THE EFFECT OF ESTRADIOL ON FSH INDUCTION OF FSH RECEPTORS IN GRANULOSA CELLS OF THE RAT

BY

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ABSTRACT

THE EFFECT OF ESTRADIOL ON FSH INDUCTION OF FSH RECEPTORS IN GRANULOSA CELLS OF THE RAT

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I examined whether estradiol is required for FSH to increase its own receptor in granulosa cells of the rat ovary and promotes follicular development. First, I characterized granulosa cells receptors for estradiol and compared these results (Scatchard analyses, sucrose density gradient analysis, effects of temperature and emzymes, steroid specificity and translocation of receptor) to that known for the uterus. Next, I blocked the synergistic effects of estradiol with FSH by pharmacologically inhibiting estradiol synthesis (cyanoketone) or estradiol binding to its receptor (CI628) in granulosa cells. Hypophysectomized rats were divided into five groups: 1) saline, 2) CI628 or cyanoketone, 3) FSH, 4) CI628 or cyanoketone--then FSH, and 5) CI628 or cyanoketone plus estradiol--then FSH. Animals were decapitated at 0, 6, 12, or 24 h post-FSH injection and granulosa receptors for FSH and LH and nuclear receptors for estradiol were measured. Finallly, I examined whether estradiol affects the FSH-induced cAMP-adenylate response system. Hypophysectomized rats were divided into five groups as described. Twelve h after the initial FSH injection, rats were injected with FSH. Animals were killed 60 min

later and granulosa cAMP measured. Granulosa estradiol receptors were identified, characterized, and found similar to those described in the uterus. After administration of CI628 or cyanoketone, LH receptors were unchanged unless estradiol was administered comcomitantly. Numbers of receptors for FSH and estradiol were similar in saline-, CI628- and cyanoketone-treated animals. In group 3, FSH and estradiol receptors increased 6-fold ($p<0.01$) over controls. In group 4, CI628 or cyanoketone administration prevented the FSH-induced increase in FSH receptors at all times. Number of estradiol receptors were similar to controls. Estradiol administration with CI628 or cyanoketone (group 5) prior to FSH reversed the inhibitory effects of the drugs on FSH and estradiol receptors. Progesterone ,testosterone, R5020 and DHT partially reversed inhibitory effects of cyanoketone prior to FSH. Estradiol appears to be required for maximal stimulation of FSH receptors after FSH injection. The interaction of estradiol and FSH appears to be past the cAMP-adenylate cyclase system since treatment with CI628 prior to FSH had no effect on FSH-stimulated cAMP production. Thus, estradiol may be required for FSH to increase its own receptor and, in turn, promote follicular growth.

To my parents

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LIST OF ABBREVIATIONS

ACS. . . . . .aqueous counting scintillant
ADH. . . . . .alcohol dehydrogenase
BSA. . . . . .bovine serum albumin
cAMP . . . . .cyclic 3', 5', adenosine monophosphate
CI628. . . . .α-4-pyrrolidino-ethoxy-phenyl-4-methoxy-α-nitrostilbene
cpm. . . . . .counts per minute
cyanoketone. .2-α-cyano-4,4,17α-tri-methylandrost-5-en-17B-ol-3-one
DES. . . . . .diethylstilbestrol
DHT. . . . . .dihydrotestosterone
E_{17B} . . . . .estradiol
FSH. . . . . .follicle stimulating hormone
h. . . . . .hour(s)
hCG. . . . . .human chorionic gonadotropin
LH . . . . . .luteinizing hormone
mg . . . . . .milligram(s)
min. . . . . .minute(s)
ml . . . . . .milliliter(s)
mM . . . . . .millimolar
NaSCN. . . . .sodium thiocyanate
P. . . . . .progesterone
PBS. . . . . .phosphate buffered saline
R5020. . . . .promegestrone
s.c. . . . . .subcutaneously
T. . . . . .testosterone
TED. . . . . .Tris-EDTA
ul . . . . . .microliter(s)

INTRODUCTION

Gonadotropins from the pituitary gland interact with ovarian steroids to stimulate growth of follicles, ovulation, and formation of corpora lutea.

Follicle stimulating hormone (FSH) and luteinizing hormone (LH) interact with their receptors in follicular tissue to increase numbers of receptors for FSH and LH and promote steroidogenesis. FSH stimulates production of progesterone and estradiol in granulosa cells while LH stimulates androgen production in thecal cells. The androgens, androstenedione and testosterone, are aromatized to estradiol by the aromatase enzymes in granulosa cells which are under the control of FSH.

Although androgens appear to be associated with atresia of follicles, estradiol promotes the formation of preantral follicles and renders the ovaries more responsive to gonadotropins, thus further promoting follicular development.

Estradiol has no effect on content of FSH receptors in granulosa cells; however, FSH can increase the number of receptors for LH and FSH. Moreover, estradiol-priming prior to administration of FSH increases levels of receptors for FSH and LH more expediently than with FSH alone. However, androgens prevent hormonal induction of receptors

for LH in antral follicles. This suggests that differentiation of granulosa cells is a hormonally-regulated event involving synergism of estradiol and gonadotropins.

Since FSH increases endogenous levels of estradiol and estradiol can enhance gonadotropin action, a synergistic action of estradiol and FSH may be required for follicular maturation.

The first objective of my research was to determine if granulosa cells have receptors for estradiol. The second objective was to determine if estradiol is required for FSH action. The third objective was to attempt to determine if the interaction of estradiol and FSH occurs on the cAMP-adenylate cyclase response system of granulosa cells. In the following review of the literature, a brief history is given of the research on ovarian function and an overview of the roles that estradiol, LH and FSH have on follicular development.

LITERATURE REVIEW

Although the ovarian follicle was identified by de Graaf in 1672 and ovum described by von Baer in 1827, it was not until the early 1900's that the reproductive organs were identified as having an endocrine function (Asdell, 1969). At first, phases of the female cycle were described and ovarian follicles and corpora lutea identified (Heape, 1990; Marshall, 1903; Ancel and Bouin, 1909). Next, crude extracts of gonads were injected into animals, but results were discouraging as uterine weight increased only occassionally. However, in 1917, Stockard and Papunicolaou published their results from guinea pigs showing that changes in vaginal epithelium corresponded with changes in days of the estrous cycle.

Through the use of vaginal smears, Allen and Doisy (1923) isolated and identified a follicular hormone, estrogen. Isolation of estrogens was quickly followed by that of progesterone from corpora lutea of pigs by Corner and Allen (1929). About this time, testosterone, considered the male hormone, was isolated by Gallagher and Koch (1929).

The interaction of substances from the gonads with those from the pituitary in the control of follicular growth was suggested as early as 1905 (Bellerby, 1929). Injection of extracts from the anterior pituitary induced follicular growth and ovulation. Pituitary extracts were semi-purified and found to contain two hormones, follicle stimulating hormone and luteinizing hormone (Fevold *et al.*, 1931;

Evans et al., 1936; Fevold, 1939; 1943). Due to the purity of these hormones, however, some results from early experiments are questionable. For example, using intact female rats, injections of FSH markedly increased ovarian weight (Fevold et al., 1933), however, more recent studies demonstrate that FSH alone has little effect on ovarian weight (Dorrington and Armstrong, 1979). LH in large quantities produces pseudolutein bodies in follicles by hypophysectomized rats and causes ovulation (Greep et al., 1942; Fraenkel-Conrat et al., 1943). Generally, LH augments FSH stimulation of ovarian weight in intact and hypophysectomized rats, and this is associated with an increase in estrogen production and follicular growth (Simpson et al., 1941; Bates and Schooley, 1942; Greep et al., 1942).

FSH plays an important role in follicular development. Although administration of estrogen alone promotes growth of preantral follicles, FSH is required for preantral follicles to form antral follicles (Lane and Greep, 1935; Simpson et al., 1941). Further, FSH administration after estrogen pretreatment causes more expedient differentiation of preantral follicles into mature follicles (Goldenberg et al., 1972a,b; Richards et al., 1976; Richards and Kersey, 1977).

Most of the actions of ovarian and pituitary hormones on follicular growth were elucidated after development of the technique of hypophysectomy, parapharangeal surgical removal of the pituitary gland, by P.E. Smith (1930). Hypophysectomy results in immediate atrophy of the gonads. This was reversed with either pituitary implants or injections of extracts from the pituitary (Smith, 1930).

The hypophysectomized rat continues to be a good model for testing the effects of steroids and gonadotropins, either alone or in combinations, on ovarian function. This model has been used to study the effects of hormones on numbers of receptors for gonadotropins and steroids, changes in levels of cAMP and protein kinases, and mechanisms of hormone action in the ovary.

After hypophysectomy, administration of estradiol increases mitotic activity (Bullough, 1942) and prevents atrophy of ovaries (Williams, 1940; 1944; 1945; Pencharz, 1940). Estradiol also increases ovarian and uterine weights and renders ovaries more responsive to injections of gonadotropins (Williams, 1940; 1944; 1945; Pencharz, 1940). Although estradiol increases mitotic activity and ovarian weight, estradiol alone promotes development of only preantral follicles, with little effect on antrum formation (Lane and Greep, 1935; Simpson et al., 1941; Richards et al., 1978).

Administration of estradiol or progesterone during follicular development causes atresia in monkeys (Clark et al., 1981). Also, estradiol implanted into one ovary of rats can decrease the number of ovulations from that ovary without any effect on the other ovary (Dierschke et al., 1983). This suggests a direct inhibitory effect of estradiol on ovulation. The mechanism by which estradiol causes these inhibitions is unknown.

Estradiol can have inhibitory effects on steroidogenesis. In culture, estradiol inhibited progesterone secretion by granulosa cells from small (Thanki and Channing, 1976) and medium (Schomber et al., 1976) but not large preovulatory follicles (Goldenberg et al., 1972a;

Schomberg et al., 1976; Haney et al., 1978). Fortune and Hansel (1979) demonstrated that estradiol inhibits progesterone secretion by bovine granulosa cells, however, if LH is added to the culture, estradiol has no effect.

In intact animals, estradiol has no effect on progesterone production (Leung et al., 1978; Leung and Armstrong, 1979), but inhibits androgen production (Leung et al., 1978; Leung and Armstrong, 1979; Leung et al., 1979). This inhibition by estradiol on androgen synthesis appears to be a direct effect on the ovary (Leung et al., 1979). Estradiol implanted in one ovary can inhibit androgen production without any effect on the contralateral ovary (Leung et al., 1979).

In hypophysectomized animals, estradiol prevents LH-stimulated progesterone production (Leung et al., 1978; Leung et al., 1979; Leung and Armstrong, 1979) however this inhibitory effect by estradiol can be prevented by administering FSH and LH suggesting an interaction of estradiol with gonadotropins (Leung et al., 1979).

Administration of testosterone to rats causes atresia of follicles (Payne and Runser, 1958; Schreiber and Ross, 1976; Farookhi, 1980) unless FSH is administered (Ireland and Richards, 1978). Then, growth of follicles occurs. Dihydrotestosterone (DHT), a nonmetabolizable androgen, prevents the induction of receptors for LH in antral follicles after FSH administration (Farookhi, 1980). Androgens do, however, stimulate progesterone synthesis in granulosa cells, but not as well as FSH (Hillier et al., 1977; Lucky et al., 1977).

Although progesterone can have an inhibitory effect on follicular

development in primates (Goodman et al., 1977; Goodman and Hodgens, 1977), hamsters (Greenwald, 1977; Moore and Greenwald, 1974) and rats (Kalra and Kalra, 1974; Schreiber et al., 1980; Schreiber et al., 1981; Schreiber et al., 1982), Richards and Bogovich (1982) have shown that elevated progesterone in rats can enhance responses of small antral follicles to subtle changes in LH. The increased progesterone levels override the need for a sustained rise in LH and allow small antral follicles to grow and develop when LH is low as during most of an estrous cycle and early pregnancy. Thus, the exact role of progesterone in follicular development is controversial.

Rats injected with progesterone have decreased levels of gonadotropins (Beattie and Corbin, 1975; Goodman, 1978; Richards et al., 1980; Taya et al., 1981), inhibition of FSH-induced increases in LH receptors (Schreiber et al., 1982) and decreased levels of androgens and estradiol (Kalra and Kalra, 1974; Saidapur and Greenwald, 1979; Richards et al., 1980; Schreiber et al., 1980; Taya et al., 1981; Richards and Bogovich, 1982). Progesterone depresses FSH induction of the aromatase enzyme system in granulosa cells thus preventing conversion of androgens to estradiol (Schreiber et al., 1981; Fortune and Vincent, 1983). Besides regulating estradiol synthesis, progesterone inhibits estradiol action by preventing retention of the receptor-hormone complex in the nucleus which is necessary for hormone action (Evans et al., 1980; Okulicz et al., 1981; Leavett et al., 1982).

Estradiol, progesterone, and testosterone are in follicular fluid of follicles. Cystic and atretic follicles have a higher proportion of

androgens and progesterone to estradiol whereas healthy follicles have a larger concentration of estradiol (Short and London, 1961; Short, 1962; Ireland and Roche, 1983).

Estradiol, testosterone and progesterone in conjunction with the gonadotropins stimulate follicular growth. Testosterone plus FSH stimulates follicular development. FSH apparently increases aromatase activity which converts testosterone to estradiol. However, testosterone alone inhibits follicular maturation by causing atresia (Payne and Runser, 1958). Progesterone plus hCG administered to intact rats promotes follicular growth, however an interaction of hCG and/or progesterone with FSH and estradiol present in the ovary can not be ruled out. Progesterone alone stimulates development of small antral follicles in rats, however, this hormone has a negative effect on induction by FSH of the aromatase system and estradiol synthesis. As levels of estradiol are high in non-atretic, healthy follicles, progesterone ultimately would decrease follicular growth. Although estradiol inhibits progesterone production in cultures of granulosa cells and hypophysectomized rats, it has little effect in vivo. Estradiol does decrease levels of androgens, however androgens in large amounts cause atresia. Therefore, estradiol either alone or in conjunction with gonadotropins generally promotes follicular growth and maturation.

Because estradiol is a steroid, it is capable of moving freely through cell membranes. Thus, estradiol is found in non-target and target tissues. However, in target tissues such as ovaries and uteri, estradiol is retained and is found in higher concentrations than in

non-target tissues (Jensen and Jacobson, 1962; Stone et al., 1963; Stone and Baggett, 1965; Jensen et al., 1966; Terenius, 1966; Jensen et al., 1967). Binding of tritiated estradiol to subcellular fractions of the rat uterus was competitive only in the soluble and nuclear-myofibrillar fractions (Noteboom and Gorski, 1965) and the tritiated steroid was released when fractions were treated with proteases. This suggested that the hormone was bound to proteins in the cytosol and nucleus (Toft and Gorski, 1966; Jensen et al., 1971).

