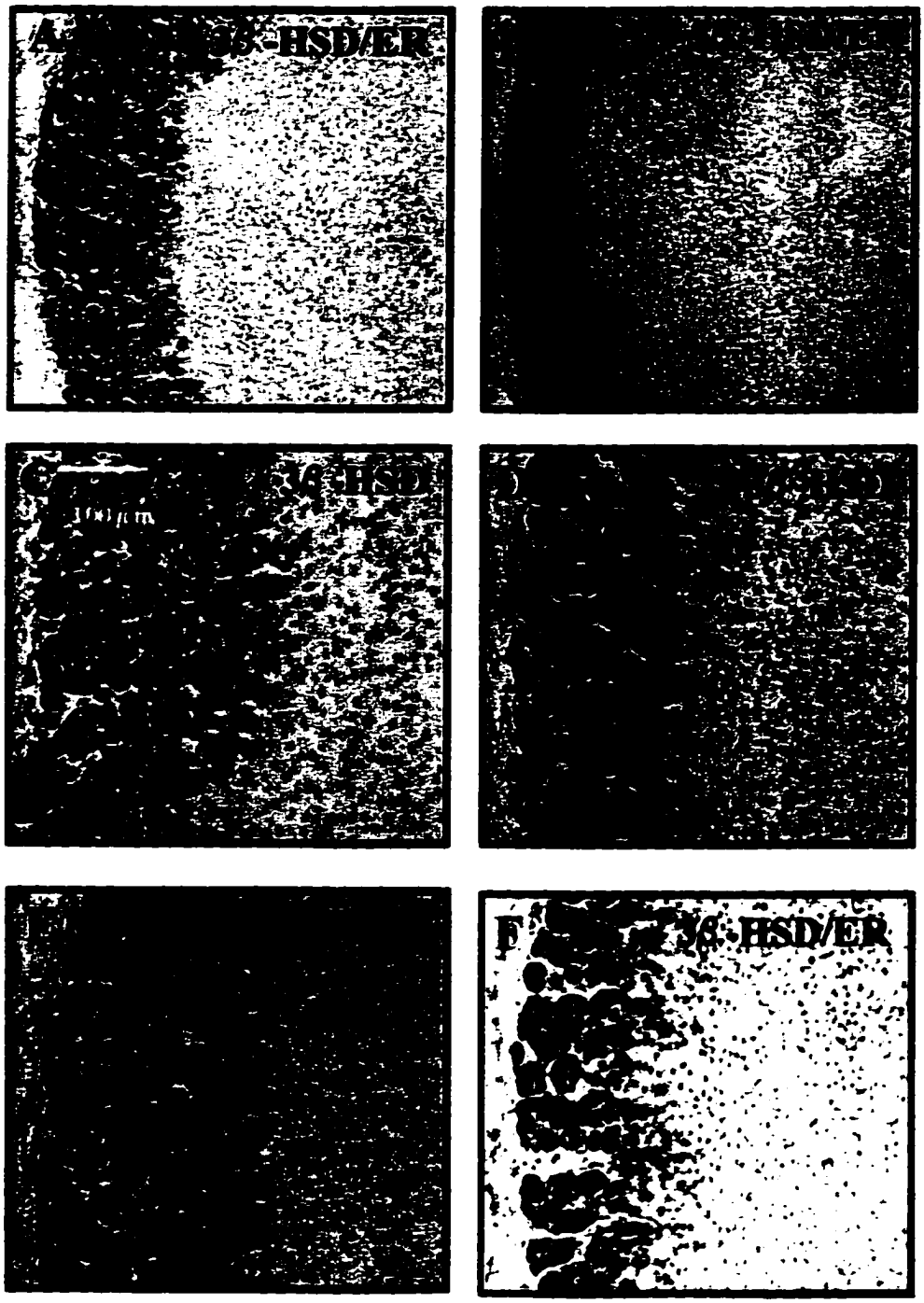
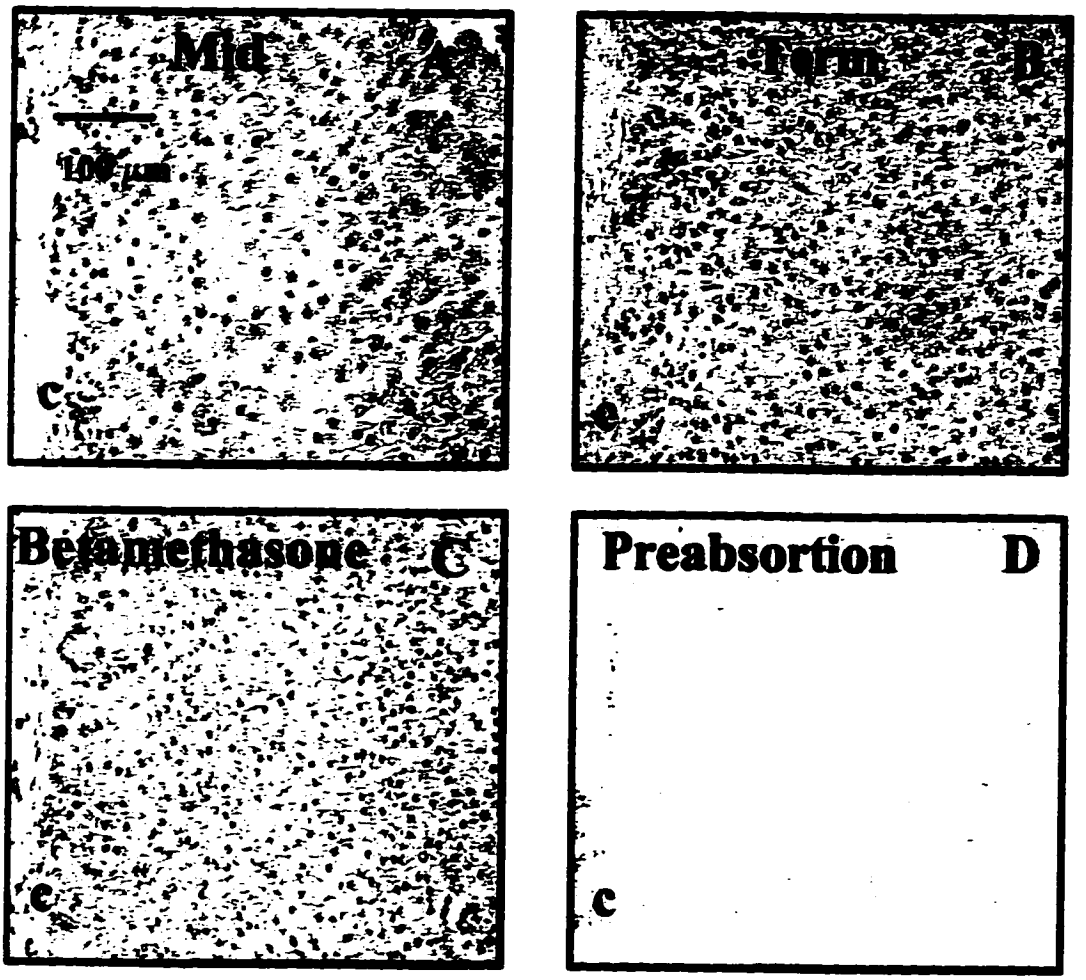


Figure 5-6. Localization of nuclear GR immunostaining in adrenal gland sections from representative fetuses of mid untreated (A), term untreated (B) or betamethasone treated baboons from days 150 to 164 of gestation (C). The specificity of ER staining was demonstrated by lack of immunoreactivity in adrenal sections (D), (day 164) treated with ER antibody that has been preabsorbed with 100-fold excess of peptide (c = capsule; original magnification=X100).

Immunolocalization of Adrenal Glucocorticoid Receptor



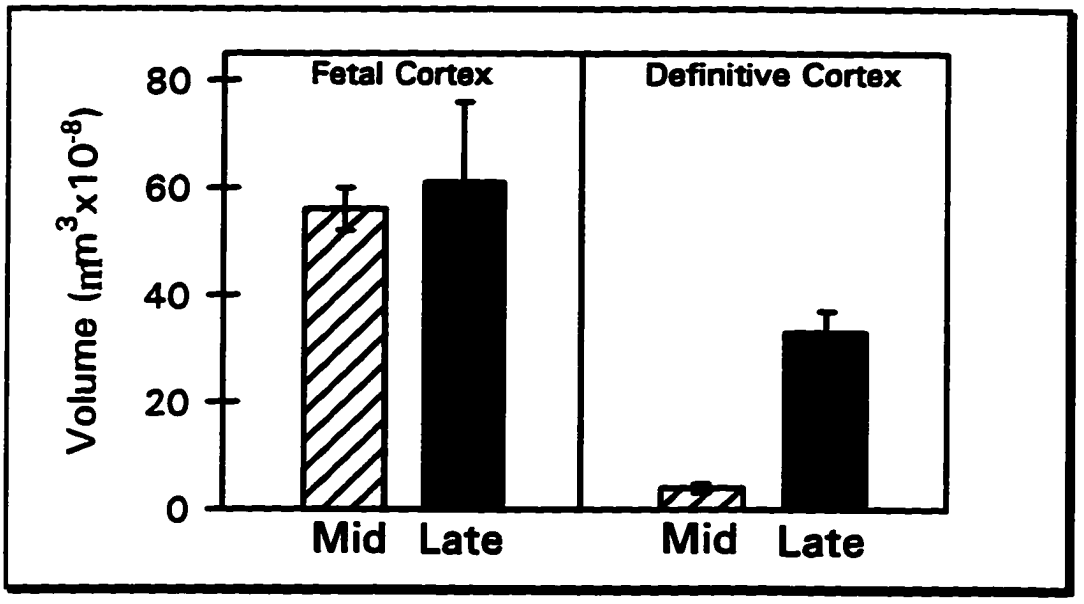


Fig. 5-7: Volume of the fetal and definitive zones of the baboon fetal adrenal cortex. Width (μm) of both zones determined by image analysis and the volume calculated as 4/3 πr^3.

CHAPTER VI

CONCLUSIONS AND FUTURE DIRECTIONS

We examined the role of fetal pituitary ACTH in regulating cellular proliferation, maturation, and steroidogenic potential of each cortical zone in the primate fetal adrenal and the modulation of ACTH-induced growth and maturation by placental estrogen during mid and late gestation by determining adrenal gland growth, cellular remodeling (i.e. apoptosis) and mRNA and/or protein levels/distribution of the enzymes 3βHSD, P450c17, P450scc and the membrane receptors for LDL and ACTH. Regardless of the stage of gestation, high glucocorticoids levels and thus low fetal ACTH levels induce atrophy and a decrease in cellular size and integrity of the fetal adrenal. This suggests that growth of both the fetal and the definitive zone in response to increased endogenous ACTH secretion occurs primarily by hypertrophy and a more limited proliferation. Glucocorticoid suppression of fetal pituitary POMC mRNA/ACTH also resulted in a reduced fetal adrenal ACTH receptor mRNA expression during both mid and late gestation. Thus the inhibitory effects of betamethasone treatment in fetal adrenal development are probably mediated not only by a reduction in fetal circulating ACTH levels but also by subsequent decrease in adrenal ACTH receptor content.

Reduced levels of mRNA expression for the enzymes P450c17, P450scc and the membrane receptors for LDL were also observed in adrenals of betamethsone-treated animals. The corresponding decline in ACTH-depleted baboons of mRNA expression for P-450scc and P-450_{C17}, enzymes expressed in the fetal zone (Mesiano S et al., 1993) and stimulated *in vitro* by ACTH (Mesiano S et al., 1996; Mesiano S et al., 1993; DiBlasio AM et al.,

1987; Voutilainen R and Miller WL, 1988; Ilvesmaki V and Voutilainen R, 1991), support the concept of a role for the receptor-mediated action of ACTH *in vivo* in regulating tissue differentiation and those enzymes critical to fetal adrenal C_{19}-steroid biosynthesis and thus estrogen production at mid and term gestation. Prior observations of a decrease in fetal adrenal P-450scc and P-450_{C17} mRNA levels in rhesus monkeys treated with betamethasone (Coulter CL et al., 1996) are also consistent with the findings in baboons of the present study.

Consistent with the reduction of steroidogenic enzyme mRNA by Northern blot analyses, was the reduced immunocytochemical expression of 3βHSD in the definitive zone after betamethasone treatment both at mid and near term gestation indicating that fetal pituitary ACTH has a major role in stimulating growth and differentiation of the fetal definitive zone during at least the second half of gestation. Simultaneous reduction in immunoexpression of P450c17 in the fetal zone and of 3βHSD in the definitive zone in betamethasone treated-animals near term resulted in the virtual elimination of the transitional zone (cells positive for both 3βHSD and P450c17 at the interface between fetal and definitive zone). These data indicate that the increased endogenous ACTH secretion late in gestation induces differentiation of the transitional zone and thus the potential of these cells to synthesize cortisol. Collectively, these observations indicate that the ACTH receptor mRNA is regulated coordinately with other ACTH-regulated genes required to maintain fetal adrenal differentiation and responsiveness to ACTH, *in utero* in the primate fetus as recently suggested by Mesiano et al (Mesiano S et al., 1996) based on studies of human adrenal cells in culture.

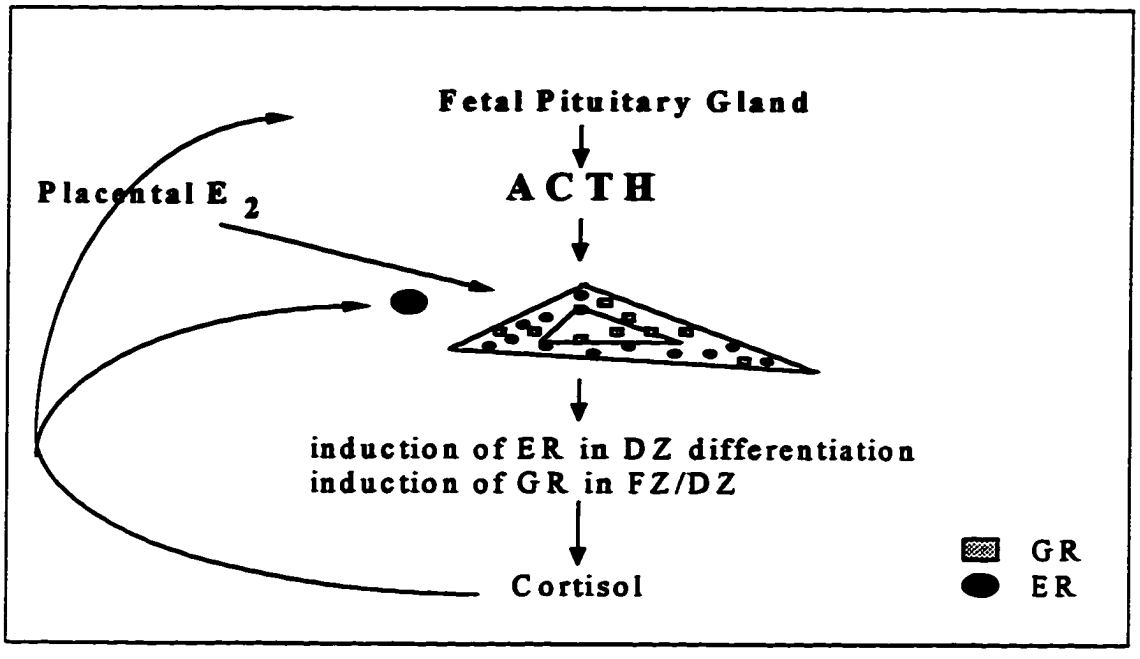
Recently, Aberdeen and colleagues (Pepe GJ et al., 1994; Aberdeen WA et al., 1997), have shown that ACTH receptor mRNA levels declined in the fetal zone with

advancing gestation even though secretion of pituitary ACTH is increasing during this time. Although it is not known if ACTH protein levels parallel these mRNA patterns, as discussed in Chapter 5, basal as well as ACTH-stimulated DHA production by the baboon fetal adrenal *in vitro* is lower per cell at term than at midgestation; whereas basal and ACTH stimulated cortisol synthesis and ACTH receptor mRNA levels in the definitive zone at term were higher than that at midgestation (Berhorn KA et al., 1995; Leavitt et al., 1997). Moreover, we demonstrated (Chapter 4), that betamethasone treatment late in gestation elicited a reduction in the mRNA levels for ACTH receptor in the definitive zone but had no significant effect in the fetal zone, suggesting reduced ACTH-regulation of the fetal zone as pregnancy advanced (Aberdeen GW et al., 1997). This is paralleled by the reduced rate of growth of the fetal zone compared to that of the definitive zone in the second half of baboon pregnancy. These results indicate that the definitive zone cells are more sensitive to ACTH than cells of the fetal zone late in pregnancy. Thus, while ACTH appears to be the principal regulator of the fetal adrenal definitive zone, ACTH modulation of the adrenal fetal zone may be influenced by the presence or absence of other factors making this zone less sensitive to ACTH action. The presence of the glucocorticoid receptor, preferentially in the fetal zone at term gestation, provides a mechanism for a possible paracrine effect of cortisol secreted by the fetal transitional zone cells, modifying the sensitivity of the fetal zone. Indeed, there is considerable evidence that growth factors, acting in an autocrine and/or paracrine fashion, mediate the secretion and action of ACTH, i.e. estrogen, prolactin, insulin-like growth factors, and epidermal growth factor (Jaffe RB et al, 1988; Crickard K and Jaffe RB, 1981; Coulter et al, 1996; Pepe GJ and Albrecht ED, 1985; Albrecht ED and Pepe GJ, 1987; Albrecht ED and Pepe GJ,

1995; Voutilainen R and Miller WL, 1988; Mesiano S and al., 1993). In the fetus these growth factors seem to modulate preferentially *in utero* growth of the fetal zone. In support of the latter concept, estrogen administration at midgestation suppressed the responsivity of the baboon fetal adrenal to ACTH with respect to the secretion of fetal zone-specific DHA, but not of definitive zone-specific cortisol, as determined *in vitro* (Albrecht et al., 1987; Albrecht et al., 1990) and *in vivo* (Pepe GJ et al., 1989). Based on these results Albrecht et al., (1996) proposed that estrogen normally feedsback to restrain ACTH-regulated growth and steroidogenesis in the fetal zone, while simultaneously in an indirect way (Pepe et al., 1988), via the placental 11βHSD system, enhances ACTH-regulated development and function of the definitive zone. Our results of estrogen receptor distribution in the fetal adrenal support a direct action of estrogen on the definitive zone but leave unanswered how estrogen modulates ACTH activity in the fetal zone or transitional zone in the presence of minimal amounts of ACTH (re: betamethasone and CGS, Chapter 5). Localization of the GR at midgestation in both the fetal and the definitive zones could also explain why the mRNA for the ACTH receptor, P-450scc, and P-450_{C17}, were only restored in 2 of the 4 fetuses injected with ACTH to betamethasone-suppressed baboons although fetal adrenal weight/growth, cellular integrity and 3β-HSD expression were uniformly restored to normal (Chapter3). Betamethasone acting via a receptor-mediated mechanism could have a direct effect on the fetal adrenal gland itself. This proposed inhibitory effect of betamethasone on the expression of the mRNA for the ACTH receptor, P-450scc, and P-450_{C17} may not be reversed by the simultaneous administration of ACTH.

As demonstrated by Pepe and Albrectch (Pepe GJ and Albrecht ED, 1995, for review) the baboon placental oxidation of cortisol to cortisone is induced by estrogen. As placental estrogens are derived from adrenal precursor androgens, this represents a positive feedback loop where a further increase in placental estrogen late in gestation leads to increased conversion of cortisol to cortisone, decreasing the levels of cortisol in the fetus derived from the mother which decreases the inhibition of the hypothalamus-pituitary and thus increases fetal pituitary ACTH secretion. This in turn stimulates fetal adrenal androgen production providing more substrate for placental estrogen formation. The estrogen-mediated ACTH increase eventually promotes growth and differentiation of the definitive and transitional zones and the ability of the fetal adrenal to synthesize cortisol. The results of the present study employing the aromatase inhibitor CGS demonstrated that in the presence of reduced placental estrogen the ACTH-induced growth of the adrenal fetal zone appears to be preferentially enhanced at the expense of the definitive/transitional zones. These results with CGS indirectly support previous observations by Pepe and Albrecht (1988) demonstrating that placental estrogens reduced the responsivity of the baboon fetal adrenal gland to ACTH with respect to the formation of DHA, presumably by inhibiting P450c17 expression. In addition, estrogen appears to also be necessary to control excessive ACTH-induced growth. We can hypothesize that E_2 modulates ACTH-induced hypertrophy and differentiation of the fetal adrenal rather than hyperplasia, contributing to the maturity of the definitive zone cells at term. Although additional studies remain to be performed to demonstrate reduced levels of fetal pituitary POMC/ACTH after CGS administration, presumably low levels of fetal pituitary POMC/ACTH would be consistent with the reduced expression of 3βHSD. Increased

growth of adrenals after CGS treatment supports the concept that estrogen acts in conjunction with ACTH to prevent uncontrolled increased of adrenal cortical growth. Thus, our results support previous studies by Pepe and Albrecht (Albrecht et al., 1987; Albrecht et al., 1990) demonstrating that the developmental increase in placental estrogen secretion may lead to inhibitory effects of estrogen on fetal zone growth and Δ^5C_{19}-steroid production while enhancing cortisol synthesis by inducing expression of 3βHSD in the definitive zone cells. Estrogens also contribute to definitive zone differentiation acting through a receptor mediated mechanism. As explained above, the ACTH-induced growth of the definitive zone is mediated by placental estrogens which rise gradually in human (Tulchinsky D et al., 1972), and baboon (Townsley JD, 1972) over several weeks prepartum. Late in gestation, as the fetal adrenal begins to express 3βHSD, it may acquire the capability of *de novo* glucocorticoid production. Thus, increased endogenous levels of cortisol acting in a paracrine/autocrine fashion via a GR-mediated mechanism increase ACTH-induced estrogen receptor expression. In this way placental estrogen may exert a direct effect on the differentiation of the fetal definitive zone. This action used represent a positive feedback loop whereby further increases in adrenal cortisol secretion lead to increased expression of ER within the definitive zone thus increasing responsiveness to estrogen-induced differentiation. Thus, full maturation and differentiation of these cells is linked to estrogen modulation of ACTH action through ER mediation. The suggested mechanism of ACTH-induced and estrogen-modulated differentiated definitive zone cells is presented diagrammatically below:



Future research directions

During the past decade our laboratory has established the baboon as a valid model for the study of human pregnancy and the perinatal period (Chapter2). Based on the results of the present study, the need of the *in vivo* model is further emphasized. We have discussed previously that one important physiological function of the primate fetal adrenal cortex is to provide C19 substrate to the placenta for the formation of estrogens. This strategy for estrogen formation in pregnancy is unique to primate species and is accomplished by the "fetoplacental unit" requiring the use of the *in vivo* model. In addition, the maturation of the fetal definitive zone is a complex process involving the effect of several factors, , i.e. estrogen, ACTH, glucocorticoid, and other factors, which modulate adrenal cortical growth and differentiation. Furthermore, we demonstrated induction of ER requires additional ontogenic development of the adrenal and the presence of glucocorticoid and the GR are not sufficient in themselves to induce

expression of the ER. Thus, these considerations confirm that *in vivo* studies provide a more advantageous system to elucidate the physiological interactions between the placenta and the fetal adrenal. The following studies are suggested to define with more precision the modulation by estrogen of ACTH action using the *in vivo* model: 1) Simultaneous administration of betamethasone and ACTH during the second half of gestation to test if ACTH can reverse the effects of betamethasone on growth, differentiation, and expression of ER in adrenal cortical zones. This should help distinguish what effects are due to decreased ACTH and what are possibly direct glucocorticoid effects. 2) Additional *in situ* studies to demonstrate that reduction in levels of placental estrogen after CGS administration induce a concomitant decrease in levels of fetal pituitary POMC/ACTH secretion. The proposed study will help to elucidate the indirect way by which estrogen, via placental oxidation of cortisol to cortisone, enhances ACTH-regulated development and function of the definitive and transitional zones while preventing expression of P450c17 in the definitive zone. These results will be compared with *in situ* studies where simultaneous administration of CGS and estrogen should reverse the proposed decrease in fetal pituitary POMC/ACTH by CGS alone. If levels of pituitary POMC/ACTH are not reduced after CGS treatment, this suggest that estrogen may only be regulating ACTH action in a direct way in the adrenal gland itself via a receptor mediated mechanism. In this case, it is possible that the growth of the fetal zone late in gestation is modulated indirectly in a paracrine manner by the action of estrogen in the outer definitive zone and that specific growth factors may mediate estrogen-regulated ACTH trophic actions. 3) Preliminary studies in our laboratory indicate that the inhibin molecule, a herodimeric glycoprotein related to the TGF-β family of peptides, plays a role in the functional

zonation of the fetal adrenal cortex. ACTH regulates expression of the α-subunit of inhibin, and its localization through gestation appears be predominantly in the fetal zone. Additional studies are necessary to establish if lack of α-inhibin in the definitive zone is linked to the ACTH-induced and estrogen-modulated differentiated definitive zone cells.