It is now known from studies with uteri that estradiol binds to its receptor in the cytoplasm. This receptor then undergoes a transformation, and the receptor is rapidly translocated into the nucleus (Shayamala and Gorski, 1969; Jensen et al., 1969a; Jensen et al.,1969b; Jensen et al., 1971; Jensen et al., 1972; Williams and Gorski, 1972; Notides et al., 1975; Notides and Nielsen, 1975). The nuclear receptor binds to specific acceptor sites on chromosomes and there is an increase in mRNA synthesis (Aizawa and Mueller, 1961; Gorski and Nicolette, 1963; Hamilton et al., 1965; Means and Hamilton, 1966; Billing et al., 1969; Luck and Hamilton, 1972; Mohla et al., 1972; Yamamoto and Alberts, 1975; Kon and Spelsberg, 1982). The amount of growth of tissue has been associated with the level of estradiol receptors in the nucleus (Jensen et al., 1968; Anderson et al., 1975). After nuclear action, receptors from the nucleus can be recycled back into the cytoplasm (Kassis and Gorski, 1981).

Although early studies have established a single binding site for estradiol in the cytosol and nucleus of the uterus, recently, studies have demonstrated two binding sites for estradiol in the uterus (Clark

et al., 1978). The type I site in the cytosol has a dissociation constant (Kd) similar to the classical uterine estradiol receptor (1nM) with a limited binding capacity (Ro~1pM). This receptor is translocated to the nucleus. The type II site has a lower Kd, 4-fold more binding sites and is not translocated from the cytoplasm into the nucleus. The function of the type II binding sites is unknown.

Two binding sites for estradiol have been identified in the nucleus of uteri (Markaverich and Clark, 1979). As in the cytosol, the type I site is similar to the classical uterine receptor (Kd= 1nM, Ro= 1pM/uterus). The type II site persists after levels of type I sites are lower and is thought to be associated with long term uterine growth (Clark and Markaverich, 1981; Markaverich et al., 1981; Clark et al., 1982).

Two distinct high affinity, low capacity binding proteins have been demonstrated in the cytoplasm and nucleus of chick oviduct and human uteri (Smith et al., 1979) and the cytosol of rat uteri (Erickson et al.,1978). The importance of these two receptors biologically remains to be elucidated.

Receptors for estrogen have been identified and characterized in uteri and ovarian homogenates (Saiddudin and Zassenhaus, 1977). However, only binding of estrogen has been demonstrated in granulosa cells which both synthesize and respond to estrogens (Fortune and Armstrong, 1978). Whether a single, specific, high affinity receptor for estradiol is present in granulosa cells has not been determined.

Follicular development is under the control of estradiol and the gonadotropins. Estradiol increases responsiveness of follicles to

gonadotropins (Pencharz, 1940; Williams, 1945), increases proliferation of granulosa cells (Rao et al., 1978; Goldenberg et al., 1972a) and induces follicular growth of preantral follicles. FSH alone promotes estrogen production and formation of antra in ovarian follicles (Moon et al., 1975; Zeleznik et al., 1974). However, estrogen priming prior to FSH increases ovarian and uterine weights and promotes follicular growth above that with FSH alone (Lane and Greep, 1935; Simpson et al., 1941; Richards et al., 1976). As FSH increases estradiol production and estradiol, in turn, can further enhance FSH action, there appears to be an interaction between estradiol and FSH during follicular development.

Estradiol and FSH have a synergistic effect on induction of receptors for estradiol and gonadotropins in granulosa cells. Administration of estradiol alone increases numbers of receptors for estradiol and gonadotropins per ovary but has no effect on numbers of receptors for FSH or LH per granulosa cell (Louvet and Vaitukaitis, 1976; Richards and Midgley, 1976; Richards, 1978; Richards and Kersey, 1979). Administration of FSH alone increases receptors for estradiol and FSH but has little effect on numbers of receptors for LH in granulosa cells (Richards et al., 1976). However, pretreatment with estradiol prior to FSH causes a significant increase in numbers of receptors for estradiol, FSH and LH per granulosa cell compared to animals receiving FSH alone (Richards and Midgley, 1976; Richards et al., 1976; Richards and Kersey, 1979). The interaction of estradiol and FSH on induction of receptors for FSH was shown to be time dependent by Ireland and Richards (1978). Estradiol priming 12 or 24 h

prior to FSH significantly increased numbers of receptors for FSH compared to levels after FSH alone.

Estradiol also enhances FSH stimulation of cAMP accumulation and binding sites in granulosa cells (Richards, 1978; Richards et al., 1976; 1979). Although estradiol alone had no effect on cAMP accumulation or number of binding sites for cAMP, it enhances the ability of FSH to increase production of cAMP 2-fold and increases numbers of cAMP binding proteins 10-20 fold compared to FSH alone. Further, the increase in cAMP accumulation occurs without a change in number of FSH receptors per granulosa cell (Richards, 1978; Richards et al., 1979). Thus, estradiol appears to enhance FSH induction of cAMP production independent of increases in numbers of FSH receptors.

Since estradiol priming prior to FSH increases ovarian and uterine weights, follicular growth, numbers of estrogen and gonadotropin receptors per granulosa cell and cAMP accumulation over an injection of FSH alone, and FSH increases levels of estradiol, estrogen may have a role in FSH action. Estradiol synthesis or action through its receptor may be required for FSH to increase its own receptor or increase cAMP accumulation in granulosa cells. Because growth of preovulatory follicles requires the presence of estradiol plus FSH, control of synthesis of estradiol may control development of antral follicles.

Although the theca cells were originally considered the site of synthesis of estradiol (Allen and Doisy, 1923; Corner, 1938), only after the unique studies of Falck (1959) using autotransplants of cells from the ovary of rats to the eye chamber was there evidence that estrogen biosynthesis requires the interplay of at least two cell

types, theca and granulosa. The theca cells are the major source of androgens which are required for synthesis of estrogen (Erickson and Ryan, 1976; Fortune and Armstrong, 1977; Moor, 1977; Tsang et al., 1979). The androgens are released from the theca cells and diffuse into granulosa cells. The conversion of androgens to estrogens occurs in granulosa cells and is under the control of the aromatase enzyme system. FSH can increase estradiol accumulation by granulosa cells by increasing aromatase activity (Moon et al., 1975; Dorrington et al., 1975; Erickson and Hsueh, 1978; Daniel and Armstrong, 1980; Adashi and Hsueh, 1982). Furthermore, estradiol augments FSH stimulation of aromatase activity (Watson and Howson, 1977; Adashi and Hsueh, 1982; Veldhuis et al., 1982).

Follicular growth of preovulatory follicles depends on the interaction of estradiol and FSH. The FSH-cAMP response system in small antral follicles can maintain some aromatase activity, however, with increased estradiol production, this activity is increased. Acting via its receptor, estradiol further modifies granulosa cell function. It enhances the ability of FSH to stimulate cAMP production and increases cAMP binding sites and responsiveness of granulosa cells to gonadotropins. Thus, it appears that once estradiol production begins, it assumes an important role in follicular development.

In this dissertation, I attempted to establish if a single class of high affinity receptors for estradiol are present in granulosa cells and if synthesis of estrogens or action of estrogens through the receptor is required for follicular development.

CHAPTER I

IDENTIFICATION OF ESTROGEN RECEPTORS IN GRANULOSA CELLS OF IMMATURE RATS

Introduction

Estradol plays a major role in ovarian follicular growth and development. Estradiol directly increases ovarian weight and causes a proliferation of granulosa cells (Bradbury, 1961). Estrogen also synergizes with gonadotropins to cause maturation of follicles in preparation for ovulation (Richards et al., 1976; Ireland and Richards, 1978).

In developing follicles, granulosa cells both synthesize and respond to estrogens (Fortune and Armstrong, 1978). Estrogen receptors are found in the corpus luteum of various species (Yuh and Keyes, 1979; Richards, 1974) and in whole ovarian homogenates (Saiddudin and Zassenhaus, 1977). Although binding of estrogen has been demonstrated in granulosa cells (Richards, 1975), the specific receptor for estrogen in granulosa cells has not been fully characterized.

In this study, I characterized estrogen receptors in granulosa cells of rats. The properties of these receptors were compared to

those of uterine estrogen receptors.

Materials and Methods

Materials

The following reagents were used: (2,4,6,7,16,17-^{3}H)-estradiol-17-B (137.1 Ci/mmol: $^{3}H\text{-}E_{17B}$); New England Nuclear; sodium thiocyanate (NaSCN, analytical grade), Trizma-HCl (reagent grade), diethylstilbestrol (DES), estradiol-17B (E_{17B}), testosterone, progesterone, androstenedione, corticosterone, cortisone, 20B-hydroxy- pregnenolone, 17 -hydroxy-progesterone, estrone, sucrose (Grade 1), DNA from calf thymus, DNase from bovine pancreas, RNase from bovine pancreas, protease, and charcoal (Norit A); Sigma Chemical Co.; Dextran T-70: Pharmacia; aqueous counting scintillant (ACS): Amersham; propylene glycol: J.T. Baker Chemical Co..

Animals

Immature (21-day-old) female Sprague Dawley rats were obtained from Spartan Research Animals (Haslett, Michigan), exposed to 12L:12D (24^{o}C), and given food and water ad libitum. Rats were decapitated between 22 and 28 days of age.

Tissue preparation

Ovaries and uteri were dissected, trimmed of fat, and placed in TED buffer (10 mM Tris, 1.5 mM EDTA, pH 7.4) on ice.

For each Scatchard analysis, granulosa cells were obtained from ovaries of 100 rats according to the method of Zeleznik et al., (1974); gentle pressure was applied to follicles and expressed cells were collected after centrifugation at 800 x g (20 min, 4^{o}C). Cells were

washed three times in TED (2 ml/wash), homogenized in a glass Dounce homogenizer (2 ml TED), and centrifuged at 4000 x g for 20 min at 4°C. Pellets were washed 3 times in 2 ml TED buffer, then resuspended in TED (granulosa cells from 2 ovaries/tube, 0.1 ml volume) for the estradiol nuclear receptor assay. The supernatant was further centrifuged at 106,000 x g (Type 30 rotor, Beckman) for 1 h (4°C) to obtain a cytosol preparation for the cytosol receptor assay.

Uterine tissue from 50 rats was homogenized in ice-cold TED buffer (2 uteri/5 ml) using a Servall omni-mixer S-1515. The homogenate was centrifuged at 4000 x g for 20 min at 4°C. Pellets were washed 3 more times with buffer then resuspended in TED for the nuclear receptor assay. The supernatant was further centrifuged at 106,000 x g for 1 h. After centrifugation, the supernatant was removed using a Pasteur pipette, taking care to exclude the fat layer floating on top, and used in the estradiol cytosol receptor assay.

Exchange assays for Scatchard analysis

Two assays for estradiol nuclear receptors were used. The method of Anderson et al. (1972) involved incubation of nuclei from granulosa cells at 37°C for 1 h in TED buffer containing various concentrations of $^{3}H\text{-}E_{17B}$ (1 to 20 nM). Total reaction volume was 0.5 ml. After incubation, nuclear samples were washed 3 times with TED then extracted (2 times) with 1 ml of methanol. Extracts were placed in scintillation vials, dried, and reconstituted in 10 ml of ACS. The method of Sica et al. (1981) involved incubation of nuclear preparations from granulosa cells in TED buffer with various concentrations of $^{3}H\text{-}E_{17B}$ (1 to 20 nM) at 4°C. Total volume equaled 0.25 ml. After 1 h, 0.05 ml of TED

buffer containing 3 M NaSCN which solubilizes the nuclear estradiol binding site was added to each tube for a final volume of 0.3 ml (final NaSCN concentration = 0.5 M). After overnight incubation at 4°C, 0.3 ml of Dextran-coated charcoal (DCC; 1% charcoal, 0.05% Dextran in TED) was added to separate free from bound estradiol in the nuclear extract. Fifteen min after adding DCC, samples were centrifuged at 8,000 x g for 10 min to precipitate the charcoal and nuclei. The supernatant was then removed and placed in scintillation vials containing 10 ml of ACS. Radioactivity was quantified in an Isocap/300 6872 Liquid Scintillation System (Searle Analytic, Inc.). Specific binding was calculated as the difference in counts bound in the presence (non-specific) or absence (total) of a 500-fold excess of DES.

The two methods utilized for the estradiol cytosol receptor assay were similar. The method of Richards (1974) utilized TED buffer with a 24 h incubation, while the method of Sica et al. (1981) required addition of 0.5 M NaSCN to the TED buffer and incubation for 3 h. Aliquots of cytosol (0.2 ml) were added to tubes containing 1 to 20 nM $^{3}H\text{-}E_{17\beta}$. To determine non-specific binding, 500-fold excess DES was added to a parallel set of tubes. After incubation for 3 h (Sica et al., 1981) or 24 h (Richards, 1974) at 4°C, 1 ml of DCC was added to each tube. The suspension was incubated for 15 min (4°C) and was centrifuged at 8,000 x g for 10 min. The supernatant was added to scintillation vials along with 10 ml of ACS and counted.

Because Scatchard analysis of estradiol binding to uterine nuclei or granulosa and uterine cytosol using the method of Anderson et al. (1972) or Sica et al. (1981) gave similar results, the method of Sica

et al. (1981) was used for all characterizations and assays.

Exchange assays for saturation analysis

Nuclear or cytosol preparations were added to 19 nM ^{3}H-E_{17B} with or without 500-fold DES plus 0.5 M NaSCN-Tris. After incubation for 16-24 h (nuclear assay) (4°C) or 3 h (cytosol assay), DCC was added (0.3 ml-nuclear assay; 1 ml-cytosol assay). Tubes were further incubated for 15 min then centrifuged for 10 min at 3000 x g (4°C). Supernatant was removed, placed in vials containing 10 ml ACS, then counted. Amount of tissue in samples was estimated by measuring DNA (for nuclear receptor assay; Burton [1956]) or protein content (for cytosol receptor assay; Lowry et al.[1951]).

Sucrose density gradient

Aliquots (0.2ml) of either cytosol or nuclear preparations (4 rats total) were layered onto 4.8 ml of 5-20% linear sucrose density gradient (cellulose nitrate tubes) made in TED-0.5 M NaSCN buffer (4°C). Gradients were centrifuged in a Beckman L8-70 ultracentrifuge using a SW 50.1 rotor at 175,000 x g for 24 h (4°C). Alcohol dehydrogenase (ADH) from yeast and bovine serum albumin (BSA) were used as standards to estimate sedimentation constants (S). Individual fractions were obtained by puncturing tubes through the bottom and collecting fractions of approximately 0.3 ml in a series of tubes. Excess DES was used to demonstrate specificity of binding. Fractions were assayed for either nuclear or cytosol receptor as stated above (Sica et al., 1981).

Steroid specificity

Cytosol or nuclear preparations (2 ovaries/tube) were added to tubes containing 19 nM $^{3}H\text{-}E_{17B}$. Excess (1000-fold) of DES, E_{17B}, testosterone, progesterone, androstenedione, corticosterone, cortisone, 20B-hydroxy-pregnenolone, 17 -hydroxy-progesterone, or estrone were added to determine binding specificity. Assays were completed as stated above.

Effects of treatment of receptors with various enzymes

Nuclei were prepared and preincubated with $^{3}H\text{-}E_{17B}$ with or without DES for 1 h at 4°C. Then 0.6 mg/ml RNase, 1.2 mg/ml protease, or 0.75 mg/ml DNase (Saiddudin and Zassenhaus, 1977) was added to the incubation mixture. Control tubes contained only $^{3}H\text{-}E_{17B}$ with or without DES. Nuclei were incubated for an additional 30 min at 37°C and number of nuclear estradiol binding sites were determined by the method of Sica et al. (1981).

For cytosol, each tube contained cytosol and 3 ng $^{3}H\text{-}E_{17B}$ with or without DES (300 ng). After pre-incubation for 1 h on ice, one of the following enzymes was added: 0.9 mg/ml RNase, 1.8 mg/ml protease, or 0.37 mg/ml DNase (Saiddudin and Zassenhaus, 1977). Controls had only $^{3}H\text{-}E_{17B}$ with or without DES. Tubes were then incubated for an additional 30 min at 25°C and processed by the method of Sica et al. (1981).

Translocation of receptor

Rats were injected subcutaneously with 4 mg of estradiol in propylene glycol (4 mg/rat). At 0, 15, 30, 45, 60, and 120 min, rats (n=12 or 12/interval) were killed and cytosol and nuclear estrogen

receptors in granulosa cells were measured. Nuclear and cytosol preparations were added to tubes containing 19 nM $^{3}H\text{-}E_{17B}$. Assays were performed as stated above.

Statistical analysis

Scatchard plots (Scatchard, 1949) were analyzed by linear regression. Curvilinear Scatchard plots were analyzed by the computer program ISIS-59 developed by Thakur et al. (1980) for NICHD, Biophysical Endocrinology Section. Each Scatchard was repeated 3 times. Other experiments were repeated 2 times.

Results

Characterization of binding assays

In the nuclear assay, the method of Anderson et al. (1972) resulted in a curvilinear Scatchard (see insert, Figure 1). The dissociation constant (K_d) of the higher affinity binding component was 3.4×10^{-10}M with a binding capacity (R_o) of 133 pM. The lower affinity site had a K_d of 4.9×10^{-9}M and a R_o of 872 pM. Two component Scatchard plots were not found for uterine nuclei or for granulosa cell or uterine cytosol using this method (data not shown). Use of dithiothreitol as a reducing agent which prevents binding to type II sites (0.1 mM, [Markaverich et al., 1981]; Figure 2) or sodium molybdate which minimizes denaturation and loss of receptor (100 mM, [Krozowski and Murphy, 1981]; Figure 3) in TED buffer during nuclear exchange assays gave similar curvilinear results (e.g., the K_d of the high affinity component was 1.5×10^{-10}M). However, the lower affinity binding site was in the range of a binding protein in serum ($K_d = 10^{-8}$ to 10^{-7}M).

As shown in Figure 1A, a single class of high affinity ($K_d = 1.9 \times 10^{-10}$M) binding sites for estradiol with a R_o of 80 pM (1.4 fmol/ug DNA) was found in NaSCN extracts of granulosa cell nuclei. After extraction of nuclei with NaSCN, no specific binding of estradiol was observed in the remaining nuclear pellet (Table 1). When estrogen receptors from rat uteri were prepared and treated in a fashion identical to that already described, estradiol binding sites with a K_d similar to that of receptors of granulosa cell nuclei ($K_d = 2.5 \times 10^{-10}$M) was observed. A binding capacity of 63 pM (5.0 fmol/ug DNA)

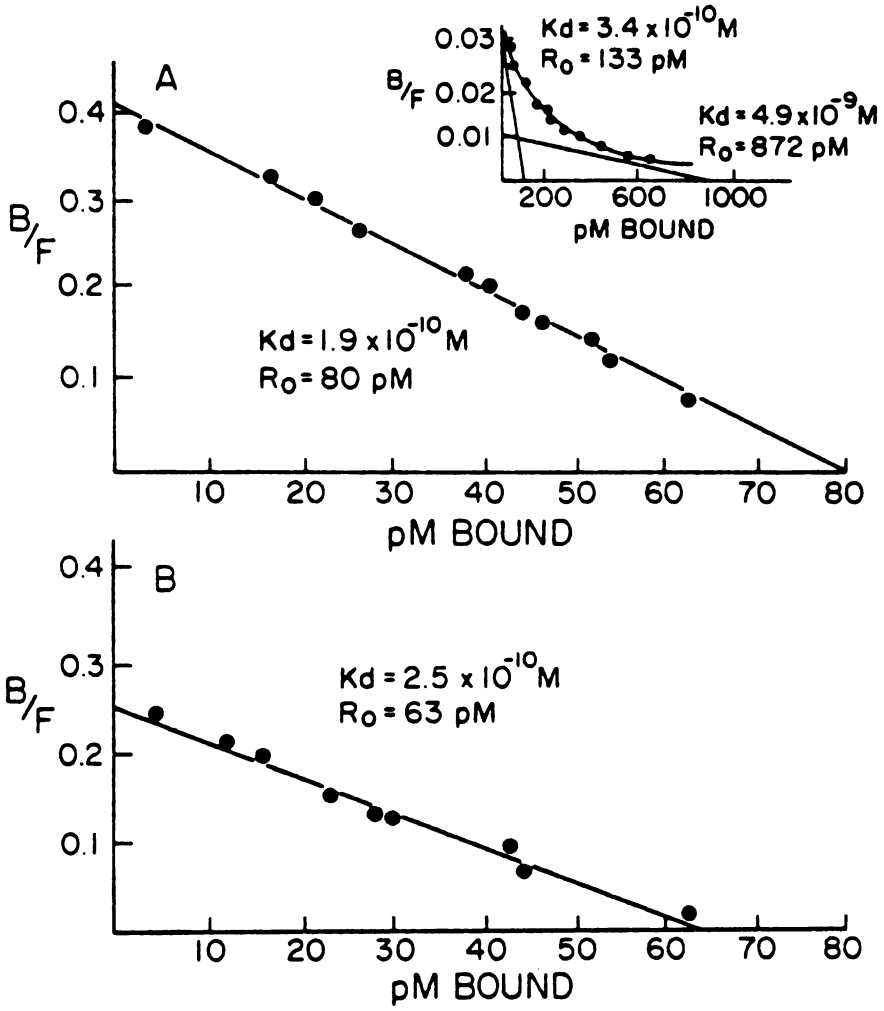

Figure 1. ESTRADIOL BINDING TO PARTIALLY PURIFIED NUCLEI OF GRANULOSA CELLS AND UTERI. Ovaries (A) and uteri (B) from 100 rats were removed and granulosa cells expressed. Nuclear exchange assays were conducted according to the method of Anderson et al. (1972)(A insert) or Sica et al. (1981).

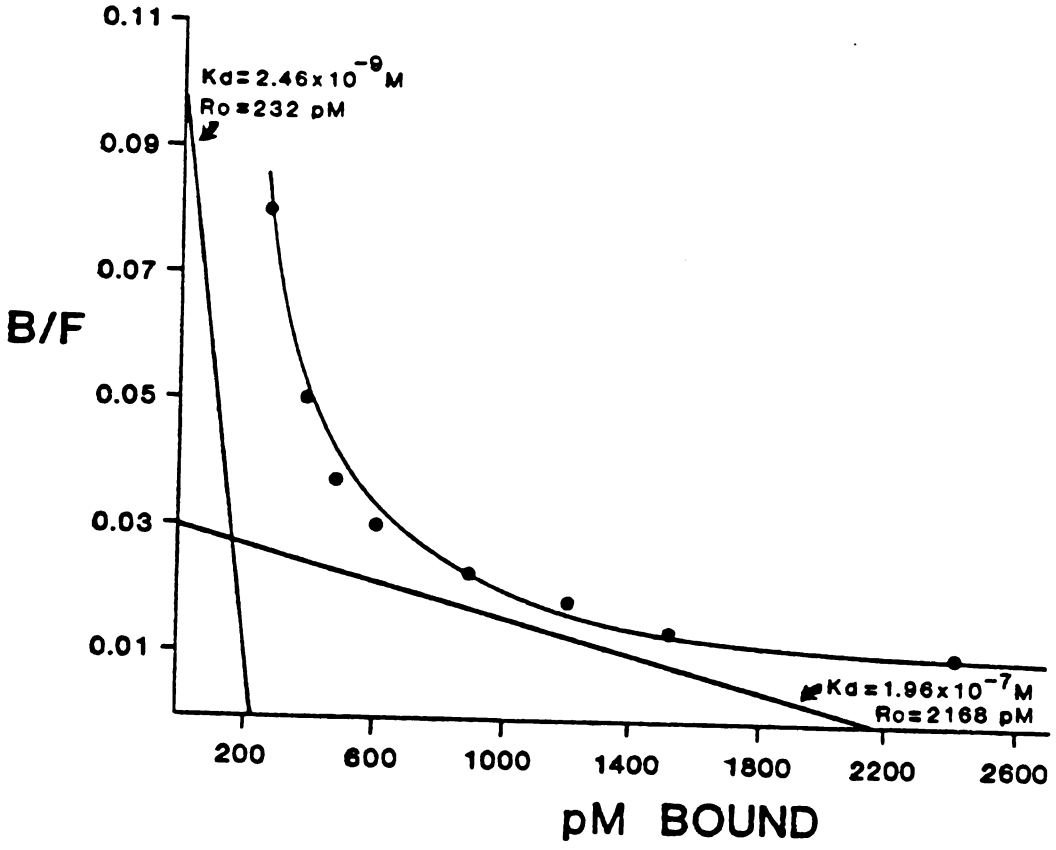

Figure 2. EFFECT OF DITHIOTHREITOL ON BINDING OF $^{3}H\text{-}E_{17\beta}$ TO GRANULOSA CELL NUCLEI. Ovaries from 100 rats were removed and granulosa cells expressed into a buffer containing 0.1 M dithiothreitol, 10 mM Tris-HCl, and 1.5 mM EDTA, pH 7.4.

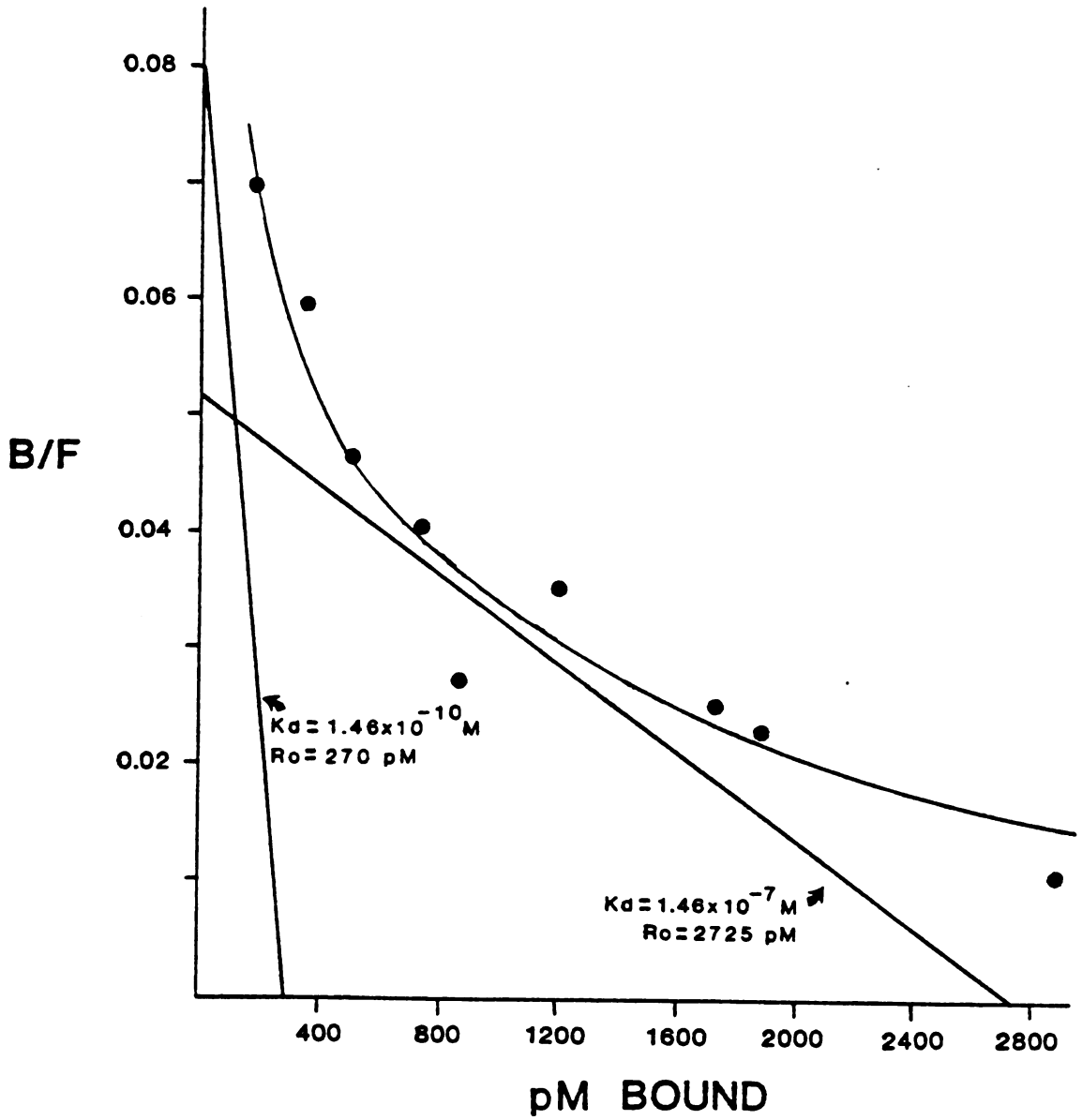

Figure 3. EFFECT OF SODIUM MOLYBDATE ON BINDING OF $^{3}H\text{-}E_{17B}$ TO NUCLEI OF GRANULOSA CELLS. Ovaries from 100 rats were removed and granulosa cells were expressed into a buffer containing 100 mM sodium molybdate, 10 mM Tris-HCl, and 1.5 mM EDTA, pH 7.4.

was demonstrated for uterine nuclei (Figure 1B) using the method of Sica et al. (1981).

TABLE 1

ESTRADIOL BINDING TO RESIDUAL TISSUE FROM GRANULOSA CELLS AND UTERI

Tissue	cpm in nuclear extract	% of total binding
granulosa residual	475	2.40%
uterine residual	570	0.29%

Data are expressed as means.