The mechanism(s) by which ACTH regulates fetal zone development has been extensively documented using primary cultures of fetal cells. The correlation between the *in vitro* results in this laboratory (Albrecht et al., 1987; Albrecht et al., 1987) and the *in vivo* pattern demonstrated in the present study of growth and differentiation of the adrenal fetal and definitive zones, primarily regulated by estrogen modulation of fetal pituitary ACTH actions, indicates that the regulatory mechanism(s) is maintained in culture. Thus the following experiments are proposed, using cell culture methodologies as a result of the present *in vivo* studies, to confirm our results and to elucidate with more precision *in vivo* observation at the cellular level: 1) Regulation by glucocorticoids of ER expression. We have demonstrated upregulation of ER expression *in vivo* by betamethasone administration. In the proposed study, primary cultures of fetal and definitive zone cells of mid and term gestation will be incubated in the presence or absence of glucocorticoids to determine time-and dose-dependent regulation. Regulation of estrogen receptor will be studied using Northern blot analysis of total RNA extracted from these primary cultures of adrenal cell as well as immunohistochemistry. These studies will provide insight as to whether glucocorticoids act directly on definitive zone cells to induce the ER or whether indirectly by stimulating the fetal zone cells to produce a ER inducing factor. 2) Glucocorticoid modulation of ACTH-induced ER expression. The present study has demonstrated that ACTH is presumably necessary for the induction of the ER in fetal

definitive zone in late gestation which is glucocorticoid-upregulated. Thus the proposed study will involved addition of physiological concentrations of ACTH, cAMP analogs (e.g., 8-bromo-cAMP) or products which increase adenylate cyclase activity (e.g., forskolin), to determine actions of ACTH in the presence or absence of betamethasone. Regulation of estrogen receptor and P-450 enzyme mRNA will be studied using Northern blot analysis of total RNA extracted from these primary cultures of adrenal cells.

3) Involvement of ER in maturation of the definitive zone cells. We have hypothesized that estrogen modulates ACTH action via a receptor mediated mechanism. To study the direct involvement of estrogen we propose the establishment of a definitive zone cell line in which the ERE could be stably integrated. In this cell line, we could demonstrate activation of the ERE by the ER present in the definitive zone cells. Moreover, transfected cell lines will allow us to study growth and steroidogenesis (e.g., cortisol, DHA) in the absence/presence of estrogen. The transfected cell line could be used to investigate if the ERE/ ER system can be activated by factors other than the specific ligand.

REFERENCES

1. **Aberdeen GW, Babischkin JS, Davies WA, Pepe GJ, Albrecht ED** 1997 Decline in Adrenocorticotropin receptor messenger ribonucleic acid expression in the baboon fetal adrenocortical zone in the second half of pregnancy. Endocrinology138:1634-1640

2. **Aberdeen GW, Leavitt MG, Pepe GJ, Albrecht ED** 1997 *In vivo* regulation of baboon fetal adrenal gland development and ACTH receptor expression at midgestation by ACTH. Endocrinology, submitted.

3. **Albiston AL, Obeyesekere V, Smith R, Krozowski Z** 1994 Cloning and tissue distribution of the human 11 β-hydroxysteroid type 2 enzyme. Mol Cell Endocrinol 105: R11-R17

4. **Albrecht DE, Pepe GJ** 1995 Supression of maternal adrenal dehydroepiandrosterone and dehydroepiandrosterone sulphate production by estrogen during baboon pregnancy. J Cli Endocrinol Metab 80:201-3207

5. **Albrecht ED** 1980 A role for estrogen in progesterone production during baboon pregnancy. Am J Obstet Gynecol 136:569-574.

6. **Albrecht ED, Aberdeen GW, Babischkin JS, Tilly JL, Pepe GJ** 1996 Biphasic developmental expression of adrenocorticotropin receptor messenger ribonucleic acid levels in the baboon fetal adrenal gland. J Cli Endocrinol Metab 137:1292-8

7. **Albrecht ED, Crenshaw Jr MC, Pepe GJ** 1988 The effect of estrogen on placental delivery after fetectomy in baboons. Am J Obstet Gynecol 160:237

8. **Albrecht ED, Henson MC, Walker ML, Pepe GJ** 1990 Modulation of adrenocorticotropin-stimulated baboon fetal adrenal dehydroepiandrosterone

formation in vitro by estrogen at mid-and late gestation. Endocrinology 126:3083-3088

9. **Albrecht ED, Pepe GJ** 1980 The utilization of placental substrates for cortisol synthesis by the baboon fetus near term. Steroids 35:591

10. **Albrecht ED, Pepe GJ** 1987 Effect of estrogen on dehydroepiandrosterone formation by baboon fetal adrenal cells *in vitro*. Am J Obstet Gynecol 156:1275-1278.

11. **Albrecht ED, Pepe GJ** 1988 Endocrinology of pregnancy. In: Brans YW, Kuelhl TJ (eds) Nonhuman Primates in Perinatal Research. John Wiley & sons, Inc, New York,p13

12. **Albrecht ED, Pepe GJ** 1990 Placental steroid hormone biosynthesis during primate pregnancy. Endocrine Reviews 11:124-150.

13. **Albrecht ED, Townsley JD** 1976 Metabolic clearance rates of progesterone in nonpregnant and pregnant baboons (*Papio papio*) Endocrinology 99:1291-4

14. **Albrecht ED, Townsley JD** 1976 Serum progesterone in the pregnant baboon (*Papio papio*) Biol Reprod 14:610-2

15. **Albrecht ED, Townsley JD** 1978 Serum stradiol in mid and late gestation and estradiol/progesterone ratio in baboons near parturition. Biol Reprod 18:247-50

16. **Albrecth ED, Haskins AL Pepe GJ** 1980 The influence of fetectomy at midgestation upon the serum concentrations of progesterone, estrone, and estradiol in baboons. Endocrinology 107: 766-70

17. **Arnold SF, Obourn JD, Jaffe H, Notides AC** 1995 Phosphorilation of the human estrogen receptor on Tyrosine 537 in vivo and by src family tyrosine kinases in vitro. Mol Endocrinology 9:24-33

18. **Auricchio F, Domenico MD, Migliaccio A, Castoria G, Bilancio A** 1995 The role of estradiol receptor in the proliferative activity of vanadate on MCF-7 cells. Cell Growth and Differenciation 6:105-113

19. **Baggia S, Albrecht ED, Babischkin JS, Pepe GJ** 1990 Interconversion of cortisol and cortisone in baboon trophoblast and decidua cells in culture. Endocrinology 127:1735-1741

20. **Baggia S, Albrecht ED, Pepe SED** 1990 Regulation of 11-β-hydroxysteroid dehydrogenase activity in the baboon placenta by estrogen. Endocrinology 126:2742-2748

21. **Baker PL, Jaffe RB** 1975 The genesis of cell types in the adenohypophysis of the human fetus as observed with immunocytochemistry. Am J Anat 143:137-62

22. **Ballard PL, Gluckman PD, Liggins GC, Kaplan SL, Grumbach MM** 1980 Steroid and growth hormone levels in premature infants after perinatal betamethasone therapy to prevent respiratory distress syndrome. Pediat Res 14:122-27

23. **Ballard PL, Hovey ML, Gonzales LK** 1984 Thyroid hormone stimulation of phophatidylcholine synthesis in cultured fetal rabbit lung. J Clin Invest 74:898-905

24. **Ballard PL, Hovey ML, Gonzales LK** 1984 Thyroid hormone stimulation of phosphatidylcholine synthesis in cultured fetal rabbit lung. J Clin Invest 74:898-905

25. **Bayard F, Damilano S, Robel P, Baulieu EE** 1978 Cytoplasmic and nuclear estradiol and progesterone receptor in human endometrium. J Clin Endocrinol Metab. 46:635-48

26. **Beato M** 1989 Gene regulation by steroid hormones. Cell 56:335-344

27. **Begeot M, Dubois MP, Dubois PM** 1977 Growth hormone and ACTH in the pituitary of normal and anencephalic human fetuses: Immunocytochemical evidence for hypotalamic influences during development. Neuroendocrinology 24:208-20

28. **Beitins IZ, Bayard F, Ances IG, Kowarski A, Migeon CJ** 1973 The metabolic clearance rate, blood production, interconversion, and transplacental passage of cortisol and cortisone in pregnancy near term. Pediatr Res 7:509-519

29. **Beitins IZ, Bayard F, Levitsky L, Ances IG, Kowarski A, Migeon CJ** 1972 Plasma aldosterone concentration at delivery and during the newborn period. J Clin Invest 51:386-94

30. **Benirschke K** 1956 Adrenal in anencephaly and hydrocephaly. Obtet Gynecol 8:412-425

31. **Berdusco ETM, Kaiping Y, Challis JRG, Hammond GL** 1993 Tissue distribution of a_1-proteinase inhibitor messenger ribonucleic acid and its regulation by glucocorticoids in fetal and neonatal sheep. Biology of Reproduction 49:816-821

32. **Berghorn KA, Albrecht ED, Pepe GJ** 1995 Activation of the baboon fetal pituitary-adrenocortical axis at midgestation by estrogen: Responsivity of the fetal adrenal gland to adrenocorticotropic hormone in vitro. Biol Reprod 53:996-1002

33. **Bergman MD, Schachter BS, Karelus K, Combatsiaris EP, Garcia T, Nelson JF** 1992 Upregulation of the uterine estrogen receptor and its messenger ribonucleic

acid during the mouse estrous cycle: The role of estradiol. Endocrinlogy 130:1923-1930

34. **Bianchini RP, Kumar VL, Chambon SP, Senno LD** 1988 Estrogen induced increase of estrogen receptor RNA in human breast cancer line. Biochem Biophys Res Commun 155:943-949

35. **Bigsby RM, Aixin L, Luo K, Cunha GR** 1990 Strain differences in the ontogeny of estrogen receptor in murine uterine epithelium. Endocrinology 126:2592-2596

36. **Billiar RB, Loukides JA, Miller MM** 1992 Evidences for the presence of the estrogen receptor in the ovary of a no-human primate, the baboon (*Papio annubis*). J Clin Endocrinol Metab. 75:1159-1165

37. **Boggaram V, Mendelson CR** 1988 Transcriptional regulation of the gene encoding the major surfactant protein (SP-A) in rabbit fetal lung. J Biol Chem 263:19060-19065

38. **Boggaram V, Smith ME, Mendelson CR** 1989 Regulation of expression of the gene encoding the major surfactant protein (SP-A) in human fetal lung *in vitro*. Disparate effects of glucocorticoids on transcription and on mRNA stability. J Biol Chem 264:11421-11427

39. **Bolte E, Mancuso S, Eriksson G, Wiqvist N, Diczfalusy E** 1964 Aromatisation of C-19 steroids by placentas perfused *in situ*. Acta Endocrinol. 45: 535-59

40. **Boshier DP and Holloway H** 1989 Morphometric analyses of adrenal gland growth in fetal and neonata sheep. I. The adrenal cortex.J Anat 167:1-14

41. **Boshier DP, Holloway H, Liggens GC** 1981 Effects of cortisol and ACTH on adrenal growth and cytodifferenciation in the hypophysectomized fetal sheep. Journal of Developmental Physiology 3:355-373

42. **Braems GA, Matthews SG, Challis JR** 1996 Differential regulation of proopiomelanocortin messenger ribonucleic acid in the pars distalis and pars intermedia of the pituitary gland after prolonged hypoxemia in fetal sheep. Endocrinology 137: 2731-8