As shown in Figure 4, Scatchard analysis of estradiol binding to cytosol of granulosa cells and uteri using the method of Sica et al. (1981) also resulted in a single class of binding sites with a K_d = 3.5 x 10^{-10}M (R_o = 45 pM, 0.8 fmol/ug DNA) for granulosa cells and a K_d = 1.4 x 10^{-10}M for uterine tissue (R_o = 20 pM, 1.6 fmol/ug DNA).

At 37°C, a steady state was achieved with nuclear exchange assays by 45 min, and binding was stable until 90 min (Figure 5). For the NaSCN extraction at 0-4°C, steady state was achieved at 16 h. Similar studies were performed for cytosol assays. Utilizing the method of Richards (1974), steady state occurred after 16 h. With the method of Sica et al. (1981), steady state occurred after 2.5 h.

Sucrose density gradient analysis

Estradiol was specifically bound by a macromolecule which sedimented in approximately the 5S region in NaSCN-treated nuclear

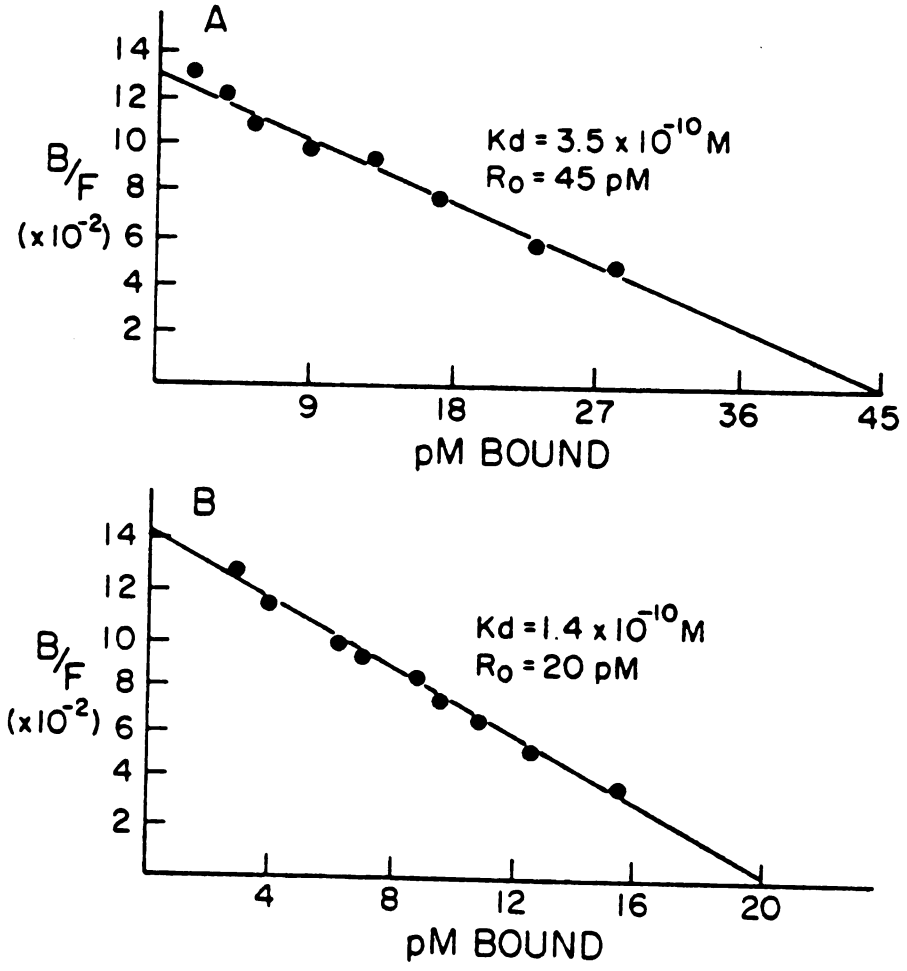

Figure 4. ESTRADIOL BINDING TO CYTOSOL FROM GRANULOSA CELLS AND UTERI. Ovaries and uteri were removed from 100 rats and granulosa cells were expressed. Estradiol binding to cytosol suspensions of granulosa cells of ovary (A) or uteri (B) were measured.

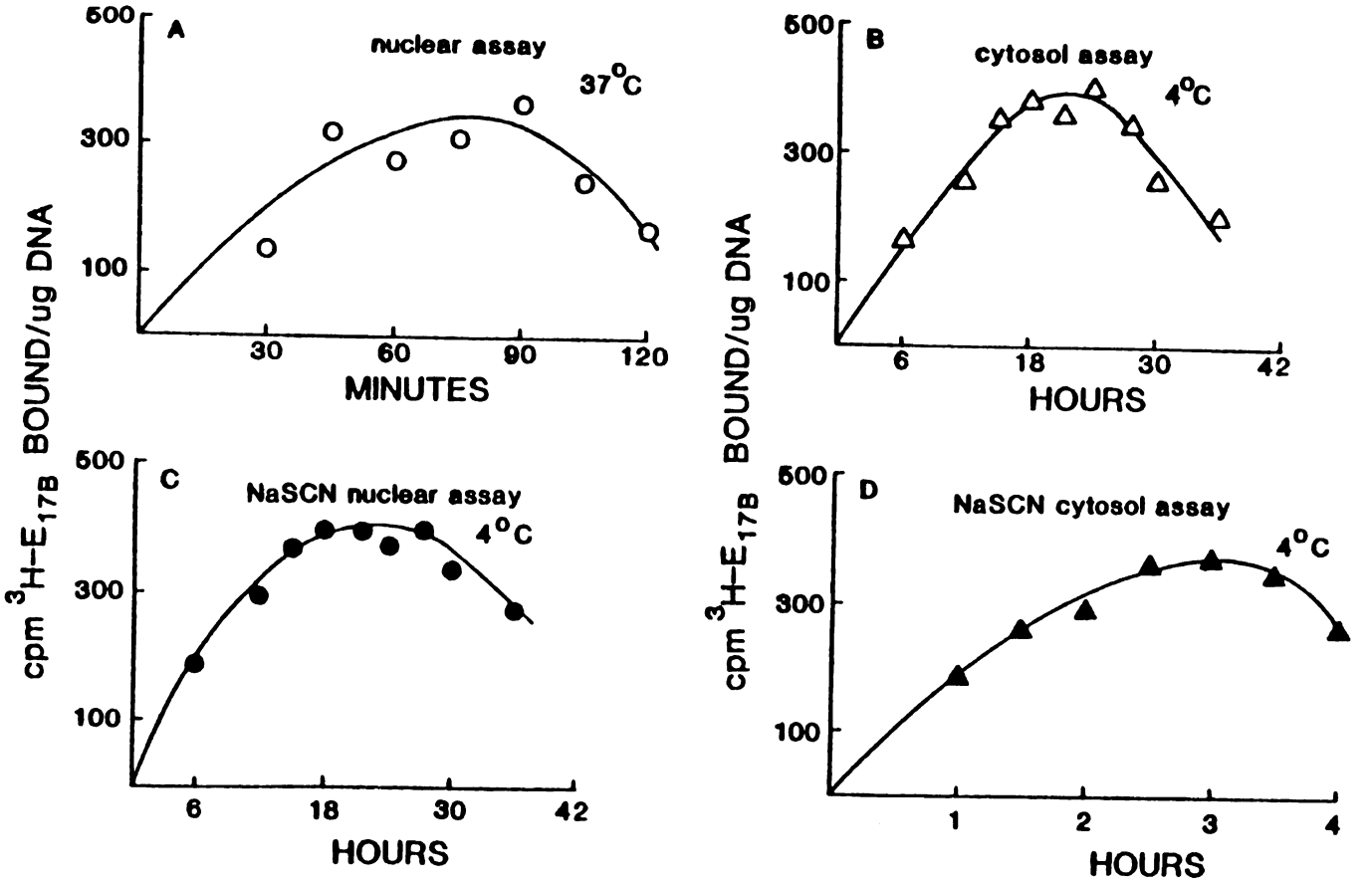

Figure 5. OPTIMUM TIME OF INCUBATION FOR NUCLEAR AND CYTOSOL ASSAYS FOR ESTRADIOL. Crude nuclear and cytosol preparations from granulosa cells were incubated with ^{3}H-E$_{17B}$ for various times according to the methods of Anderson et al. (1972) (A insert), Richards (1974) (B insert) or Sica et al. (1981) (C and D inserts) described in the Materials and Methods. All data are expressed as specific binding.

preparations of granulosa and uterine cells (Figure 6). Estrogen receptors prepared from cytosol sedimented as an 8S form with a much smaller peak at approximately 5S. Since the smaller peak coincided with the nuclear peak, the 5S in the cytosol could be due to a small amount of nuclear receptor contamination rather than disaggregation of the cytosol receptor which appears at 4S (DeSombre et al., 1969; Notides and Nielson, 1975) as previously demonstrated (Puca and Bresciani, 1970).

Steroid specificity

Specificity of binding of estradiol to estrogen receptors in the nucleus and cytosol was examined (Table 2). Maximum competition occurred with estradiol and DES. Estrone was able to compete for receptor, but to a lesser extent than E_{17B} or DES. All other hormones examined had little effect on binding of estradiol to its nuclear or cytosolic binding site.

Effects of temperature and enzyme treatments on binding of estradiol

Estrogen receptors in granulosa cells were shown to be proteins and heat labile (Figure 7). After preincubation of receptor at 37°C, specific binding of estrogen was very low (<10% in nucleus; <5% in cytosol). As incubations of assays after enzyme treatments are at 37°C, receptors were pre-incubated with $^{3}H\text{-}E_{17B}$ to prevent degradation due to temperature. Treatment with protease greatly diminished (<30%) estrogen binding in both nuclear and cytosol preparations, whereas RNase and DNase had little effect.

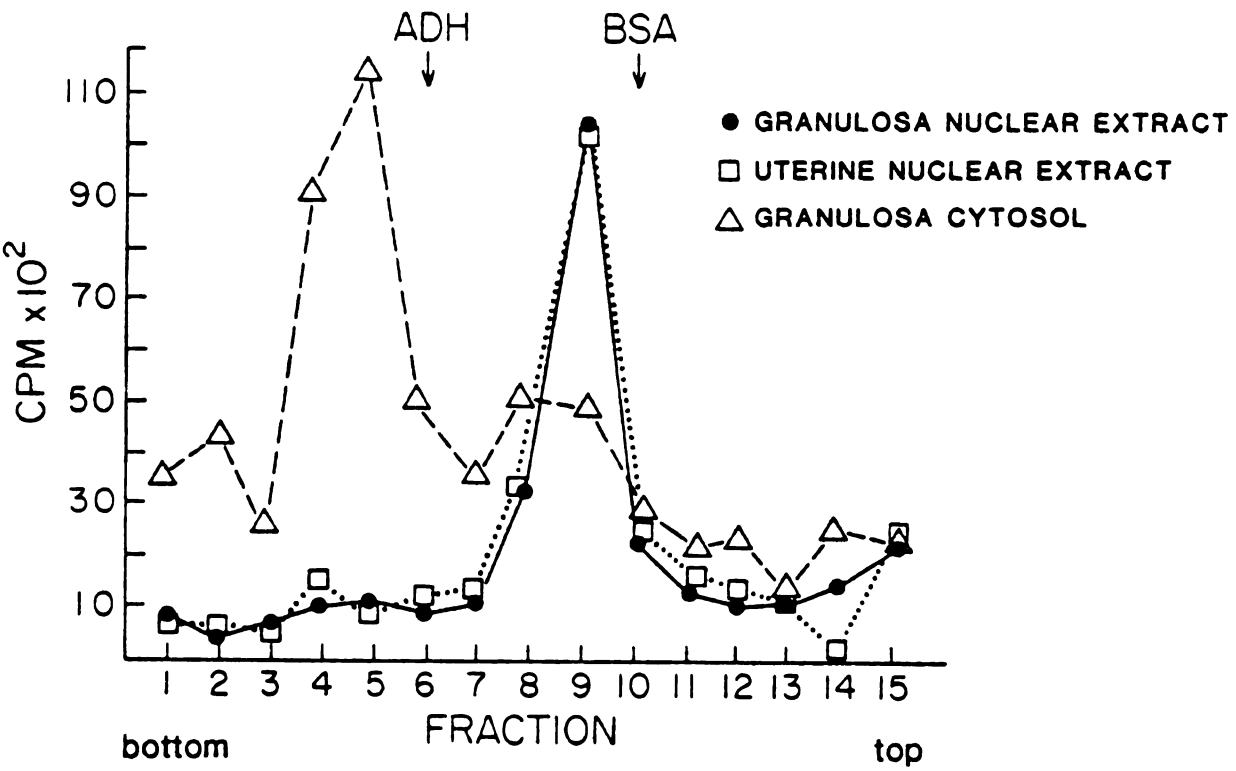

Figure 6. SUCROSE DENSITY GRADIENT SEDIMENTATION PROFILE FOR NUCLEAR AND CYTOSOLIC ESTROGEN RECEPTORS FROM GRANULOSA CELLS AND NUCLEAR RECEPTORS FROM UTERI. Crude nuclear and cytosol preparations from granulosa cells were extracted with 10 mM Tris-1.5mM EDTA-0.5 M NaSCN. Uterine nuclear extractions were used as a control. All data are expressed as specific binding. Arrows indicate the fractions where ^{3}H-BSA (4.4 S) or ^{3}H-ADH (7.4 S), markers for molecular weight, were collected.

TABLE 2

BINDING SPECIFICITY OF NUCLEAR AND CYTOPLASMIC RECEPTORS

Competing Steroid	Specifically Bound in Nucleus (% control)	Specifically Bound in Cytoplasm (% control)
Control	100	100
Cortisone	98	103
Corticosterone	100	101
Progesterone	97	99
20B-OH-pregnenolone	110	102
17α-OH-progesterone	92	95
Testosterone	110	101
Androstenedione	104	105
DES	9	12
Estradiol	10	8
Estrone	22	25

Nuclear exchange assays were as described in Appendix. Suspensions of nuclei or cytosol from granulosa cells were added to 19.9 nM ^{3}H-E_{17B} ± 19.9 uM of cortisone, corticosterone, progesterone, 20-B-hydroxypregnenolone, 17-α-hydroxyprogesterone, testosterone, androstenedione, DES, estradiol, or estrone. Data are expressed as percent of specific binding.

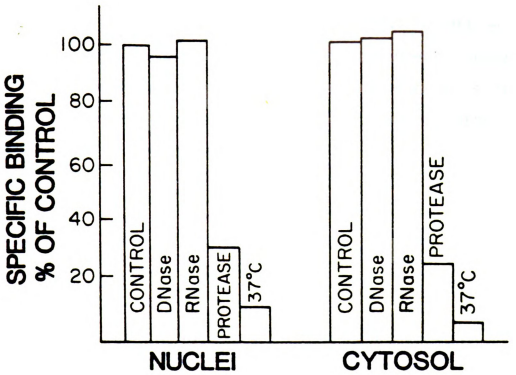

Figure 7. EFFECT OF TEMPERATURE AND VARIOUS ENZYMES ON NUCLEAR AND CYTOPLASMIC RECEPTORS. Nuclei and cytosol were prepared and incubated with $^{3}H\text{-}E_{17\beta}$. After extraction, nuclei were incubated with $^{3}H\text{-}E_{17\beta}$ with or without DES identical to the receptor assay except 0.6 mg/ml RNase, 1.2 mg/ml protease, or 0.75 mg/ml DNAse was also added. Nuclei were then incubated for 30 min at 37°C, then processed. For cytosol, tubes contained cytosol, 3 ng $^{3}H\text{-}E_{17\beta}$ ± DES (300 ng) plus one of the following enzymes: 0.9 mg/ml RNAse, 1.8 mg/ml protease, or 0.37 mg/ml DNAse. For heat lability, nuclear and cytosol preparations were incubated at 37°C for 1 h prior to the exchange assays.

Translocation of receptor

As shown in Figure 8, cytosol receptors appeared to be translocated into the nucleus by 15 min after a 4 mg injection of E_{17B} *in vivo*. Nuclear receptor levels continued to increase until 45 min post-injection, when they reached a plateau. At 120 min, numbers of nuclear receptors were still high with cytosol receptors starting to rise slightly. Basal levels of cytosol and nuclear receptors were not restored by 2 h.

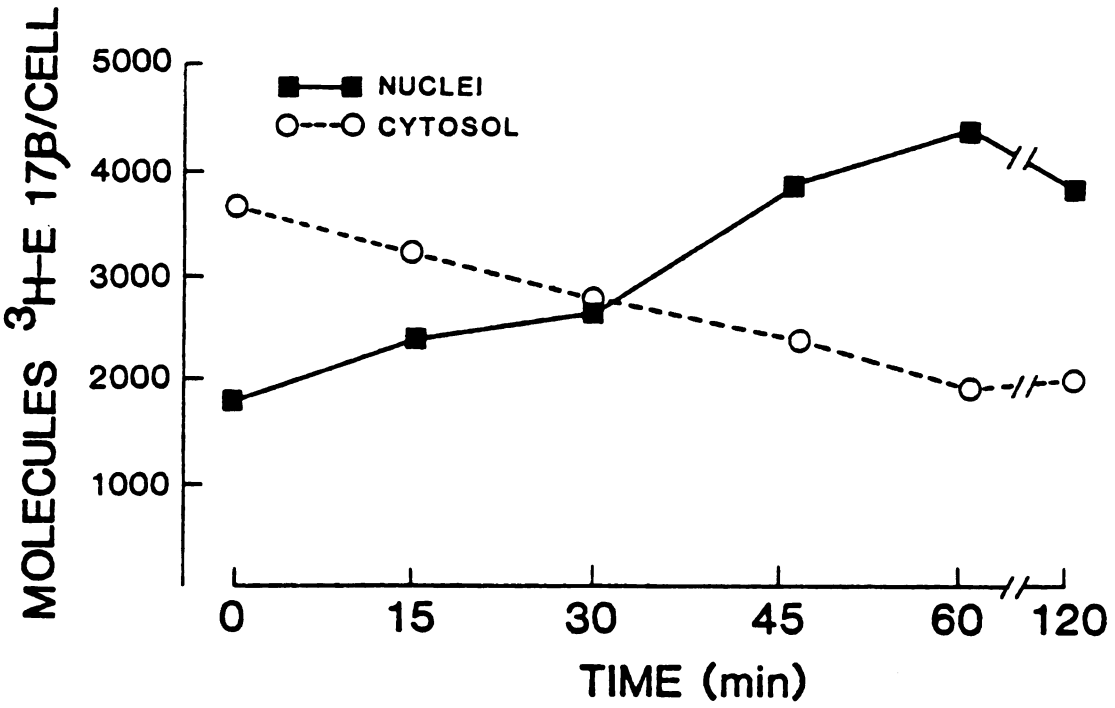

Figure 8. TRANSLOCATION OF THE ESTROGEN RECEPTOR. Rats were injected subcutaneously with 4 mg of estradiol. At 0, 15, 39, 45, 60, and 120 min after injection, rats were killed and cytosol and nuclear estrogen receptors measured. All data were expressed as molecules of (^{3}H)-estradiol/cell, using 6 pg DNA/cell.

Discussion

Reports of multiple binding sites for estradiol in the nuclear and cytosol preparations of uteri are well documented (Erickson et al., 1978;. Clark et al., 1978, Smith et al., 1979; Gibbons et al., 1979). Due to these studies, I utilized various methods to examine estrogen receptors in granulosa cells. As demonstrated in Figure 1 (insert), a curvilinear Scatchard plot for estradiol binding to granulosa but not uterine nuclei was observed when I used the method of Anderson et al. (1972). This could have been the result of another class of binding sites for estradiol in the granulosa cell nucleus. However, a curvilinear Scatchard plot was not observed when I examined estradiol binding in NaSCN nuclear extracts of granulosa cells. In addition, no binding of estradiol occurred to the nuclear pellet of granulosa cells after NaSCN extraction. Thus, it is unlikely that a second class of E_{17B} binding sites exist in nuclei of granulosa cells. Also, de Boer and Notides (1981a, 1981b) suggest that high concentrations of steroids which would be present in granulosa but not in uterine cells interact with hydrophylic sites on the receptor, exerting a non-specific detergent action on the receptor. Perhaps NaSCN minimizes this effect. Binding of estradiol to the nuclear receptor may be altered depending upon whether the nuclear receptor is bound to chromatin or is free (Yamamoto and Alberts, 1975; de Boer and Notides, 1981b). NaSCN extraction may eliminate this interference. Although the reason for curvilinear Scatchards when the method of Anderson (1972) is used is not known, alterations in binding of E_{17B} to its nuclear receptor in

granulosa cells which were reflected in Scatchard plots (Figure 1), may be eliminated when nuclear exchange assays are done with solubilized receptor preparations at cold temperatures.

Utilizing the NaSCN extraction, the estrogen receptor in granulosa cells had a high affinity, low capacity for estradiol. The binding affinities of receptors in the nucleus and cytosol were in the range of those reported for rat uteri (K_d = 2 to 5 x10^{-10}M; [Saiddudin and Zassenhaus, 1977; Anderson et al., 1972; Sica et al., 1981; Pavlik and Rutledge, 1980]), rat endometrium (K_d = 3 to 8 x 10^{-10}M; [Farookhi and Sonnenschein, 1976]), and hypothalamus and pituitary (K_d = 2 x 10^{-10}M; [Kelner and Peck, 1981]). Saiddudin and Zassenhaus (1977) examined nuclear and cytosol estrogen receptors in ovarian and uterine homogenates from immature rats. The affinities of the cytosol estrogen receptors in the ovary (K_d = 4.13 x 10^{-10}M) and uterus (K_d = 1.22 x 10^{-10}M) were very similar to the cytosol estrogen receptors of granulosa cells. However, the binding affinity of estrogen nuclear receptors from whole ovarian homogenates (K_d = 1.26 x 10^{-9}M) was found to be much higher when only granulosa cells were used (K_d = 1.9 x 10^{-10}M).

Estrogen receptors in granulosa cells have biochemical characteristics similar to uterine estrogen receptors. Sucrose density gradient analysis has been previously used to characterize estrogen receptor complexes. The radioactive hormone in the cytosol sediments as a discrete band with a coefficient of 8S (Puca et al., 1971; Notides et al., 1972) and the estradiol-receptor complex in the nucleus sediments at 5S (DeSombre et al., 1969; Jensen et al., 1968). The

results in this study were similar to those previously described for estrogen receptors in uteri and other tissues (Saiddudin and Zassenhaus, 1977; Notides and Nielsen 1975; DeSombre et al., 1969; Puca and Bresciani, 1970). Binding specificity of estradiol to its receptor in granulosa cells is similar to that in ovarian homogenates (Saiddudin and Zassenhaus, 1977), corpora lutea (Yuh and Keys, 1979; Richards, 1974), and uteri (Cidlowski and Muldon, 1978; Richards, 1980). Also, estradiol receptor in granulosa cells was found to be proteinaceous and heat labile as demonstrated for estrogen receptors in ovarian homogenates (Saiddudin and Zassenhaus, 1977) and uterine preparations (Krozowski and Murphy, 1981; Cidlowski and Muldon, 1978). Finally, translocation of cytosol estrogen receptors into the nucleus after a bolus injection of estradiol was demonstrated in granulosa cells (Figure 8). Results were comparable to those found in uterine (Jensen et al., 1968) and ovarian (Saiddudin and Zassenhaus, 1977) tissue.

In conclusion, receptor sites specific for estradiol are present in nuclei and cytosol of granulosa cells from immature rats. Thus, many of the effects of gonadotropins, which stimulate estrogen synthesis, or of estradiol on ovarian follicular growth and development (Richards, 1980) may be mediated via the estrogen receptor in granulosa cells.

I next examined the affect of blocking steroidogenesis on the ability of FSH to increase receptors for FSH and estradiol.

CHAPTER II

EFFECT OF CYANOKETONE ON FSH-INDUCTION OF FSH RECEPTORS IN GRANULOSA CELLS OF THE RAT

Introduction

Although estradiol increases numbers of granulosa cells in ovaries, estradiol alone does not increase number of receptors for FSH per granulosa cell. FSH, however, can increase number of receptors for LH and FSH (Richards et al., 1976). Moreover, estradiol priming followed by administration of FSH increases levels of FSH receptors more quickly than FSH alone (Richards et al.,1976). Since FSH alone can increase production of estradiol (Moon et al., 1975; Zeleznik et al., 1974; Armstrong et al., 1979; Richards et al., 1982) and estradiol can enhance FSH action (Pencharz, 1940; Williams, 1945), a synergistic action of estradiol and FSH may be required for maturation of ovarian follicles. As an injection of estradiol and/or FSH increases numbers of nuclear estradiol receptors in granulosa cells (Richards, 1975) and uterine and ovarian weights (Pencharz, 1040; Williams, 1945), these effects of FSH are probably mediated via an FSH-induced increase in estradiol. I hypothesize that an increase in intraovarian levels of estradiol is required before FSH can increase its own receptor. I

attempted to test this hypothesis through the use of cyanoketone (2α-cyano-4, 4, 17α-tri-methylandrost-5-en-17B-ol-3-one), a potent antagonist to steroidogenesis which blocks the conversion of pregnenolone to progesterone. Cyanoketone also binds to the cytosolic receptor for estrogen thus blocking estrogen action (Wolfson et al., 1983).

Rather than attempt to determine the direct effects of cyanoketone on intraovarian production of estradiol, I examined whether cyanoketone pretreatment prevented FSH-induced increases in numbers of receptors for FSH and estradiol in granulosa cells as well as ovarian and uterine weights.

Materials and Methods

Materials

The following reagents were used: (2, 4, 6, 7, 16, 17-^{3}H)-estradiol-17B (136.1 Ci/mmol; ^{3}H-E_{17B}), ^{125}Iodine (IMS 30), promegestrone (R5020, NLP-004B, lot 1461-129), New England Nuclear; ovine FSH (S1390-2BR,100x FSH S10,0.056 units LH-S19/mg, oFSH), human chorionic gonadotropin (CR-119,11600 IU/mg, hCG); sodium thiocyanate (NaSCN, analytical grade), chloramine-T, Trizma-HCl (reagent grade), ethylenediamine tetraacetic acid (EDTA, Sigma grade), calf thymus DNA, diethylstilbestrol (DES), estradiol-17B (E_{17B}), testosterone, progesterone, dihydrotestosterone (DHT), charcoal (Norit A), Sigma Chemical Co.; Dextran T-70, Parmacia; aqueous counting scintillant (ACS), Amersham; propylene glycol, J.T. Baker Chemical Co.; sodium metabisulfite (reagent grade), Matheson, Coleman and Bell; Bio-Gel P-60 (100-200 mesh), Bio Rad Laboratories; 2 -cyano-4, 4, 17 -trimethylandrost-5-en-17B-ol-3-one (cyanoketone, R-103-UH), Sterling-Winthrop Research Institute (Renssilaer, N.Y.).

Animals

Immature female Sprague-Dawley rats, hypophysectomized on day 24 of age, were obtained from Hormone Assay Labs (Chicago, Illinois). Rats were housed under 12L:12D (24°C), and given food and water ad libitum. Rats were decapitated between 26 and 30 days of age.

Injection regime

Cyanoketone (0.05 mg, dose obtained from dose response), progesterone (2 mg), testosterone (2 mg), estradiol-17B (2 mg), R5020 (2 mg), or DHT (2 mg) dissolved in 0.2 ml propylene glycol were injected subcutaneously (s.c.) into rats (20 rats/treatment group, each experiment repeated twice). Twenty-four hours later, FSH (200 ug/0.2 ml PBS) was administered s.c. with or without cyanoketone, estradiol-17B, progesterone, testosterone, DHT, or R5020. Animals were then decapitated 12 or 24 h later.

The dose and preparation of FSH chosen in my study (200 ug/0.2 ml PBS) provided maximum increase in FSH receptor with little change in number of LH receptors (Table 3).

Tissue preparation

Ovaries and uteri were dissected, trimmed of fat, and weighed on a Mettler balance. After weighing, ovaries were placed in 0.01 M PBS buffer (pH 7.0) on ice.

Granulosa cells were obtained from rat ovaries (2 ovaries/tube) according to the method of Zeleznik et al. (1974); gentle pressure was applied to follicles to express cells. After centrifugation at 800 x g (20 min, 4°C), granulosa cells were washed three times in PBS buffer (2 ml/tube/wash). Cells were then resuspended in PBS buffer (2 ovaries /tube, 1 ml volume) and aliquots of 0.1 ml were used for FSH and LH receptor assays.