43. **Branchaud CL, Goodyer CG, Shore P, Lipowski LS, Lefebre Y** 1985 Functional zonation of the midgestation human fetal adrenal cortex: fetal vesus definitive zone use of progesterone for cortisol synthesis. Am J Obstet Gynecol 151:271

44. **Branchaud CT, Goodyer CG, Hall SGC, Arato JS, Silman RE, Giroud CJP** 1978 Steroidogenic activity of ACTH and related peptides on the human neocortex and fetal adrenal cortex in organ culture. Steroids 31:557-72

45. **Bransome EJ** 1968 Regulation of adrenal growth. Differences in the effects of ACTH in normal and dexamethasone-supressed guinea pigs. Endocrinology 83:956-964

46. **Brenner RM, Resko JA, West NB** 1974 Cyclic changes in oviductal morphology and residual cytoplasmic estradiol binding capacity induced by sequential estradiol-progesterone treatment of spayed rhesus monkeys. Endocrinology 95:1094-1104

47. **Brown MS, Kovanen PT, Goldtein JL** 1979 Receptor-mediated uptake of lipoprotin-cholesterol and its utilization for steroid synthesis in the adrenal cortex. Recent Prog Horm Res. 35:215

48. **Brown RW, Capman KE, Kotelevtsev Y, Yau JLW, Lindsay RS, Brett L, Leckie C, Murad P, Lyons V, Mullins JJ, Edwards CRW, Seckl JR** 1996 Cloning and production of antisera to human placetal 11β-hydroxysteroid dehydrogenase type 2. Biochem J 313:1007-1017

49. **Campen CA, Gorski J** 1986 Anomalous behavior of protein inhibitors on the turnover of the estrogen receptor as measured by density labeling. Endocrinology 119:1454-1461

50. **Carr BR** 1986 Adenylate cyclase activity in membrane fractions of adrenal tissue of human anencephalic fetuses. J Clin Endocrinol Metab 63:51

51. **Carr BR, Ohashi M, MacDonald PC, Simpson ER** 1981 Human anencephalic adrenal tissue: Low density lipoprotein metabolism and cholesterol synthesis. J Clin Endocrinol Metab 53:406

52. **Carr BR, Parker CR, Milewich L, Porter JC, MacDonald PC, Simpson ER** 1980 The role of low density, high density, and very low density lipoproteins in steroidogenesis by the human fetal adrenal gland. Endocrinology 106:1854

53. **Carr BR, Parker CR, Porter JC, MacDonald PC, Simpson ER** 1980 Regulation of steroid secretion by adrenal tissue of a human anencephalic fetus. J Clin Endocrinol Metab 50:870

54. **Carr BR, Porter JC, MacDonald PC, Simpson ER** 1980 Metabolism of low density lipoprotein by human fetal adrenal tissue. Endocrinology 107:1034

55. **Carr BR, Simpson ER** 1981 *De novo* synthesis of cholesterol by the human fetal adrenal gland. Endocrinology 108:2154-61

56. **Carr BR, Simpson ER** 1982 Cholesterol synthesis in human fetal tissues. J Clin Endocrinol Metab 55:447

57. **Casey ML, MacDonald PC** 1988 Decidual activation: the role of protaglandin in labor. In: McNellis D, Challis JRG, MacDonald PC, Nathanielsz PW, Roberts JM

(eds) Reproductive and Perinatal Medicine. The Onset of Labor: Cellular and Integrative Mechanisms. Perinatology Press, New York p141

58. **Challis JR, Lye SJ, Welsh J** 1986 Ovine fetal adrenal maturation at term and during fetal ACTH administration: evidence that the modulting effect of cortisol may involve cAMP. Can J Physiol Pharmacol 64:1085-90

59. **Challis JRG, Brooks AN** 1989 Maturation and activation of hypothalamic-pituitary-adrenal function in fetal sheep. Endocr Rev 10:182-204

60. **Challis JRG, Davis IJ, Benirschke K, Hendrickx AG, Ryan KJ** 1974 The effects of dexamethasone on plasma steroid levels and fetal adrenal histology in the pregnant rhesus monkey. Endocrinology 95:1300

61. **Challis JRG, Huhtanen D, Sprague C, Michell BF, Lye SJ** 1985 Modulation by cortisol of adrenocorticotropin-induced activation of adrenal function in fetal sheep. Endocrinology 116: 2267-2272

62. **Challis JRG, Lye SJ** 1994 Parturition. In: The Physiology of Reproduction. Knobil E, Neill JD. New York: Raven Press pp. 985-1031.

63. **Chang RJ, Buster JE, Blakely JL, Adada DM, Hobel CJ, Abraham GE, Marshall JR** 1976 Silmutaneous comparison of Δ5-3βhydroxysteroid levels in the fetoplacental circulation of normal pregnancy in labor and not in labor. J Clin Endocrinol Metab 42: 744-51

64. **Chint HAJ, Pepe GJ, Chez RA and Townsley JD** 1978 Contribution of dehydroepiandrostenedione and its sulfate to estradiol in baboon pregnancy. Am J Physiol 235:E78-E81

65. **Chu AJ, Rooney SA** 1985 Stimulation of cholinephosphate cytidylyltransferase activity by estrogen in fetal rabbit lung is mediated by phospholipids. Biochim Biophys Acta 834:346-356

66. **Coulter CL, Martin MC, Voytek CC, Hofmann JI, Jaffe RB** 1993 Response to hemorrhagic stress in the rhesus monkey fetus *in utero*: Effects on the pituitary-adrenal axis. J Clin Endocrinol Metab 76:1234-1240.

67. **Coulter CL, Read LC, Carr BR, Tarantal AF, Barry S, Styne DM** 1996 A role for epidermal growth factor in the morphological and functional maturation of the adrenal gland in the fetal rhesus monkey *in vivo*. J Clin Endocrinol Metab 81:1254-1260.

68. **Coulter CL, Young IR, Browne CA, McMillen IC** 1991 Different roles for the pituitary and the adrenal cortex in the control of opiod peptide localization and cortico-medullary interaction in the sheep adrenal gland during development. Neuroendocrinology 53: 281-286

69. **Coulter LC, Goldsmith PC, Mesiano S, Voytek CC, Martin MC, Mason JI** 1996 Functional maturation of the primate fetal adrenal *in vivo*. II. Ontogeny of corticosteroid synthesis is dependent upon specific zonal expression of 3-βHydroxysteroid dehydrogenase/ isomerase. Endocrinology 137:4953-59

70. **Crickard K, III CR, Jaffe RB** 1981 Control of proliferation of human fetal adrenal cells *in vitro*. J Clin Endocrinol Metab 53:790-796

71. **Cunha GR, Chung LWK, Shannon JM, Taguchi O, Fuji H** 1983 Hormone-induced morphogenesis and growth: role of mesenchymal-epithelial interactions. Recent Prog Horm Res 39:559-595

72. **Cuthell WV, Rose JC, Meis** PJ 1990 The effect of adrenocorticotropic hormone infusion on subsequent pituitary response in the sheep fetus. Am J Obstet Gynecol 163:170-174

73. **Dallman MF** 1984-85 Control of adrenocortical growth in vivo. Endocr Res 10:213-42

74. **Davies WA, Berghorn KA, Albrecht ED, Pepe GJ** 1993 Developmental regulation of protein kinase-A and -C activities in the baboon fetal adrenal. Endocrinology 132:2941-2401

75. **Dawood MY, Fruchs F** 1980 Estradiol and progesterone in the maternal and fetal circulation in the baboon. Biol Reprod 22:179-84

76. **Dean DM, Sanders MM** 1996 Ten years after: reclassification of steroid-responsive genes. Molecular Endocrinology 1489-95

77. **Diblasio AM, Fujii D, Yamamoto M, Martin M, Jaffe RB** 1990 Maintenance of cell proliferation and steroidogenesis in cultured human fetal adrenal cells chronically exposed to adrenocorticotropic hormone: rationalization of *in vitro* and *in vivo* findings. Biol Reprod 42:683-691

78. **Diblasio AM, Voutilainen R, Jaffe RB, Miller WL** 1987 Hormonal regulation of messenger ribonucleic acids for P450 (cholesterol side-chain cleavage enzyme) and P450c17 (17α-hydroxylase/17,20-lyase) in cultured human fetal adrenal cells. J Clin Endocrinol Metab 65:170

79. **Diczfalusy E** 1964 Endocrine function of the human fetal placental unit. Fed Proc 23:791

80. **Diczfalusy E** 1974 Endocrine function of the human fetus. Am J Obstet Gynecol 119:419-33

81. **Doody KM, Carr BR, Rainey WE, Byrd W, Murry BA, Strickler RC, Thomas JL, Mason JI** 1990 3β-hydroxysteroid dehydrogenase/ isomerase in the fetal zone and neocortex of the human fetal adrenal gland. Endocrinology 126:2487-2492

82. **Ducsay CA, Hess LH, McClellan MC, Novy MJ** 1991 Endocrine and morphological maturation of the fetal and neonatal adrenal cortex in baboons. J Clin Endocrinol Metab 73:385-95

83. **Ducsay CA, Stanczyk FZ, Novy MJ** 1985 Maternal and fetal production rates of progesterone in rhesus macaques: placental transfer and conversion to cortisol. Endocrinology 117:1253-58

84. **Dupont E, Luu-The F, Labrie F, Pelletier G** 1990 Ontogeny of 3β-hydroxysteroid dehydrogenase/Δ^5-Δ^4 isomerase (3βHSD) in human gland performed by immunocytochemestry. Mol and Cell Endocrinol 74:R7-R10

85. **Easterling WE, Simmer HH, Digman WJ, Frankland MV, Naftolin F** 1966 Neutral C19-steroids and steroid sulfates in human pregnancy. II. Dehydroepiandrosterone sulfate, 16a-hydroxydehydroepiandrosterone sulfate in maternal and fetal blood of pregnancies with anencephalic and normal fetuses. Steroids 8:157

86. **Eisen HJ, Goldfine ID, Glinsmann WH** 1973 Regulation of hepatic glycogen synthesis during fetal development: role of hydrocortisone, insulin, and insulin receptors. Pro Natl Acad Sci USA 70:3454

87. **Farrell PM and Zachman RD** 1973 Induction of choline phosphotransferase and lecithin synthesis in the fetal lung corticosteroids. Science 179: 297-8

88. **Feinberg AP, Vogelstein B** 1983 A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. Anal Biochem 132:6-13.

89. **Flint AP, Anderson AB, Goodson JD, Steele PA, Turnbull AC** 1976 Bilateral adrelactomy of lambs *in utero*: effect on maternal hormone levels at induced parturition J Endocrinol 69:433-44

90. **Freyschuss MO, Staverus-Evers A, Sahlin L, Eruksson H** 1993 Induction of the estrogen receptor growth hormone and glucocorticoid substitution in primary cultures of rat hepatocyte. Endocrinology 133:1548-1554

91. **Fujieda K, Faiman C, Feyes FI, Winter JSD** 1982 The control of steroidogenesis by human fetal adrenal cells in tissue culture. IV. The effects of exposure to placental steroids. J Clin Endocrinol Metab 54:89-94

92. **Furlow JD, Ahrens H, Mueller G, Gorski J** 1990 Antisera to a synthetic peptide recognize native and denatured rat estrogen receptors. Endocrinology 127:1028-1032

93. **Gell JS, Rainey WE, Carr BR** 1997 Effects of estradiol on DHEAS production in the human adrenocortical cell line, H295R. In: Program of the 44th annual meetting of the Society for Gynecologic Investigation; San Diego, California. Abstract 593

94. **Giannopoulos G** 1975 Early events in the action of glucocorticoids in developing tissues. J Steroid Biochem 6:623

95. **Goldzieher JW, Axelrod LR** 1969 Urinary metabolites of 4-14C progesterone in the baboon (*Papio sp.*) Gen Comp Endocrinol 13:201-5

96. **Golos TG, Strauss JF III** 1988 8-Bromo adenosine cyclic 3'5' phosphate rapidly increases 3-hydroxy-3-methylglutaryl coenzyme A reductase mRNA in human granulosa cells: Role of cellular sterol balance in controlling the response to tropic stimulation. Biochemistry 27:3503

97. **Gonzales LW, Ballard PL** 1981 Identification and characterization of nuclear 3, 5, 3'-triiodothyronine-binding sites in fetal human lung. J Clin Endocrinol Metab 53:21-28472.