For estradiol receptor assays, granulosa cells were washed three times in TED buffer (1 mM Tris, 1.5 mM EDTA, pH 7.4), homogenized in a glass Dounce homogenizer, and centrifuged at 4,000 x g for 20 min at

TABLE 3

EFFECTS OF VARIOUS PREPARATIONS OF FSH ON FSH AND LH RECEPTORS IN GRANULOSA CELLS OF HYPOPHYSECTOMIZED IMMATURE RATS [a]

Type of FSH	Ovarian Weight (mg)	FSH Receptor (cpm/ug DNA)	LH Receptor (cpm/ug DNA)
Controls	7.1 ± 0.6	166.3	118.9
Estradiol	20.3 ± 1.7	358.3	292.3
Bovine FSH, NIH B1			
(200 ug)	23.2 ± 1.7	1040.1	2.7
Porcine FSH			
(100 ug)	35.7 ± 3.2	1135.8	192.9
(200 ug)	26.0 ± 5.3	1609.3	134.4
Rat FSH, NIAMDD B-1			
(100 ug)	48.0 ± 3.0	1245.9	68.5
(200 ug)	57.0 ± 4.1	831.9	302.7
Ovine FSH, Abbott			
(100 ug)	31.7 ± 3.5	1321.6	364.9
(200 ug)	22.5 ± 3.0	1468.8	-----
(400 ug)	24.2 ± 2.8	2109.5	-----
Human FSH, LER-1951			
(2 ug)	25.3 ± 1.8	2466.9	272.3
(10 ug)	49.3 ± 6.6	1927.9	1859.1
(50 ug)	50.0 ± 7.7	348.1	111.6
Ovine FSH, NIH S-9			
(100 ug)	35.4 ± 3.5	1993.6	282.7
(200 ug)	34.0 ± 2.8	615.1	1138.0

[a] Rats, hypophysectomized on day 24, were injected for 2 days with each of the FSH preparations listed in Table 1. Rats were killed 24 h after the last injection of FSH, ovaries were weighed, and granulosa cells collected. Saturation analysis was used to determine specific binding of ^{125}I-oFSH to granulosa cells. Data are expressed as cpm bound/ug DNA.

4°C. Supernatant was further centrifuged at 106,000 x g (type 30 rotor, Beckman) for 1 h (4°C) to obtain a cytosol preparation. Pellets were washed 3 times in TED buffer and resuspended in 0.5 ml TED for the nuclear receptor assay.

Measurement of specific FSH and LH binding sites

Ovine FSH (10 ug/10 ul) or hCG (5 ug/10 ul) was iodinated in the presence of chloramine-T and purified by gel filtration on a column of Bio-Gel P-60 as described previously (Spicer et al., 1981). Specific activity of radioactive oFSH and hCG was 24 cpm/pg and 19 cpm/pg, respectively.

The procedures used to measure specific binding sites in vitro have been described previously (Spicer et al., 1981). Briefly, aliquots of resuspended cells were incubated in triplicate at 27°C for 24 h with radioactive oFSH or hCG (200,000 cpm) in the presence or absence of unlabeled crude preparation of oFSH (kindly provided by Abbott Laboratories, Chicago, IL, 4 mg/ml, 0.02 ml/aliquot) or hCG (1 mg/ml, 0.02 ml aliquot). After incubation, samples were washed twice with PBS (4°C), centrifuged at 3,000 x g for 10 min (4°C), and radioactivity counted in a gamma counter. Specific binding, expressed as cpm ^{125}I-oFSH or ^{125}I-hCG/ug DNA, was calculated as the difference in counts bound in the presence (non-specific) or absence (total) of an excess of unlabeled hormone. DNA values were determined by the diphenylamine method of Burton (1956).

Estradiol receptor assays

I have previously identified the presence of cytosolic and nuclear estradiol receptor sites for rat granulosa cells (Chapter I). Assays

for estrogen receptors were performed as described in Chapter I (also see Appendix).

Radioimmunoassays for progesterone and testosterone

Trunk blood was collected and serum stored at -20°C until assayed. Serum progesterone (Louis et al., 1973, as modified by Convey et al., 1977) and serum testosterone (Mongkonpunya et al., 1975) were measured using radioimmunoassays previously validated in our laboratory.

Statistical analyses

One way analysis of variance and Bonferroni-t statistics (Gill, 1978) were used to test for significant changes in concentrations of progesterone, testosterone, weights of ovaries and uteri, and amounts of specific binding of ^{125}I-oFSH, ^{125}I-hCG, and ^{3}H-$E_{17\beta}$ to granulosa cells.

Results

Cyanoketone dose response

FSH increased ($p<0.01$) levels of its own receptor compared with control values (Figure 9). Cyanoketone at all doses tested (0.01-1 mg) reduced ($p<0.05$) the FSH-induced increase in levels of FSH receptor with maximal suppression at 0.05 mg. Ovarian and uterine weights were similar in the control and cyanoketone-treated groups (Table 4). In all subsequent experiments, 0.05 mg of cyanoketone was used.

Effects of cyanoketone on FSH-induced increases in estradiol, LH, and FSH receptor levels

Ovine FSH increased ($p<0.01$) FSH and estradiol receptor levels in granulosa cells by 12 and 24 h post injection (Figure 10). Cyanoketone suppressed the FSH-induced increase in FSH and estrogen receptors to values below or near controls at 12 and 24 h. Cyanoketone alone had no effect on estradiol or FSH receptor numbers in granulosa cells.

Levels of estradiol receptors in the cytosol were similar in saline- and cyanoketone+FSH-treated rats (Table 5). Numbers of cytosol receptors were slightly depressed in FSH-treated rats compared to controls at 12 and 24 h.

Number of LH receptors remained unchanged throughout the experiment (Table 6).

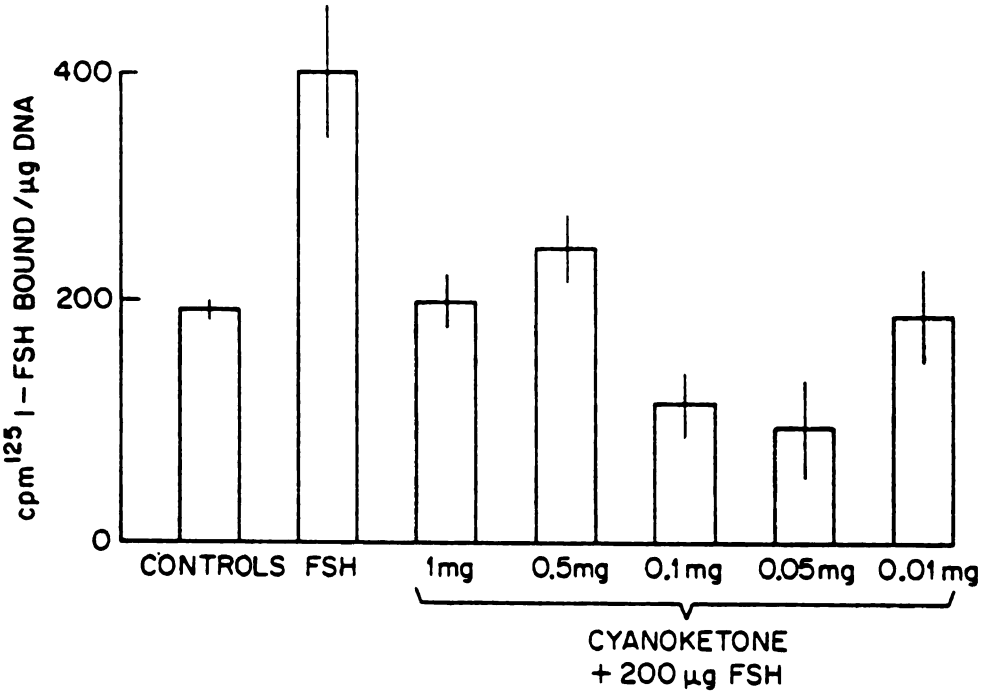

Figure 9. EFFECT OF VARIOUS DOSES OF CYANOKETONE ON SPECIFIC BINDING OF ^{125}I-oFSH TO GRANULOSA CELLS OF RATS. Immature rats, hypophysectomized on day 24, were injected with cyanoketone (0.01 to 1 mg) and oFSH 24 h later. Rats were killed 24 h after FSH administration. Granulosa cells were collected, and saturation analysis was used to determine specific binding of ^{125}I-oFSH to granulosa cells. Bars represent means $\pm$ SEM (n=10 rats/group).

TABLE 4

EFFECT OF VARIOUS DOSES OF CYANOKETONE PLUS FSH ON OVARIAN AND UTERINE WEIGHTS[a]

Treatment	Ovarian Weight (mg)	Uterine Weight (mg)
Controls	9.22 ± 0.53	36.84 ± 1.80
FSH	13.40 ± 1.06	43.34 ± 1.93*
FSH + Cyanoketone		
(0.01 mg)	10.01 ± 0.63	32.75 ± 2.12
(0.05 mg)	8.24 ± 0.52	32.94 ± 1.99
(0.10 mg)	8.18 ± 0.46	32.36 ± 1.97
(0.50 mg)	7.86 ± 0.44	29.92 ± 1.58
(1.00 mg)	8.96 ± 0.49	31.25 ± 1.89

[a]Experiment was conducted as described in Materials and Methods. Data are expressed as mean ± SEM, n = 20 rats/group.
*Statistically significant at the 0.05 level compared with controls.

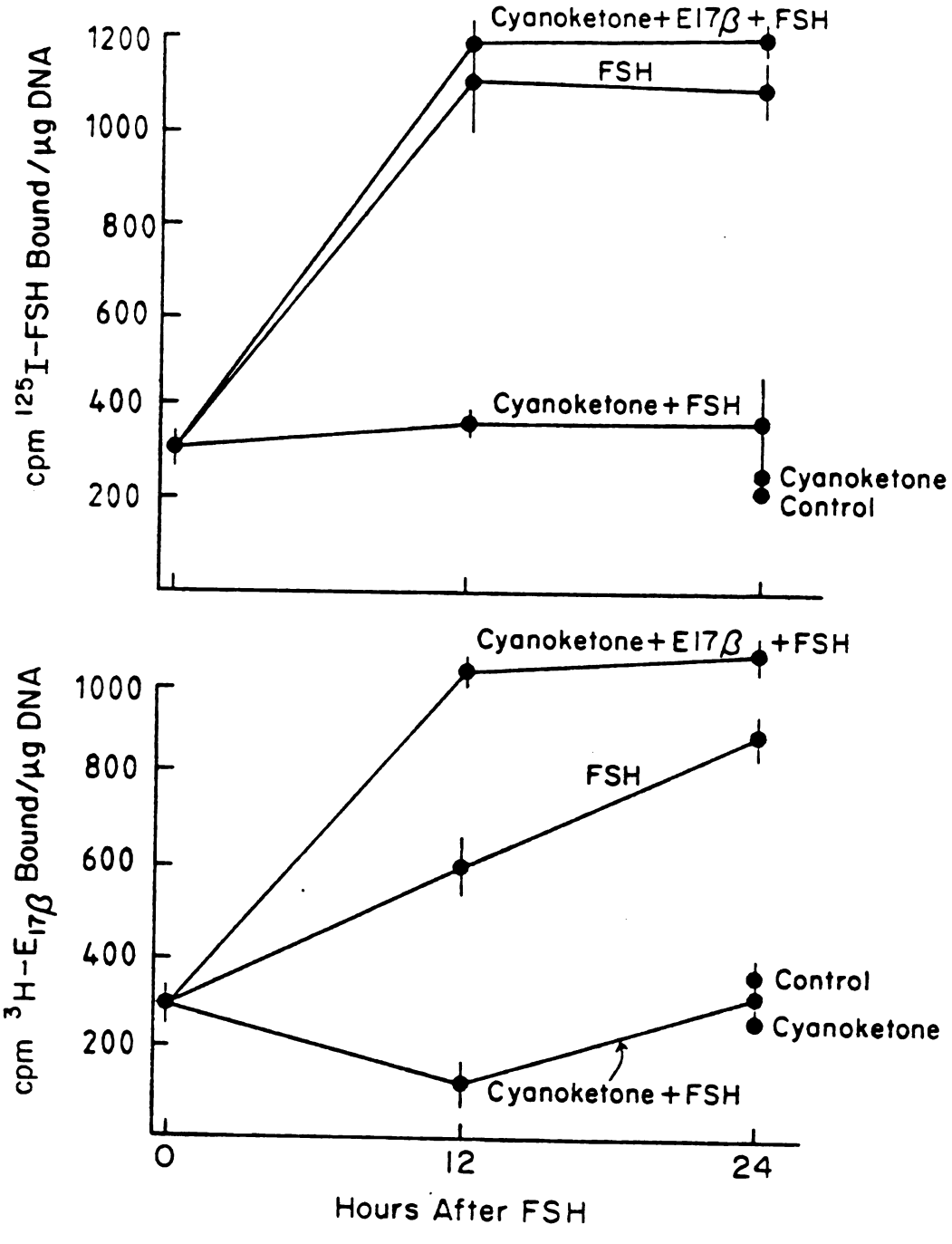

Figure 10. EFFECT OF CYANOKETONE ON FSH INDUCTION OF FSH AND ESTRADIOL BINDING SITES IN GRANULOSA CELLS OF RATS. Hypophysectomized rats (20/group) were injected with 0.05 mg of cyanoketone (s.c.). Twenty-four h later, rats were injected s.c. with 200 ug of oFSH. Dots represent means ± SEM (n=10 rats/group).

TABLE 5

EFFECT OF CYANOKETONE ON ESTRADIOL BINDING TO CYTOSOL OF GRANULOSA CELLS

Treatment	cpm/mg protein
Control	380
FSH	200
Cyanoketone	383
Cyanoketone + FSH	392

Data are expressed as means. N = 20 rats/group.

TABLE 6

EFFECT OF CYANOKETONE ON FSH INDUCTION OF LH BINDING SITES ON GRANULOSA CELLS

Treatment	LH Receptor (cpm/ug DNA)
Control	63.7 ± 15.3
FSH	69.8 ± 6.9
Cyanoketone	68.3 ± 16.7
Cyanoketone + FSH	71.6 ± 17.1

Data are expressed as mean ± SEM. N = 20 rats/group.

Effect of estrogen, progestin, or androgen replacement in cyanoketone-treated rats

Estradiol reversed the cyanoketone-induced blockage of FSH-stimulated increases in FSH (Figure 11) and estradiol receptors (Figure 12).

Progesterone partially reversed the effects of cyanoketone on the FSH-induced increase in FSH receptors, while receptors for estradiol were increased to levels above that after FSH administration (Figures 11 and 12, panel B). R5020 alone had no effect on levels of FSH or estradiol receptors compared with controls (Figures 11 and 12, panel B). Administration of R5020 with cyanoketone prior to FSH did not increase levels of FSH or estradiol receptors above controls. Testosterone, like progesterone, partially reversed the effects of cyanoketone on FSH-induced increases in FSH receptors and increased numbers of estrogen receptors to levels similar to that with FSH alone (Figures 11 and 12, panel C). DHT either alone or in combination with cyanoketone, had little effect on estrogen receptors; however, FSH receptors were increased to levels intermediate to controls and FSH-treated groups (Figures 11 and 12, panel C).

As shown in Table 7, cyanoketone alone had no effect on ovarian or uterine weights, or progesterone or testosterone levels in serum. FSH caused a slight increase in progesterone production and a significant ($p<0.05$) increase in ovarian weight by 24 h. Addition of cyanoketone prior to FSH prevented these increases. Estradiol administration concomitant with cyanoketone prior to FSH significantly increased ($p<0.01$) ovarian and uterine weights by 24 h, but had no effect on serum concentrations of progesterone or testosterone.

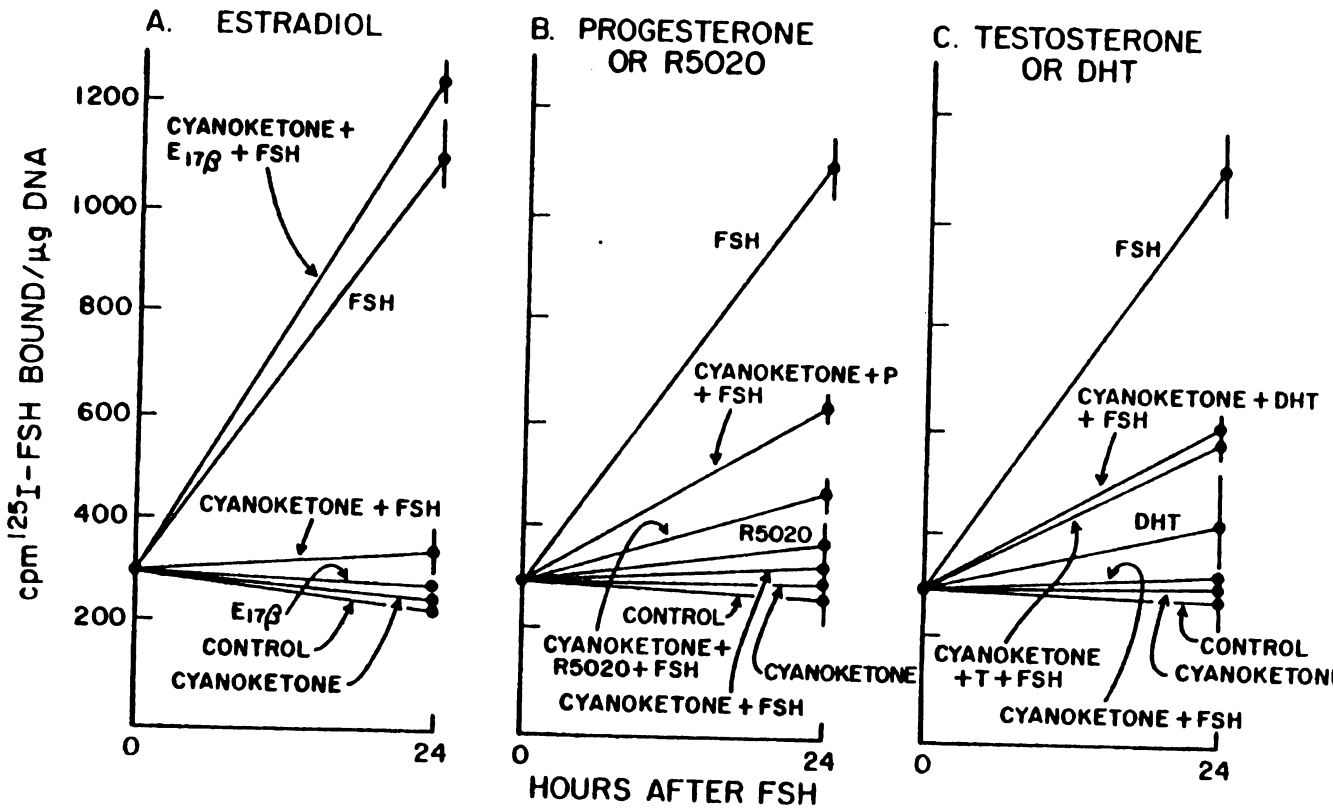

Figure 11. EFFECT OF ESTRADIOL, PROGESTIN, OR ANDROGEN REPLACEMENT ON ^{125}I-oFSH BINDING IN GRANULOSA CELLS OF CYANOKETONE-TREATED RATS. Hypophysectomized rats (20 rats/group) were injected with 0.05 mg cyanoketone and 2 mg of progesterone, testosterone, estradiol, R5020, or DHT (sc). Twenty-four h later, rats were injected sc with 200 ug oFSH. Dots represent means ± SEM.

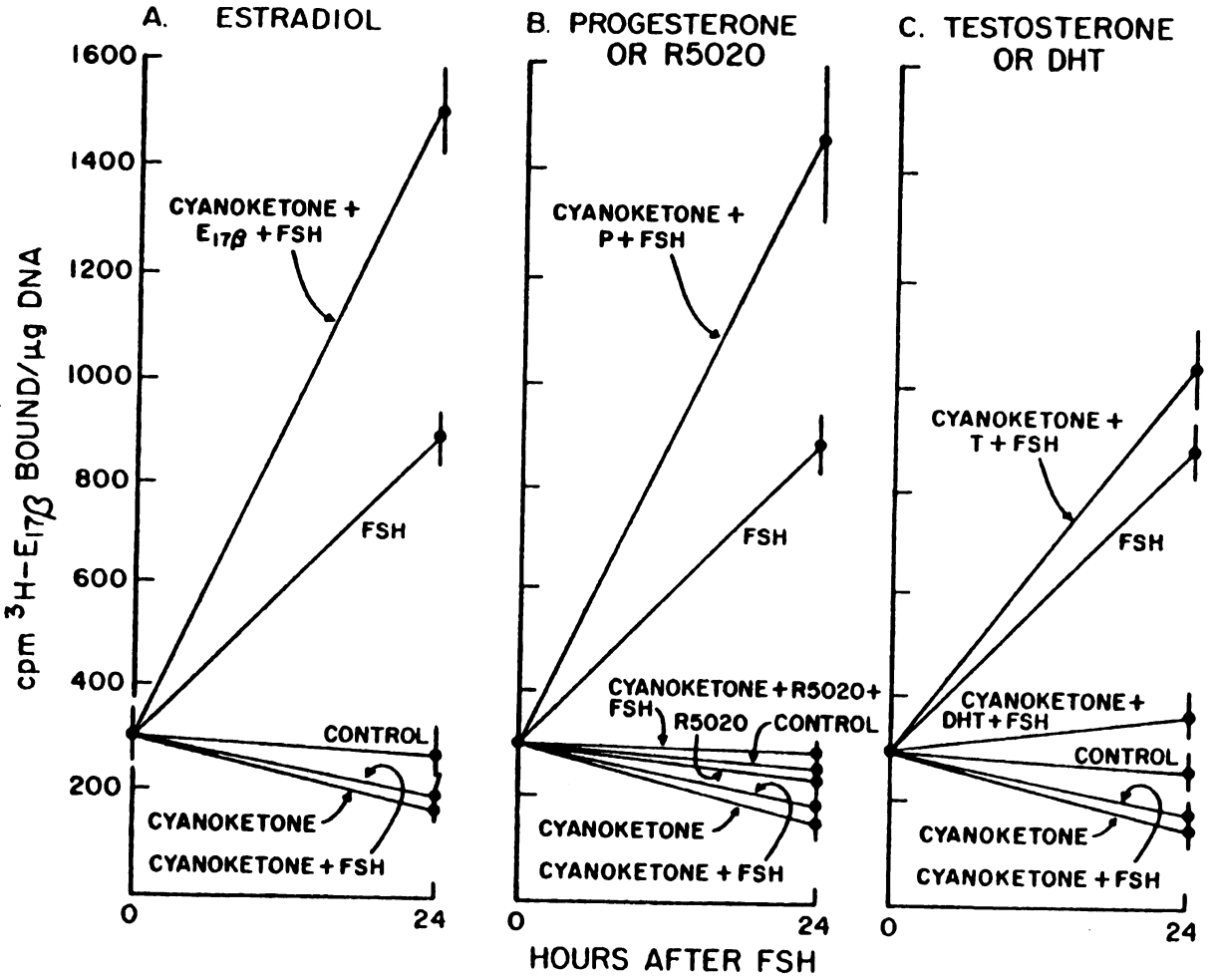

Figure 12. EFFECT OF ESTRADIOL, PROGESTIN, OR ANDROGEN REPLACEMENT ON BINDING OF ^{3}H-E$_{17B}$ TO NUCLEI OF GRANULOSA CELLS OF CYANOKETONE-TREATED RATS. Hypophysectomized rats (20/group) were injected with 0.05 mg cyanoketone and 2 mg progesterone, testosterone, estradiol, R5020, or DHT (sc). Twenty-four h later, rats were injected sc with 200 ug oFSH. Dots represent means ± SEM.

TABLE 7

EFFECT OF CYANOKETONE, oFSH, AND STEROIDS ON OVARIAN AND UTERINE WEIGHTS AND LEVELS OF PROGESTERONE AND TESTOSTERONE IN SERUM[a]

Treatment	Ovarian Weights (mg)	Uterine Weights (mg)	Progesterone (ng/ml)	Testosterone (ng/ml)
Controls	9.2 ± 0.6	24.0 ± 1.7	0.68 ± 0.03	0.27 ± 0.03
Cyanoketone	10.4 ± 1.5	24.8 ± 1.5	0.64 ± 0.08	0.26 ± 0.05
oFSH	15.1 ± 1.5*	34.9 ± 3.2*	0.96 ± 0.19	0.23 ± 0.03
oFSH + Cyanoketone	9.3 ± 0.7	34.6 ± 5.7	0.51 ± 0.07	0.33 ± 0.08
oFSH + E_{17B} + Cyanoketone	23.2 ± 2.7**	105.9 ± 12.2**	0.62 ± 0.04	0.36 ± 0.03
oFSH + R5020 + Cyanoketone	7.8 ± 0.6	30.6 ± 3.4	13.5 ± 3.4*	0.37 ± 0.03
oFSH + DHT + Cyanoketone	6.2 ± 0.8	25.3 ± 1.5	0.47 ± 0.09	27.10 ± 4.00*
oFSH + T + Cyanoketone	13.7 ± 1.4	33.3 ± 1.9	-------	-------
oFSH + P + Cyanoketone	13.3 ± 1.0	27.8 ± 1.7	-------	-------

[a]The experiment was conducted as described in Materials and Methods. Data are expressed as mean ± SEM, n = 20 rats/group. Cross reactivity of progesterone and testosterone assays with R5020 and DHT were 20% and 60%, respectively.

*Statistically significant at the 0.05 level compared with controls.

**Statistically significant at the 0.01 level compared with controls.

In summary, cyanoketone can block FSH-induced increases in FSH receptors. Although progesterone, testosterone, R5020 and DHT can partially reverse the inhibitory effects of cyanoketone, estradiol can completely reverse these inhibitory effects.

Discussion

A single injection of oFSH alone increased levels of receptors for FSH and estradiol, and ovarian and uterine weights within 24 h. However, if rats were pretreated with cyanoketone, a potent inhibitor of progesterone synthesis, all of the above actions of FSH were blocked. Estradiol replacement was able to fully reverse the effects of cyanoketone on FSH action. Replacement of other steroids either had no effect or partially reversed the effects of cyanoketone.

The effects of cyanoketone on blocking FSH-induced increases in the FSH and estradiol receptor were partially reversed with progesterone or testosterone. One reason for the increase in FSH receptor after replacement with progesterone or testosterone is that these steroids could be converted directly to estradiol. Also, progesterone and testosterone in high concentrations can interact with the estrogen receptor in the cytosol and cause translocation of the estrogen receptor to the nucleus, thus possibly mimicking estrogen action (Evans and Leavett, 1979; Farookhi, 1980; Nilsson et al., 1974). Therefore, the effects of testosterone, progesterone, and especially estradiol on FSH induction of FSH receptors may be mediated via the estrogen receptor. Nonmetabolizable forms of progesterone and testosterone, R5020 and DHT, were much less effective in reversing the effects of cyanoketone than progesterone and testosterone. Thus, the conversion of progesterone and testosterone to estradiol could explain some of these results. However, I cannot rule out the possible involvement of these steroids along with

estradiol in FSH action.

Although testosterone or progesterone could partially reverse the effects of cyanoketone, synthesis of estradiol appears to be required for FSH to maximally increase its own receptor. I have also shown that changes in number of estrogen receptors parallel changes in number of FSH receptors after FSH administration (Figures 11 and 12).

From my study, it appears that estradiol is the primary steroid involved in FSH induction of the FSH receptor in the rat. Although the biochemical action of estradiol is unknown, these data support my hypothesis that control of estradiol production may be important for FSH action, which, in turn, may promote follicular development.

Next, I examined more specifically the role of estradiol in FSH action by attempting to block estradiol action through its receptor rather than through synthesis of estradiol.

CHAPTER III

CI628 INHIBITS FSH-INDUCED INCREASES IN RECEPTORS FOR FSH IN THE OVARY OF THE RAT

Introduction

Differentiation of ovarian granulosa cells is hormonally regulated by an interaction of estradiol with the gonadotropins. Estradiol increases responsiveness of follicles to gonadotropins (Pencharz, 1940; Williams, 1945) while FSH promotes estrogen production and formation of antra in ovarian follicles (Moon et al., 1975; Zeleznik et al., 1974).

Although estradiol has no effect on FSH receptors, FSH can increase numbers of receptors for LH and FSH (Richards et al., 1976) per granulosa cell. However, estradiol priming prior to administration of FSH results in numbers of receptors for FSH which are significantly greater than when FSH is given alone (Richards et al., 1976). Since FSH promotes estrogen synthesis by granulosa cells (Moon et al., 1975), a synergistic action of estradiol with FSH on granulosa cells may be required for FSH to induce its own receptor.

If estradiol is required for FSH to increase its own receptor, blocking the action of estradiol should prevent the FSH-induced increase in FSH receptor. The anti-estrogen CI628 (α-4-pyrrolidino-ethoxy-phenyl-4-methoxy-α-nitrostilbene) was used to block the effects

of estradiol. Thus, I could evaluate if blocking the action of the estradiol receptor has an effect on FSH-induced stimulation of the FSH receptor.

Materials and Methods

Materials

The following reagents were used: (2, 4, 7, 16, 17-^{3}H)-estradiol-17B (137 Ci/mmol; $^{3}H\text{-}E_{17B}$), cyclic adenosine 3', 5'-monophosphate (cAMP) ^{125}I-radioimmunoassay kit (lot D0227AK), ovine FSH (S1390-2BR,100x FSH S10, 0.56 units LH-S19/mg, oFSH); human FSH (LER 1951, LH 100 IU/mg, FSH 6781 IU/mg, hFSH); human chorionic gonadotropin (CR-119, 11600 IU/mg, hCG); bovine luteinizing hormone (NIAMDD-bLH-4, bLH); sodium thiocyanate (NaSCN, analytical grade), chloramine-T, Trizma-HCl (reagent grade), ethylenediamine tetracetic acid (EDTA, Sigma grade), calf thymus DNA, diethylstilbestrol (DES), estradiol-17B (E_{17B}), charcoal (Norit A), Sigma Chemical Co.; Dextran T-70, Pharmacia; aqueous counting scintillant (ACS), Amersham; propylene glycol, J.T. Baker Chemical Co.; sodium metabisulfite (reagent grade), Matheson, Coleman and Bell; Bio-Gel P-60 (100-200 mesh), Bio-Rad Laboratories; α-4-pyrrolidino-ethoxy-phenyl-4-methoxy-α-nitrostilbene (CI628), Park-Davis Corp. (Ann Arbor, MI).