98. **Gorski J, Sarff M, Clark JH** 1970 Receptors for reproductive hormones. In: O'Malley BW, Means AR (eds) Adv. Exp. Med. Biol. Plenum, New York, p36

99. **Gradien K, Martin B, Ljunggren O, Gustafssin J, Berkenstam A** 1995 Estrogen target tissue determines alternative promoter utilization of the human estrogen receptor gene in osteoblast and tumor cell lines. Endocrinology 136:2223-29

100. **Gray ES, Abramovich DR** 1980 Morphologic features of the anencephalic adrenal gland in early pregnancy. Am J Obstet Gynecol 137:491:95

101. **Greco TL, Furlow DJ, Duello TM, Gorski J** 1991 Immunodetection of estrogen receptor in fetal and neonatal female mouse reproductive tracts. Endocrinology 129:1326-1332

102. **Greene GL, Fitch FW, Jensen EV** 1980 Monoclonal antibodies to estrophilin: Probes for the study of estrogen receptors Proc Nat Acad Sci. USA 77:157-161

103. **Greengard O** 1973 Effects of hormones on development of fetal enzymes. Clin Pharmacol Ther 14:721

104. **Greengard O, Dewey HK** 1970 The premature deposition or lysis of glycogen in livers of fetal rats infected with hydrocortisone or glucagon. Dev Biol 21:452

105. **Gronemeyer H** 1992 Control of transcription activation by steroid hormone receptors. FASEB J 6:2524-2529

106. **Gross I, Ballard PL, Ballard RA, Jones CT, Wilson CM** 1983 Corticosteroid stimulation of phosphatidylcholine synthesis in cultured fetal rabbit lung: evidence for *de novo* protein synthesis mediated by glucocorticoid receptors. Endocrinology 112:829-837.

107. **Gulyas BJ, Hodgen GD, Tullner WW, Ross GT** 1977 Effects of fetal or maternal hypophysectomy on endocrine organs and body weight in infant rhesus monkeys (Macaca mulatta): With particular emphasis on oogenesis. Biol Reprod 16:216

108. **Gwynne JT, Strauss JF III** 1982 The role of lipoproteins in steroidogenesis and cholesterol metabolism in steroidogenic glands. Endocr Rev 3:299

109. **Hayashi K, Sala G, Catt KJ, Dufau ML** 1979 Regulation of steroidogenesis by adrenocorticotropic hormone in isolated adrenal cells. J Biol Chem 254:6678

110. **Hendrickz AG** 1967 The menstrual cycle of the baboon as determined by the vaginal smear, vaginal biopsy and perineal swelling. Eds. Harold Vagtborg. The baboon in medical research Vol 11 p437-59

111. **Hirst JJ, Neal BW, Brenner RM, Novy MJ** 1992 Steroid hormone receptor in the adrenal gland of fetal and adult rhesus monkeys. J Cli Endocrinol Metab 75:308-14

112. **Homes RL** 1968 The adrenal glands of Macaca mulatta, with special reference to the cortico-medullary zone. J Anat 103:4711-77

113. **Hornsby PJ, Gill GN** 1977 Hormonal control of adrenocortical cell proliferation. J Clin Invest 60:342

114. **Hornsby PJ, Gill GN** 1978 Characterization of adult bovine adrenocortical cells throughout their life span in tissue culture. Endocrinology 102:926

115. **Hristic M, Kalafatic D, Plecas, Jovanovic V** 1995 The effect of dexamethasone on the adrenal gland in the fetal and neonatal rats. J Exp Zool 272:281-90

116. **Hsueh AJW, Peck EJ, Clark JH** 1974 Progesterone antagonism of the estrogen receptor and estrogen-induced uterine growth. Nature 254:337-339

117. **Hundertmark S, Ragosch V, Schein B, Buhler H, Lorenz U, Fromm M, Weitzel HK** 1994 Gestational age dependence of 11b-hydroxysteroid dehydrogenase and its relationship to the enzymes of phosphatidylcholine synthesis in lung and liver of fetal rat. Biochim et Biophys Acta 1210:348-354

118. **Jacobs RA, Young IR, Hollingwoth SA, Thorburn GD** 1994 Chronic administration of low doses of adrenocorticotropin to hypophysectomized fetal sheep leads to normal term labor. Endocrinology 134:1389-1394

119. **Jaffe JB, Seron-Ferre M, Huhtaniemi I** 1977 Regulation of the primated fetal adrenal gland and testis *in vitro* and *in vivo*. J Sterod Biochem 8:479-90

120. **Jaffe RB, Mulchahey JJ, Diblasio AM, Martin MC, Blumenfeld Z, Dumesic DA** 1988 Peptide regulation of pituitary and target tissue function and growth in the primate fetus. Recent Pro Horm Res

121. **Jaffe RB, Seron-Ferre M, Crickard K, Koritnik D, Mitchell BF, Huhtaniemi IT** 1981 Regulation and function of the primate fetal adrenal gland and gonad. Rec Prog Hormone Res 37:41-103

122. **Jaffe RB, Seron-Ferre M, Parer JT, Lawrence CC** 1978 The primate fetal pituitary-adrenal axis in the perinatal period. Am J Obstet Gynecol 131:164-170

123. **Jaskoll T, Choy HA, Melnick M** 1996 The glucocorticoid-glucocorticoid receptor signal transduction pathway, transforming growth factor-b, and embrionic mouse lung development *in vivo*. Pediatric Research 39: 749-759

124. **Jefcoate CR, McNamara BC, DeBartolomeis MS** 1986 Control of steroidogenic synthesis in adrenal fasciculata cells. Endocr Res 12:35

125. **Johannisson E** 1979 Aspects of the ultrastructure and function of the human fetal adrenal cortex. Contrib Gynecol Obstet 5:109

126. **John ME, Siimpson ER, Carr BR, Magness RR, Rosenfeld CR, Waterman MR, Mason JI** 1987 Ontogeny of adrenal steroid hydroxylases: evidence for cAMP-independent gene expression. Mol Cell Endocrinol 50:263

127. **Jones CT, Boddy K, Robinson JS** 1977 Changes in the concentration of adrenocorticotropin and corticosteroid in the plasma of foetal sheep in the latter half of pregnancy and during labour. J Endocrinol 72:293

128. **Jones CT, Roebuck MM** 1980 ACTH peptides and development of the fetal adrenal. J Steroid Biochem 12:77

129. **Kahri AI, Halinen H** 1974 Studies on the cortical cells of human fetal adrenals in tissue culture. Acta Anat (Basel) 88:541

130. **Kahri AI, Huhtaniemi I, Salmenpera M** 1976 Steroid formation and differentiation of cortical cells in tissue culture of human fetal adrenals in the presence and absence of ACTH. Endocrinology 98:33-41

131. **Kahri AI, Voutilainen R, Salmenpera M** 1979 Differece biological action of corticosteroids, corticosterone and cortisol, as a base of zonal function of adrenal cortex. Acta Endocrinologica 91:329-337

132. **Kassis JA, Sakai D, Wallent JH, Gorski J** 1984 Primary culture of estrogen-responsive cells from rat uteri: J Endocrinol 121:101-107

133. **Kerr GR, Kennan AL, Waisman HA, Allen JR** 1969 Growth and development of the fetal rhesus monkey. Growth 33:201

134. **King WJ, Greene GL** 1984 Monoclonal antibodies localize oestrogen receptor in the nuclei of target cells. Nature 307:745-747

135. **Kittinger GW** 1974 Feto-maternal production and transfer of cortisol in the rhesus (Macaca Mulatta). Steroids 23:229-243

136. **Kling OR, Rubin EJ Townsley JD** 1972 Estrogen biosynthesis by the pregnant baboon. J Med Primat 1:102-13

137. **Koji T, Brenner RM** 1993 Localization of estrogen receptor messenger ribonucleic acid in Rhesus monkey uterus by no-radioactive in situ hybridization with digoxigenin-labeled oligodeoxynucleotides. Endocrinology 132:382-392

138. **Kojima I, Kojima K, Rasmussen H** 1985 Role of calcium and cAMP in the action of adrenocorticotropin on aldosterone secretion. J Biol Chem 260:4248

139. **Kolanoswski J, Esselinckx W, Nagent de Deuxchaisnes C, Crabbe J** 1977 Adrenocortical response upon repeated stimulation with corticotropin in patients lacking endogenous cortictropin secretion. Acta Endocrinol (Copenh) 85:595-607

140. **Kotas RV, Avery ME** 1971 Accelerated appearance of pulmonary surfactant in the fetal rabbit. J Appl Physiol 30:358-523

141. **Krozowski Z, Maguire JA, Stein-Oakley AN, Dowling J, Smith RE Andrews RK** 1995 Immunohistochemical localization of 11β-hydroxysteroid dehydrogenase type II enzyme in human kidney and placenta. J Clin Endocrinol Metab 80:2203-09

142. **Kuiper GGJM, Enmark E, Pelto-Huikko M, Nilsson S, Gustafsson JA** 1996 Cloning of a novel estrogen receptor expressed in rat prostate and ovary. Proc Natl Acad Sci 93:5925-5938

143. **Lacaze-Masmonteil T, Fraslon C, Bourbon J, Raymondjean M, Kahn A** 1992 Characterization of the rat pulmonary surfactant proetin A promoter. Eur J Biochem 206:613-623

144. **Lachance Y, Luu-The V, Verreault H,, Dumont M, Rheaume E, Leblanc G, Labrie F** 1991: Stuctructure of the human 3β-hydroxysteroid dehydrogenase/Δ^5-Δ^4 isomerase gene, adrenal and gonadal specifity . DNA Cell Biol 10:701-11

145. **Lakshme V, Nath N, Muneyyirci-Delale O** 1993 Characterization of 11β-hydroxysteroid dehydrogenase of human placenta: evidence for the existence of two species of 11b-hydroxysteroid dehydrogenase. Biochem Mol Biol 45:391- 97

146. **Landel CC, Kushner PJ, Greene GL** 1994 The interaction of human estrogen receptor with DNA is modulated by receptor-associated proteins. Molecular endocrinology 8:1407-19

147. **Lanman JT** 1953 fetal zone of the adrenal gland. Its development course, comparative anatomy and possible physiological functions. In: Talbot JH (ed) Medicine. Williams & Wilkins, Baltimere, vol 32:389-430

148. **Laurence MC, Murray BA, Truant DIM Mason GY** 1990 Human 3β-hydroxysteroid dehydrogenase/Δ^5-Δ^4 isomerase from placenta: Expression in nosteroidogenic cells of a protein that catalyzes the dehydrogenation/isomerization of C21 and C19 steroids. Endocrinology 126:2493

149. **Leavitt MG, Aberdeen GW, Burch MG, Albrecht ED, Pepe GJ** 1997 Inhibition of fetal adrenal ACTH receptor messenger ribonucleic acid expression by betamethasone administration to the baboon fetus in late gestation. Endocrinology 138:2705-2711

150. **Lebrethon MC, Jaillard C, Naville D, Begeot M, Saez JM** 1994 Regulation of corticotropin and steroidogenic enzymes mRNAs in human and fetal adrenal cells by corticotropin, angiotensin-II and transforming growth factor b1. Mol Cell Endocrinl 106:137-143

151. **Lebrethon MC, Naville D, Begeot M, Saez JM** 1994 Regulation of corticotropin receptor number and messenger RNA in cultured human adrenocortical cells by corticotropin and angiotensin II. J Clin Invest 93:1828-1833.

152. **Lee GC, Yang IM, Kim BJ, Woo JT, Kim SW, Kim TS, Choi YK** 1996 Identification of glucocorticoid response element of the rat TRH gene. Korean J Intern Med 11:138-144

153. **Liggins GC** 1969 Premature delivery of foetal lambs infused with glucocorticoids. J Endocrinol 45:515-523

154. **Liggins GC** 1976 Adrenocortical-related maturational events in the fetus. Am J Obstet Gynecol 126:931

155. **Liggins GC** 1994 The role of cortisol in preparing the fetus for birth. Reprod Fertil Dev 6:141-50

156. **Liggins GC, Fairclough RJ, Grieves SA, Forster CS, Knox BS** 1977 Parturition in the sheep. In: Knight J, O'Connor M (eds) The Fetus and Birth. Ciba Foundation Symposium 47, Elsevier, Amsterdam, p 5

157. **Liggins GC, Fairclough RJ, Grimes SA, Kendall JZ, Knox BS** 1973 The mechanism of initiation of parturition on the ewe. Recent Pro Horm Res 29:111

158. **Liggins GC, Foster CS, Grieves SA, Schwartz AL** 1977 Control of parturition in man. Biol Reprod 16:39

159. **Lins DS, Pitkin RM, Connor WE** 1977 Placental transfer of cholesterol into the human fetus. Am J Obstet Gynecol 128:735

160. **Linstedt AD, West NB; Brenner RM** 1986 Analysis of monomeric-dimeric states of the estrogen receptor with monoclonal antiestrophilins. J Steroid Biochem 24:677-86

161. **Loose DS, Do YS, Chen TL, Feldman D** 1980 Demonstration of glucocorticoid receptor in the adrenal cortex: evidence for a direct dexamethasone supressive effect on the rat adrenal gland. Endocrinology 107:137

162. **Lopez-Bernal A, Anderson ABM, Turnbull AC** 1982 Cortisol:cortisone interconversion by human decidua in relation to parturition: Effects of tissue manipulation on 11b-hydroxysteroid dehydrogenase activity. J Endocrinol 93:141-9

163. **Lorence MC, Corbin CJ, Kaminmura N, Mahendrou MS, Mason JI** 1990 Structural analysis of the gene encoding human 3β-hydroxysteroid dehydrogenase/Δ^5-Δ^4 isomerase. Mole Endocrinol 4:1850

164. **Lund J, Faucher DJ, Ford SP, Porter JC, Waterman MR, Mason JI** 1988 Developmental expression of bovine adrenocortical streroid hydroxylasees. Regulation of P-450$_\alpha$ expression leads to episodic fetal cortisol porduction. J Biol Chem 263:16195

165. **Malendowicz L, Nussdorfer GG, Markowska A, Nowak KW** 1992 Analysis of the preventive action of ACTH on dexamethasone-induced adrenocortical atrophy in the rat Cytobios 71:191-199

166. **Malet C, Gompel HY, Cren H, Fidji N, Mowszowicz I** 1991 Estradiol and progesterone receptors in cultured normal human breast epithelial cells and fibroblats: Immunocytochemical studies. J Clin Endocrinol Metab 73:8-17

167. **Mason JI, Bird IM, Rainey WE** 1995 Adrenal androgen biosynthesis with special attention to P450c17. Ann NY Acad Sci 774:47-58

168. **Mason JI, France JT, Magness RR, Murry BA, Rosenfeld CR** 1989 Ovine placental steroid 17 alpha-hydroxylase/C-17,20-lyase, aromatase and sulphatase in dexamethasone-induced and natural parturition. J Endocrinol 122:351-9

169. **Mason JI, Murray BA, Staten JR** 1983 Pregnenolone and pregnenolone sulfate metabolism in human fetal adrenal. In: Program for the 30th Annual Meeting of the Society for Gynecological Investigation, p 137 (Abstract).

170. **Mason JI, Rainey WE** 1987 Steroidogenesis in the human fetal adrenal: A role for cholesterol synthesized de novo. J Clin Endocrinol Metab 64:140

171. **Mason JI, Ushijima K, Doody KM, Nagai K, Naville D, Head JR, Milewich L, Rainey WE, Ralph MM** 1993 Regulation of expression of the 3β-Hydroxy dehydrogenases of human placenta and fetal adrenal J Sterod Biochem Mol Biol 47:151-9

172. **Matthews SG, Challis JR** 1995 Levels of pro-opiomelanocortin and prolactin mRNA in the fetal sheep pituitary following hypoxaemia and glucocorticoid treatment in late gestation. J Endocrinol 147: 139-46

173. **McAllister JM, Hornsby PJ** 1988 Dual regulation of 3β-hydroxysteroid dehydrogenase, 17α-hydroxylase, and dehydroepiandrosterone sulfotransferase by adenosine 3',5'-monophosphate and activators of protein kinase C in cultured human adrenocortical cells. Endocrinology 122:2012-2018.

174. **McClellan M, Brenner RM** 1981 Development of the fetal adrenals in nonhuman primates: electron microscopy. In: Novy MJ, Resko JA (eds) Fetal Endocrinology. Academy press, New York, p383

175. **Mcclellan MC, West NB, Tacha DE, Greene GL, Brenner RM** 1984 Immunocytochemical localization of estrogen receptors in the macaque reproductive tract with monoclonal antiestrophillins. Endocrinology 114: 2002-14

176. **McNulty WP, Miles JN, Walsh SW** 1981 Fetal and postnatal development of the adrenal glands in Macaca Mulatta. Biology of Reproduction 25:1079-1089

177. **McNulty WP, Wals SW, Novy MJ** 1981 Fetal and postnatal development of the adrenal gland in Macaca mulatta. Biol Reprod 25:1079

178. **Meisfeld R** 1989 The structure and function of steroid receptor proteins. Crit Rev Biochem Mol Biol 24:101-117

179. **Mendelson CR, Boggaram V** 1991 Hormonal control of the surfactant system in the fetal lung. Annu Rev Physiol 53: 415-440

180. **Merkatz IR , Beling CG** 1969 Urinary excretion of oestrogen and pregnanediol in the pregnant baboon. J Rep Fert Suppl 6:129-33

181. **Mesiano S, Coulter CL, Jaffe RB** 1993 Localization of cytochrome P450 cholesterol side-chain cleavage, cytochrome P450 17α-hydroxylase/17,20-lyase, and 3β-hydroxysteroid dehydrogenase isomerase steroidogenic enzymes in human and

rhesus monkey fetal adrenal glands: reappraisal of functional zonation. J Clin Endocrinol Metab 77:1184-1189

182. **Mesiano S, Fujimoto VY, Nelson LR, Lee JY, Voytek CC, Jaffe RB** 1996 Localization and regulation of corticotropin receptor expression in the midgestation human fetal adrenal cortex:implications for *in utero* homeostasis. J Cli Endocrinol Metab 81:340-45

183. **Mesiano S, Jaffe RB** 1993 Interaction of insulin-like growth factor-II and estradiol directs steroidogenesis in the human fetal adrenal toward dehydroepiandrosterone sulfate production. J Clin Endocrinol Metab 77:754-758

184. **Mesiano S, Mellon SH, Jaffe RB** 1993 Mitogenic action, regulation and localization of insulin-like growth factors in the human fetal adrenal gland. J Clin Endocrinol Metab. 76:968-976

185. **Meyer TE, Gerard W, Julia L, Beckmann W, Habeners JF** 1993 The promoter of the gene encoding 3',5'-cyclic adenosine monophosphate (cAMP) response element binding protein contains cAMP response elements: evidence for positive autoregulation of gene transcription. Endocrinology 132:770-780

186. **Meyer TE, Habener JF** 1993 Cyclic adenosine 3',5'-monophosphate response element binding protein (CREB) and related transcription-activating deoxyribonucleic acid-binding proteins Endoc Rev 14:269-290

187. **Michell BF, Seron-Ferre M, Hess DL, Jaffe RB** 1981 Cortisol production and metabolism in the late gestation rhesus monkey fetus. Endocrinology 108:916-24

188. **Michell BF, Seron-Ferre M, Jaffe RB** 1982 Cortisol-cortisone interrelationship in the late gestation rhesus monkey fetus *in utero*. Endocrinology 11:1837-1842

189. **Migeon CJ, Bertrand J, Wall PE** 1957 Physiologic disposition of 4 ^{14}C cortisol during late pregnancy. J Clin Invest 36: 1350-1362

190. **Miller WL** 1988 Molecular biology of steroid hormone synthesis. Endocrine Reviews 9:295.

191. **Miller WL and Tyrrell JB** 1995 The adrenal cortex. In: Jeffers JD, Englis MR (eds) Endocrinology and Metabolism. Atlis Graphics & Design, Inc. Century Schoolbook, p555

192. **Miller WL, Levine LS** 1987 Molecular and clinical advances in congenital adrenal hyperplasia. J Pediatr 111:1

193. **Moog F** 1971 Corticoids and the enzymatic maturation of the intestinal surface: alkaline phosphatase, leucyl naphthylamidase and sucrase. In: Hamburg M, Barrington EJ (eds) Hormones in Development, ed 1. Appletn-Century-Crofts, Inc., New York, p143

194. **Moscona AA, Piddington R** 1966 Stimulation by hydrocortisone of premature changes in the developmental pattern of glutamine synthetase in embryonic retina. Biochem Biophys Acta 121:409

195. **Mosselman S, Polman J, Dijkema R** 1966 ERb: identification and characterization of a novel human estrogen receptor. FEBS Letters 392:49-53

196. **Mountjoy KG, Bird IM, Rainey WE, Cone RD** 1994 ACTH induces up-regulation of ACTH receptor mRNA in mouse and human adrenocortical cell lines. Mol Cell Endocrinol 99:R17-R20.

197. **Muller-Huebach E, Myers RE, Adamsons K** 1972 Effects of adrenalectomy on pregnancy length in the rhesus monkey. Am J Obstet Gynecol 112:221

198. **Murdoch FE, Byrne LM, Arizi EA, Furlow JD, Meier DA, Gorski J** 1995 Estrogen receptor binding to DNA: affinity for nopalindromic elements from the rat prolactin gene. Biochemistry 34:9144-50

199. **Murphy BEP** 1982 Human fetal serum cortisol levels related to gestational age:evidence of a midgestational fall and a steep late gestational rise,independent of sex or mode of delivery. Am J Obstet Gynecol 144:276-282

200. **Murphy BEP, Diez d'Aux RC** 1972 Steroid levels in the human fetus: Cortisol and cortisone. J Clin Endocrinol Metab 35:678-683.

201. **Neville AM O'Hare MH** 1982 Origin and development of the adrenal gland. In: The Human Adrenal Cortex. Springer-Verlag, Berlin, p12

202. **New MI, White P, Pang S, Dupont B Speiser P** 1981 The adrenal hyperplasias, in Scriver C, Beaudet A, Sly W, Valle D (eds):The Metabolic Basis of Inherited Disease. New York,McGraw-Hill, p1881

203. **Nicholson WE, Liddle RA, Puett D, Liddle GW** 1978 Adrenocorticotropic hormone biotransformation, clearance and metabolism. Endocrinology 103:1344

204. **NIH Consensus Conference.** Effect of corticosteroids for fetal maturation on perinatal outcomes. 1995 JAMA 273:413-18

205. **Novy MJ, Walsh SW, Kittinger GW** 1977 Experimental fetal anencephaly in the rhesus monkey: effect on gestational length and fetal and maternal plasma steroids. J Clin Endcrinol Metab 45:1021

206. **Oakey RE 1970** The progressive increase in oestrogen production in human pregnancy: an appraisal of the factors responsible. Vitam Horm 28:1

207. **Oakey RE** 1975 Serum cortisol binding capacity and cortisol concentration in the pregnant baboon and its fetus during gestation. Endocrinology 97: 1024-9

208. **Ohashi M, Carr BR, Simpson ER** 1981 Binding of high density lipoprotein to human fetal adrenal membrane fractions. Endocrinology 109:783.

209. **Ohashi M, Carr BR, Simpson ER** 1981 Effects of adrenocorticotropic hormone on low density lipoprotein receptors of human fetal adrenal tissue. Endocrinology 108:1237

210. **Okulicz WC** 1989 Temporal effects of progesterone inhibition of occupied nuclear estrogen receptor retention in rat uterus. J Endocrinol 121:101-7

211. **Parker CR Jr, Faye-Petersen O, Stankovic AK, Mason JI, Grizzle WE** 1995 Immunohistochemical evaluation of the cellular localization and ontogeny of 3β-hydroxy steroid dehydrogenase/Δ5-4 isomerase in the human fetal adrenal gland Endocr Res 21: 69-80

212. **Parker Jr CR, Simpson ER, Bilheimer DW, Leveno K, Carr BR, MacDonald PC** 1980 Inverse relation between LDL-cholesterol and dehydroepiandrosterone sulfate in human fetal plasma. Science 208:512

213. **Parker MG** 1993 Steroid and related receptors. Curr Opin Cell Biol 5:499-504

214. **Penhoat A, Jaillard C, Saez JM** 1989 Synergistic effects of corticotropin and insulin-like growth factor I on corticotropin receptors and corticotropin responsiveness in cultured bovine adrenocortical cells. Biochem Biophys Res Comm 165:355-359.

215. **Penhoat A, Jaillard C, Saez JM** 1994 Regulation of bovine adrenal cell corticotropin receptor mRNA levels by corticotropin (ACTH) and angiotensin-II (A-II). Mol Cell Endocrinol 103:R7-R10.

216. **Pepe ED, Waddell BJ, Albrecht ED** 1989 Effect of estrogen on pituitary peptide-induced dehydroepiandrosterone secretion in the baboon fetus at midgestation. Endocrinology 125:1519-1524

217. **Pepe GJ and Albrecht ED** 1985 Prolactin stimulates adrenal androgen secretion in infant baboons. Endocrinology 177:1968-73

218. **Pepe GJ and Albrecht ED** 1985 Regulation of baboon fetal adrenal androgen production by adrenocorticotropin, prolactin, and growth hormone. Biol Reprod 33:545-50

219. **Pepe GJ, Albrecht ED** 1980 The utilization of placental substrates for cortisol synthesis by the baboon fetus near term. Steroids 35: 391-95

220. **Pepe GJ, Albrecht ED** 1980 The utilization of placental substrates for cortisol synthesis by the baboon fetus near term. Steroids 35:591.

221. **Pepe GJ, Albrecht ED** 1984 Transuteroplacental metabolism of cortisol and cortisone during mid and late gestation in the baboon Endocrinology 115:1946-1951

222. **Pepe GJ, Albrecht ED** 1985 Comparison of cortisol-cortisone interconversion in vitro by the human and baboon placenta. Steroids 44:229-240

223. **Pepe GJ, Albrecht ED** 1987 Fetal regulation of transplacental cortisol-cortisone metabolism in the baboon. Endocrinology 120: 2529-33

224. **Pepe GJ, Albrecht ED** 1990 Activation of the baboon fetal hypothalamic-pituitary-adrenocortical axis at midgestation by estrogen:adrenal Δ5-3β-hydroxysteroid

dehydrogenase and 17α- Hydroxylase-17,20-lyase activity. Endocrinology 128:2395-2401

225. **Pepe GJ, Albrecht ED** 1990 Regulation of the primate fetal adrenal cortex. Endocr Rev 11:151-176

226. **Pepe GJ, Brendan WJ, Albrecht ED** 1989 The effects of adreno corticotropin and prolactin on adrenal dehydroepiandrosterone secretion in the baboon fetus. Endocrinology 122:646-650

227. **Pepe GJ, Davies WA, Albrecht ED** 1994 Activation of the baboon fetal pituitary-adrenocortical axis at midgestation by estrogen: enhancement of fetal pituitary proopiomelanocortin messenger ribonucleic acid expression. Endocrinology 135:2581-2587

228. **Pepe GJ, Ehrenkranz RA, Townsley JD** 1976 The metabolic clearance rates and interconversion of cortisol and cortisone in pregnant and nonpregnant baboons. Endocrinology 99:597-601

229. **Pepe GJ, Jeffrey SB, Babischkin JS, Burch M, Leavitt M, Albrecht ED** 1996 Developmental increase in expression of the messenger ribonucleic acid and protein levels of 11b-hydroxysteroid dehydrogenase type 1 and type 2 in the baboon placenta. J Cli Endocrinol Metab 46:834-37

230. **Pepe GJ, Titus JA, Townsley JD** 1977 Increasing fetal adrenal formation of cortisol from pregnenolone during baboon (Papio papio) gestation. Bio Repro 17:701

231. **Pepe GJ, Townsley JD** 1975 Cortisol metabolism in baboon during pregnancy and postpartum period. Endocrinology 96: 587-90

232. **Pepe GJ, Townsley JD** 1976 The metabolism of cortisol by term baboon neonates (*Papio papio*). Endocrinology 99: 466-9

233. **Pepe GJ, Townsley JD** 1978 Cortisol metabolism in female baboons (*Papio papio*) Endocrinolgy 95: 1658-63

234. **Pepe GJ, Waddell BJ, Albrecht ED** 1989 Effect of estrogen on pituitary peptide-induced dehydroepiandrosterone secretion in the baboon fetus at midgestation. Endocrinology 125:1519-1524

235. **Pepe GJ, Waddell BJ, Albrecht ED** 1990 Activation of the baboon fetal hypothalamic-pituitary-adrenocortical axis at midgestation by estrogen-induced changes in placental corticosteroid metabolism. Endocrinology 127:3117-3123

236. **Pepe GJ, Waddell BJ, Burch MG, Albrecht ED** 1996 Interconversion of cortisol and cortisone in the baboon placenta at midgestation:expresion of 11b-hydroxysteroid dehydrogenase type 1 messenger RNA. J Steroid Biochem Mol Biol 58:403-10

237. **Pepe GJ, Waddell BJ, Sahl SJ, Albrecht ED** 1988 The regulation of transplacental cortisol-cortisone metabolism by estrogen in pregnant baboon Endocrinology 122:78-83

238. **Piddington R, Moscona AA** 1967 Precocious induction of retinal glutamine synthetase by hydrocortisone in the embryo and in culture. Age-dependent differences in tissue response Biochim Biophys Acta 141:429

239. **Press MF, Nousek-Goebl NA, Bur M, Greene GL** 1986 Estrogen receptor localization in the female genital tract. Am J Pathol 123:280-292

240. **Raeymaekers L** 1993 Quantitative PCR: Theoretical consideration with practical implications. Anal Biochem 214:582-585

241. **Rainey WE, Bird IM, Mason JI, Bruce RC** 1992 Angiotensin II receptors on human fetal adrenal cells. Am J Obstet Gynecol 167:1679:85

242. **Rainey WE, Shay JW, Mason JI** 1986 ACTH induction of 3-hydroxy-3-methylglutaryl coenzyme A reductase, cholesterol biosynthesis and steroidogenesis in primary cultures of bovine adrenocortical cells. J Biol Chem 261:7322

243. **Rainey WE, Viard I, Saez JM** 1989 Transforming growth factor β treatment decreases ACTH receptors on ovine adrenocortical cells. J Biol Chem 264:21474-21477.

244. **Read LD, Greene GL, Katzenellenbogen BS** 1989 Regulation of estrogen receptor messenger ribonucleic acid and protein levels in human breast cancer cell lines by sex steroid hormone, their antagonists and growth factors. Mo Endocrinol 3:295-304

245. **Red LD, Greene GL, Katzenellenbogen BS** 1989 Regulation of estrogen receptor messenger ribonucleic acid and protein in levels in human breast cancer cell lines by sex steroid hormones, their antagonists, and growth factors. Mol Endocrinol 3:295-304

246. **Rheaume E, Lachance Y, Zhao H, Boeton N, Dumont M, DeLaunoit Y, Trudel C, Luu- V** 1991 Structure and expression of a new cDNA encoding the major 3β-hydroxysteroid dehydrogenase/Δ^5-Δ^4 isomerase present in human adrenals and gonads. Mol Endocrinol 5:1147

247. **Robinson PM, Comline RS, Fowden AL, Silver M** 1983 Adrenal cortex of fetal lamb: changes after hypophysectomy and effects of synacthen on cytoarchitecture and secretory activity. Q J Exp Physiol 68:15

248. **Rooney SA, Gobran LI, Marino PA, Maniscalco WM, Gross I** 1979 Effects of betamethasone on phospholipid content, composition and biosynthesis in the fetal rabbit lung. Biochim Biophys Acta 572:64-76

249. **Ruegg JC, Beinbrech B, Wiesmer RT** 1993 Quantitative PCR. Nature 366:416

250. **Saceda M, Lippman ME, Chambon P, Lindsey RL, Ponglikimongkol M, Puente M, Martin MB** 1988 Regulation of the estrogen receptor in MCF-7 cells by estradiol. Mol Endocrinol 1:1157-1162

251. **Saenger P** 1984 Abnormal sex differentiation. J Pediatr 104:1

252. **Saez JM, Durand P, Cathiard AM** 1984 Ontogeny of the ACTH receptor, adenylate cyclase and steroidogenesis in adrenal. Mol Cell Endocrinol 38:93

253. **Saez JM, Morera AM, Gallet D** 1977 Opposite effects of ACTH and glucocorticoids on adrenal DNA synthesis *in vivo*. Endocrinology 100:1268

254. **Saito E, Mukai M, Muraki T, Ichikawa Y, Homma M** 1979 Inhibirory effects of corticosterone on cell porliferation and steroidogenesis in the mouse adrenal tumor cell line Y-1. Endocrinology 104:487

255. **Seron-Ferre M, Jaffe RB** 1981 The fetal adrenal gland. Ann Rev Physiol 43:141-62

256. **Seron-Ferre M, Lawrence CC, Jaffe RB** 1978 Role of hCG in regulation of the fetal zone of the human fetal adrenal gland. J Clin Endocrinol Metab 46:834-37

257. **Seron-Ferre M, Taylor NF, Rotten D, Koritnik DR, Jaffe RB** 1983 Changes in fetal monkey plasma dehydroepiandrosterone sulfate: relationship to gestational age, adrenal weight and preterm delivery. J Clin Endocrinol Metab 57:1173

258. **Sharma A, Gonzales LW, Ballard PL** 1993 Hormonal regulation of cholinephosphate cytidylyltransferase in human fetal lung. Biochem et Biophys Acta 1170:237-244432.

259. **Sholl SA** 1981 17-Hydroxyprogesterone metabolism in the monkey fetal adrenal:C 17-20 lyase and 21-hydroxylase activities. Steroids 38:221

260. **Sholl SA** 1982 Corticosteroid formation by the monkey fetal adrenal: evaluation of 17-, 21- and 11-hydroxylase activities. Steroids 40:475

261. **Sholl SA** 1983 Patterns of 3β-hydroxysteroid dehydrogenase Δ^{5-4} isomerase activity in the rhesus monkey placenta and fetal adrenal. Steroids 41:769

262. **Shupnik MA, Gordon MS, Chin WW** 1989 Tissue-specific regulation of rat estrogen receptor mRNAs. Molecular Endocrinology 3:660-65

263. **Shutt DA, Smith IA, Shearman RP** 1974 Oestrone, oestradiol 17β and oestriol levels in human foetal plasma during gestation and at term. J Endocrinol 60:247

264. **Shyamala G, Gorski J** 1969 Estrogen receptor in the rat uterus: studies on the interaction of cytosol and nuclear binding sites. J Bio Chem 244:1097-1103

265. **Siiteri PK, MacDonald PC** 1966 Placental estrogen biosynthesis during human pregnancy. J Clin Endocrinol Metab 26:751-61

266. **Siler-Khodr TM, Morgenstern LL, Greenwood FC** 1974 Hormone synthesis and release from human fetal adenohypophyses *in vitro.* J Clin Endocrinol Metab 39:391-905

267. **Silman RE, Chard T, a PJ, Smith I, Young IM** 1976 Human pituitary peptides and parturition. Nature 260:716-18

268. **Simmer HH, Easterling WE, Pion RJ, Dignam WJ** 1964 Neutral C-19-steroids and steroid sulphates in human pregnancy. Steroids 4:125-34

269. **Simmer HH, Tulchinsky D, Gold EM, Frankland M, Greipel M, Gold AS** 1974 On the regulation of estrogen production by cortisol and ACTH in human pregnancy at term. Am J Obstet Gynecol 119: 283-97

270. **Simonian MH, Gill GN** 1981 Regulation of the fetal adrenal cortex: effect of adenocorticotropin on growth and function of monolayer cultures of fetal and definitive zone cells. Endocrinology 108:1769-1779

271. **Simpson ER, Carr BR, John ME, Parker Jr CR, Zuber MX, Okamura T, Waterman MR, Mason JI** 1985 Cholesterol metabolism in the adrenals of normal and anencephalic human fetuses. In: Albrecht ED, Pepe GJ (eds) Research In Perinatal Medicine (IV), Perinatal Endocrinology, Perinatology Press, NY, p 161

272. **Simpson ER, Carr BR, Parker Jr CR, Milewich L, Porter JC, MacDonald PC** 1979 The role of serum lipoproteins in steroidogenesis by the human fetal adrenal cortex. J Clin Endocrinol Metab 49:146

273. **Simpson ER, Parker CR, Carr BR** 1985 Role of lipoproteins in the regulation of steroidogenesis by the human fetal adrenal. In: Jaffe RB, Dell'Acqua S (eds) The Endocrine Physiology of Pregnancy and the Peripartal Period. Serono Symposia Publications, Raven Press, NY, Vol 21:7

274. **Simpson ER, Waterman MR** 1988 Regulation of the synthesis of steroidogenic enzymes in adrenal cortical cells by ACTH. Ann Rev Physiol 50:427-440.

275. **Sippell WG, Muller-Holve W, Dorr HG, Bidlingmaier F, Knorr D** 1981 Concentrations of aldosterone, corticosterone, 11-deoxy-corticosterone, progesterone, 17-hydroxy-progesterone, 11-deoxy-cortisol, cortisol, and cortisone determined simultaneously in human amniotic fluid throughout gestation. J Clin Endocrinol Metab 52:385

276. **Skipper JK, Young LJ, Bergeron JM, Tetzlaff MT, Osborn CT, Crews D** 1993 Identification of an isoform of the estrogen receptor messenger RNA lacking exon four and present in the brain.Proc. Natl Acad Sci USA 90:7172-75

277. **Slayden OD, Hirst JJ, Brenner RM** 1993 Estrogen action in the reproductive tract of rhesus monkeys during antiprostin treatment. Endocrinology 132:1845-56

278. **Smith ID, Shearman RP** 1974 Fetal plasma steroids in relation to parturition. I. The effect of gestational age upon umbilical plasma corticosteroid levels following vaginal delivery. J Obstet Gynecol Br Comm 81:11-15.

279. **Solomon S, Bird CE, Ling W, Iwamiya M, Young PCM** 1967 Formation and metabolism of steroids in the fetus and placenta. Recent Pro Horm Res 23:297

280. **Stewart and Mason JI** 1995 Cortisol to cortisone: glucocorticoid to mineralocorticoid. Steroids 60:143-6

281. **Stewart PM, Murry BA, Mason JI** 1994 Human kidney 11β-hydroxysteroid dehydrogenase is a high affinity nicotinamide adenine dinucleotide-dependent enzyme and differs from the cloned type I isoform. J Cli Endocrinol Metab 80:480-484

282. **Stewart PM, Rogerson FM, Mason JI** 1995 Type 2 11β-hydroxysteroid dehydrogenase messenger ribonucleic acid and activity in human placenta and fetal

membranes: its relationship to birth weight and putative role in fetal adrenal steroidogenesis. J Clin Endocrinl Metab 80:885-890

283. **Stewart PM, Whorwood CB, Mason JI** 1995 Type 2 11β-hydroxysteroid dehydrogenase in foetal and adult life. J Steroid Biochem Mol Biol 55:465-71

284. **Stewart PM; Murry BA, Mason JI** 1994 11β-hydroxysteroid dehydrogenase in human fetal tissues. J Clin Endocrinl Metab 78:1529-32

285. **Strauss JF III, Miller WL** 1991 Molecular basis of ovarian steroid synthesis.In Hiller SG (ed): Ovarian Endocrinology. Oxford, UK, Blackwell, 1991,p25

286. **Sucheston ME, Cannon MS** 1968 Nomenclature of a developmental zone of the adrenal cortex of mammals. General and Comparative Endocrinology 11: 603

287. **Sucheston ME, Cannon MS** 1969 Microscopic comparison of the normal and anencephalic human adrenal gland with emphasis on the transient-zone. Obstetrics and Gynecology 35:544-52

288. **Sucheston ME, Cannon MS** Development of zonular pattern in the human adrenal gland. J Morphol 126:477-92

289. **Sun K, Yang K, Matthews SG, Challis JRG** 1996 Localization of 11β-hydroxysteroid dehydrogenase activity and synthesis in human placenta and fetal membrane. J Soc Gynecol Invest 3:47

290. **Sun M, Ramirez M, Challis JRG, Gibb W** 1996 Immunohistochemical localization of the glucocorticoid receptor in human fetal membranes and decidua at term and preterm delivery. Journal of Endocrinology 149:243-248

291. **Swinyard CA** 1941 Growth of the human adrenal gland. J Morphol 126:477-92

292. **Tangalakis K, Coghlan JP, Crawford R, Hammond VE, Wintour EM** 1990 Steroid hydroxylase gene expression in the ovine fetal adrenal gland following ACTH infusion. Acta Endocrinol (Copenh) 123:371-377.

293. **Thomas JL, Myers RP, Stricler RC** 1989 Human placental 3β-hydroxy-e-ene steroid dehydrogenase and steroid-5-4-ene isomerase: purification from mitochondria and kinetic profiles, biophysical characterization of the purified mitochondrial and microsomal enzymes. J Steroid Biochem 33:209

294. **Townsley JD** 1976 Utilization of dehydroepiandrosterone and its sulphate for oestrogen production by pregnant baboons (Papio papio). Acta Endocrinol (Copenh) [Suppl] 166:191-197

295. **Townsley JD and Pepe GJ** 1977 Serum dehydroepiandrosterone and dehydroepiandrosterone sulfate in the baboon (Papio papio) pregnancy. Acta Endocrinol 85:415-21

296. **Tseng L, Gurpide E, Gusberg SB** 1977 Estradiol receptor and 17β-hydroxysteroid dehydrogenase in normal and abnormal human endometrium. Ann NY Acad Sci 286:190-198

297. **Tulchinsky D, Hobel CJ, Yeager E, Marshall JR** 1982 Plasma estrone, estradiol, estriol, progesterone and 17α-hydroxyprogesterone in human pregnancy. Am J Obstet Gynecol 112:1095

298. **Turnbull AC, Anderson ABM** 1984 Endocrine control of human parturition. Acta Obst Gynec Jpn 34:1094-6

299. **Ulisse S, Tata JR** 1994 Thyroid hormone and glucocorticoid independently regulate the expression of estrogen receptor in male Xenopus liver cells. Molecular and Cellular Endocrinology 105:45-53

300. **Voulatien R, Miller WL** 1988 Developmental and hormonal regulation of mRNA for insulin-like growth factor II and steroidogenic enzymes in human fetal adrenals and gonads. DNA 7:9-15

301. **Voutilainen R, Kahri AI, Salmenpera M** 1979 The effects of progestrrone, pregnenolone, estriol, ACTH and hCG on steroid secretion of cultured human fetal adrenals. J Steroid Biochem 10:695

302. **Voutilainen R, Miller WL** 1986 Developmental expression of genes for the steroidogenic enzymes P450scc (20,22-desmolase), P450c17 (17α-hydroxylase/17,20-Lyase), and P450c21(21-Hydroxylase) in the human fetus. J Clin Endocrinol Metab 63:1145

303. **Walker ML, Pepe GJ, Garnett NL, Albrecht ED** 1987 Effects of anesthetic agents on the adrenocortical system of female baboons. Am J Primatol 13:325-32

304. **Walker ML, Pepe GJ, Albrecht ED** 1988 Regulation of baboon fetal adrenal androgen formation by pituitary peptides at mid and late gestation. Endocrinology 122:546-551.

305. **Walker WH, Girardet C, Habener J** 1996 Alternative exon splicing controls a translational switch from activator to repressor isoforms of transcription factor CREB during spermatogenesis. J Biol Chem 271:20145-21050

306. **Walsh SW, Norman RL, Novy MJ** 1979 *In utero* regulation of rhesus monkey fetal adrenals : effects of dexamethasone, adrenocoticotropin, thyrotropin releasing

hormone, prolactin, human chorionic gonadotropin and a-melanocyte strimulating hormone on fetal and maternal plasma steroids. Endocrinology 104: 1805-13

307. **Walters MR, Clark JH** 1979 Relationship between the quantity of progesterone receptor and the antagonism of estrogen induced uterotropic response. Endocrinology 105:382-386

308. **Weaver TE and Whitsett JA** 1991 Function and regulation of expression of pulmonary surfactant-associated proteins. Biochem J 273:249-264

309. **Welshons WV, Lieberman ME, Gorski J** 1984 Nuclear localization of unoccupied oestrogen receptor. Nature 307:745-747

310. **West NB, McClellan MC, Sternfeld MD, Brenner RM** 1987 Immunocytochemestry versus binding assays of the estrogen receptor in the reproductive tract ot spayed and hormone-treated macaques. Endocrinology 121:1789-800

311. **Whorwood CB, Mason JI, Ricketts ML, Howie AJ, Stewart PM** 1995 Detection of human 11-β hydroxysteroid dehydrogenase isoforms using reverse-transcriptase-polimerase-chain reaction and localization of the type 2 isoform to renal collecting ducts. Mol Cell Endocrinol 110: R7-12

312. **Winkel CA, Snyder JM, MacDonald PC Simpson ER** 1980 Regulation of cholesterol and progesterone synthesis in human placental cells in culture by serum lipoproteins. Endocrinology 106: 1054-1060

313. **Wu WX, Derks JB, Ma XH, Nathanielsz PW** 1997 Fetal administration of β-methasone (βM) on glucocorticoid (GC) receptor (GR) mRNA expression in fetal sheep hippocampus (HIP), Hypothalamu (HYPO), pituitary (PIT) and adrenal

(ADR.) In: Program of the 44th annual meetting of the Society for Gynecologic Investigation; San Diego, California. Abstract 466

314. **Wu WX, Derks JB, Nathanielsz PW** 1996 Effects of glucocorticoids on estrogen receptor messenger ribonucleic acid in the pregnant ovine myometrium in vivo and in vitro. Biology of Reproduction 54: 230-241

315. **Wu WX, Myers DA, Nathanielsz PW** 1995 Changes in estrogen receptor messenger ribonucleic acid in sheep fetal and maternal tissues during late gestation and labor. Am J Obstet Gynecol 172:844-50

316. **Yamamoto KR** 1985 Steroid regulated transcription of specific genes and gene networks. Annu Rev Genet 19:209-252

317. **Yamashita S, Newbold RR, McLachlan JA, Korach KS** 1980 Developmental pattern of estrogen receptor expression in female mouse genital tracts. Endocrinology 125:2888-2896

318. **Zhou Y, Chorich LP, Mahesh VB, Ogle TF** 1993 Regulation of estrogen receptor protein and messenger ribonucleic acid by estradiol and progesterone in rat uterus. J Steroid Biochem Mol Biol 46:687-698

319. **Zhou Y, Mahesh VP, Ogle TF** 1994 The role of rat uterine microsomal strogen receptor in ovarian steroid hormone action. Endocr J 2:53-62

320. **Zhou Yue, Shughrue PJ, Dorsa DM** 1995 Estrogen receptor protein is differentially regulated in the preoptic area of the brain in the uterus during the rat estrus cycle. Neuroendocrinology 61:267-283

VITA

Name: Maria Gomez Leavitt
263-87-9445

Birth: June 4, 1956
Jaen, Spain

Citizenship: U.S.A.

Marital Status: Married

Address: 633 New Hampshire Ave
Norfolk, Virginia, 23508
(757) 6260614

Eastern Virginia Medical School
Department of Physiology
700 Olney Road
Norfolk, Virginia 23507-1912
(757) 446-5706
email: GJPLAB@BORG.EVMS.EDU

Education:

1997 Doctor of Philosophy
Biomedical Sciences-Cellular Endocrinology and Reproductive Biology
Eastern Virginia Medical School/Old Dominion University

1991 Master of Sciences
Biology
Old Dominion University

1984 Bachelor of Arts
Spanish Education
Old Dominion University

1979 Bachelor of Sciences
Biology
Universidad Complutense, Madrid, Spain

Awards:

EVMS Awards Committee for Biomedical Sciences Program. July 1994-to June 1996.

Predoctoral fellowship. Porter Physiology Development Committee. American Physiological Society. Bethesda . Maryland. September 1996-to August 1998.

Publications:

Pepe GJ, Babischkin JS, Burch MG, Leavitt MG, Albrecht ED 1996 Developmental increase in expression of the messenger ribonucleic acid and protein levels of 11β-hydroxysteroid dehydrogenase types 1 and 2 in the baboon placenta. **Endocrinology** 137: 5678-5684

Leavitt MG, Aberdeen GW, Burch MG, Albrecht ED, Pepe JG 1977 Inhibition of fetal adrenal ACTH receptor mRNA expression by betamethasone administration to the baboon fetus in late gestation. **Endocrinology** 138:2705-2711