Animals

Immature female Sprague-Dawley rats, hypophysectomized on day 24 of age, were obtained from Hormone Assay Laboratories (Chicago, Illinois). Rats were housed under 12L:12D and given food and water *ad libitum*. Aninals were decapitated between 26 and 30 days of age.

Tissue preparation

Ovaries and uteri were quickly dissected, trimmed of fat, and weighed on a Mettler balance. After weighing, ovaires were immediately placed in ice-cold 0.01 M phosphate buffered saline (PBS; pH 7.0) and uteri were discarded. A pair of ovaries from each treatment group was processed for histology to insure there were no gross toxic effects of CI628.

Granulosa cells were obtained according to the method of Zeleznik et al. (1974); gentle pressure was applied to follicles and expressed cells were collected after centrifugation at 800 x g (20 min, 4°C). Granulosa cells were washed three times in PBS buffer. Cells were then resuspended in ice-cold PBS (2 ovaries/ml; 0.1 ml aliquots) for FSH and LH receptor assays.

For the estradiol receptor assay, granulosa cells were washed three times in ice-cold TED buffer (10 mM Tris, 1.5 mM EDTA, pH 7.4), homogenized in a glass Dounce homogenizer, and centrifuged at 4,000 x g for 20 min at 4°C. Pellets were washed three times in ice-cold TED buffer and resuspended in 0.5 ml TED for the nuclear receptor assay.

Iodination procedures

Ovine FSH (10 ug/10 ul) or hCG (5 ug/10 ul) was iodinated by the chloramine-T method as previously described (Spicer et al., 1981). Specific activity for radioactive oFSH and hCG was 24 cpm/pg and 19 cpm/pg, respectively.

Measurement of specific FSH and LH binding sites after hormone treatments

Saturation analyses were used to measure available specific binding sites for LH and FSH. Aliquots (0.1 ml) of resuspended cells were

incubated in triplicate at 27°C for 24 h with ^{125}I-oFSH or ^{125}I-hCG (200,000 cpm) in the presence or absence of an unlabeled, crude preparation of oFSH (4 mg/ml, 0.02 ml aliquot) or hCG (1 mg/ml, 0.02 ml aliquot). After incubation, samples were washed three times with PBS (4°C), centrifuged at 3,000 x g for 10 min (4°C), and radioactivity in the pellet counted in a gamma counter. Specific binding, expressed as cpm specifically bound/ug DNA, was calculated as the difference in counts bound in the presence (nonspecific) or absence (total) of an excess of unlabeled hormone. DNA in samples was from 3 to 6 ug per tube.

Estradiol receptor assay

I have previous identified the presence of cytosolic and nuclear estradiol receptor sites for estradiol in granulosa cells of rats (Chapter I). Assays for nuclear estrogen receptors were performed as described in Chapter I (see Appendix).

To insure that CI628 did not interfere with the nuclear exchange assay, half of a pool of nuclei from granulosa cells was pre-incubated with CI628. The estrogen receptor assay was then performed. The number of receptors for estradiol were similar whether or not the tissue was pre-incubated with CI628 (Table 8). I therefore concluded that endogenous CI628 remaining after isolation of granulosa cells did not interfere with the *in vitro* exchange of ^{3}H-E_{17B} in the nuclear exchange assay.

Radioimmunoassays for progesterone and testosterone

Trunk blood was collected and serum stored at -20°C until assayed. Serum progesterone (Louis *et al.*, 1973, as modified by Convey *et al.*,

1977) and serum testosterone (Mongkonpunya et al., 1975) were measured using radioimmunoassays previously validated in our laboratory.

TABLE 8

ESTRADIOL BINDING TO GRANULOSA CELLS WITH AND WITHOUT PREINCUBATION WITH CI628

Treatment	cpm/ug DNA
with CI628 preincubation	253.3 ± 4.9
without CI628 preincubation	275.3 ± 5.7

Experiment was conducted as described in Materials and Methods. Data are expressed as mean ± SEM. N=5.

Radioimmunoassay for cAMP

Cyclic adenosine monophosphate levels in granulosa cells were determined utilizing a cAMP-^{125}I-RIA kit from New England Nuclear (lot D0227A K).

Dose response for CI628

To determine the dose of CI628 which would block estrogen action without major agonistic effects, 0.1, 1, 5, or 10 mg CI628/0.2 ml propylene glycol was given subcutaneously (s.c.). Six hours later, hFSH (2 ug/0.2 ml PBS) was administered s.c. Animals were decapitated 12 h after hFSH was injected.

Effect of CI628 on FSH induction of FSH receptors

Hypophysectomized rats were divided into five groups (20 rats/group): (1) saline-treated, (2) CI628-treated (1 mg/0.2 ml

propylene glycol), (3) hFSH-treated (2 ug/0.2 ml PBS), (4) CI628-treated, then hFSH 6 h later, and (5) CI628 plus estradiol (2 mg/0.2 ml propylene glycol), then hFSH 6 h later. Animals were decapitated 0, 6, 12, or 24 h after an injection of hFSH.

Scatchard analyses of FSH and estradiol receptors after CI628 administration

For the FSH Scatchard plot, pools of granulosa cells (20 rats/group) were incubated with 20 to 800 x 10^3 cpm of ^{125}I-oFSH with or without an excess of oFSH. For the Scatchard plots for estradiol, granulosa cell nuclei were incubated with 1 to 20 nM of $^{3}H\text{-}E_{17B}$ with or without excess DES. Assay procedures for FSH and estradiol were as described above.

Histology

Tissue was prepared for histology by fixation in Bouins fluid followed by dehydration in ethanol. Tissue was then embedded in paraffin, sectioned, and stained with hematoxylin and eosin, and evaluated under a microscope for signs of atresia.

Effect of CI628 on FSH stimulation of cAMP

Maximum stimulation of cAMP after hFSH (2 ug/rat) was determined. Rats were initially injected s.c. with saline or hFSH (2 ug/rat). Then 6 or 12 h later, rats were injected a second time with 2 ug of hFSH in the tail vein and content of cAMP in granulosa cells was determined. Animals were killed 30, 60, and 120 min after the tail vein injection of FSH. Granulosa cells were quickly expressed in 0.2 ml PBS. Expressed cells for each rat were immediately placed in a tube with 0.8 ml of distilled water at 100°C for 10 min. Tubes were then spun at 15,000 x g for 20 min (4°C). The supernatant was decanted and frozen

until assayed.

From the results of the time response (Table 9), the adenylate cyclase system was more responsive after a tail vein injection of hFSH at 12 h rather than 6 h after the initial s.c. injection of hFSH. Sixty min after the second tail vein injection of hFSH, cAMP levels were maximally increased. Therefore, these times were chosen for future experiments.

Rats (20 rats/group) were then divided into four treatment groups: (1) saline-treated, (2) CI628-treated, (3) hFSH-treated, and (4) CI628-treated plus hFSH 6 h later. Twelve h after FSH, all groups received a tail vein injection of 2 ug of hFSH. Sixty min later, rats were decapitated, and granulosa cells expressed and processed as described above.

Statistical analysis

One way analysis of variance and Bonferroni-t statistics (Gill, 1978) were used to test for significant changes in concentrations of progesterone, testosterone, cAMP content, weights of ovaries and uteri, and amounts of specific binding of ^{125}I-oFSH, ^{125}I-hCG, and ^{3}H-E_{17B} to granulosa cells. Scatchard plots (Scatchard, 1949) were analyzed by linear regression.

TABLE 9

TIME COURSE OF hFSH STIMULATION OF cAMP CONTENT IN GRANULOSA CELLS OF RATS

Treatment	cAMP (pmoles/ug DNA)
hFSH- 6 h	
30 min	1.23 ± 0.32
hFSH- 12 h	1.01 ± 0.26
30 min	3.99 ± 0.13*
60 min	5.77 ± 0.19*
120 min	1.17 ± 0.21

The experiment was conducted as described in Materials and Methods. Data are expressed as mean + SEM, n=10 rats/group.

*Statistically significant at the 0.05 level compared with controls.

Results

Dose response for CI628

There was a significant ($p<0.01$) increase in receptors for FSH (Figure 13, panel A) and E_{17B} (Figure 13, panel B) 12 h after FSH administration. These increases were suppressed ($p<0.05$) by all doses of CI628. Maximum suppression ($p<0.01$) occurred with 1 mg of CI628. CI628 alone had no significant effect on FSH or estradiol receptor numbers. To examine whether CI628 had any estrogenic effects, ovarian and uterine weights were obtained. CI628 at all doses tested had no effect on ovarian or uterine weights (Table 10). Histologically, ovaries of CI628-treated rats appeared similar to ovaries of controls. Since 1 mg of CI628 was as effective as 5 or 10 mg, the 1 mg dosage of CI628 was used in all subsequent experiments.

Effect of CI628 on FSH induction of receptors for FSH, LH, and estradiol

At 0, 6, 12, and 24 h, levels of receptors for FSH were similar in saline- and CI628-treated animals (Figure 14, top panel). From 6 to 24 h after FSH alone, numbers of FSH receptors were increased significantly ($p<0.01$) over controls. Although administration of CI628 prior to FSH prevented the FSH-induced increase in the FSH receptor, administration of estradiol concomitant with CI628 was able to reverse this effect.

Numbers of estradiol receptors in the nucleus were similar and unchanged in CI628- and saline-treated groups (Figure 14,bottom panel). Number of nuclear receptors for estradiol was significantly ($p<0.01$)

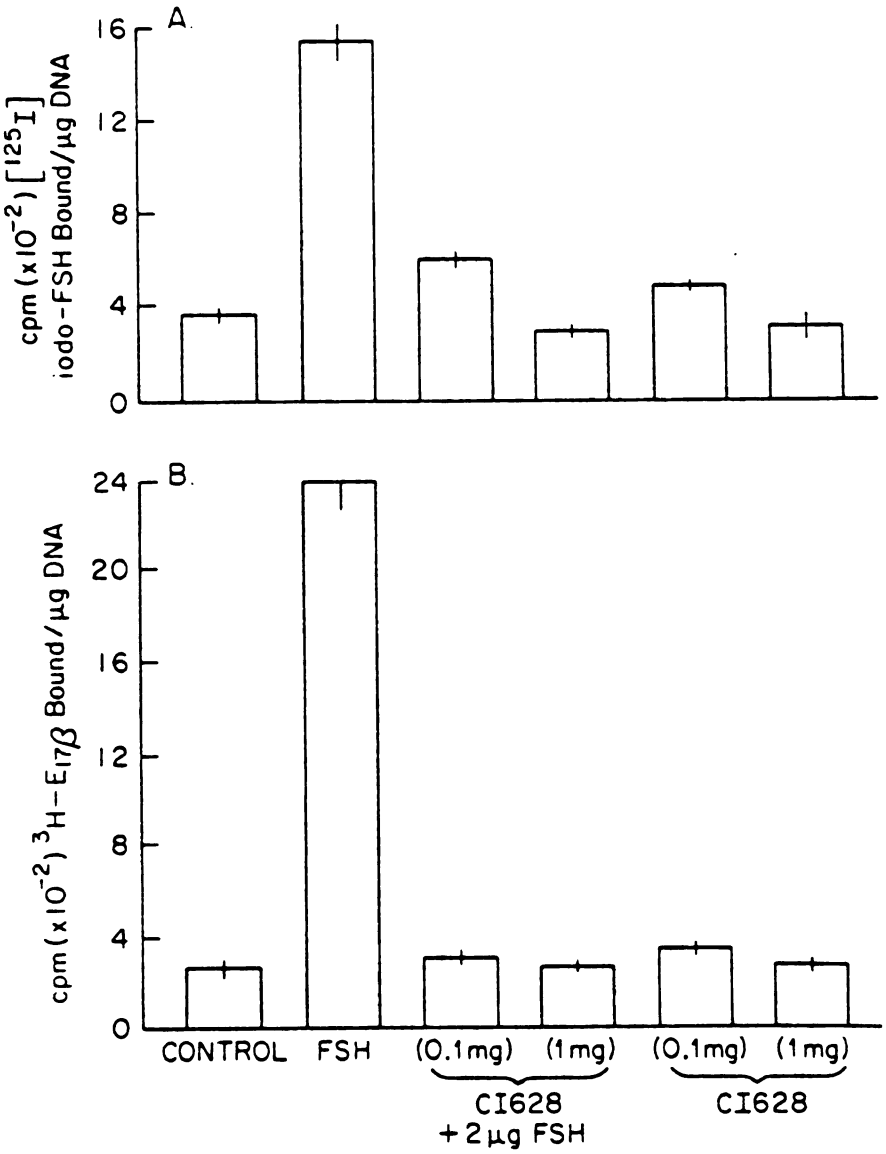

Figure 13. EFFECT OF VARIOUS DOSES OF CI628 ON SPECIFIC BINDING OF ^{125}I-oFSH TO GRANULOSA CELLS AND ^{3}H-E_{17B} TO NUCLEI OF GRANULOSA CELLS. Immature rats hypophysectomized (n=20 rats/group) on day 24 were injected with four doses of CI628 (0.1, 1, 5, or 10 mg) alone or in combination with 2 ug of hFSH. CI628 was injected 6 h prior to hFSH, and rats (20/group) were killed before (0 h) and 12 h after hFSH was injected. Granulosa cells were collected, and saturation analyses used to determine specific binding of ^{125}I-oFSH (A) or ^{3}H-E_{17B} (B) to granulosa cells. Data are expressed as cpm specifically bound/ug DNA. Bars represent means ± standard error. Results obtained with 5 and 10 mg of CI628 (data not shown) were similar to those with 1 mg.

TABLE 10

EFFECT OF VARIOUS DOSES OF CI628 AND FSH ON OVARIAN AND UTERINE WEIGHTS

Treatment		Ovarian Weight(mg)	Uterine Weight(mg)
Controls		11.0 ± 0.6	47.4 ± 3.2
FSH		9.3 ± 0.4	46.4 ± 0.5
CI628	(0.1 mg)	10.4 ± 0.4	45.1 ± 0.8
	(1.0 mg)	9.6 ± 0.8	47.4 ± 0.6
	(5.0 mg)	10.3 ± 0.6	48.4 ± 0.2
	(10.0 mg)	10.5 ± 1.1	50.1 ± 6.2
FSH+CI628	(0.1 mg)	10.2 ± 0.7	46.3 ± 3.4
	(1.0 mg)	9.4 ± 0.2	41.4 ± 3.3
	(5.0 mg)	10.4 ± 1.1	47.2 ± 0.8
	(10.0 mg)	10.3 ± 0.9	46.1 ± 0.8

Experiment was conducted as described in legend of Figure 13. Data are expressed as mean + SEM, n = 20 rats/group.

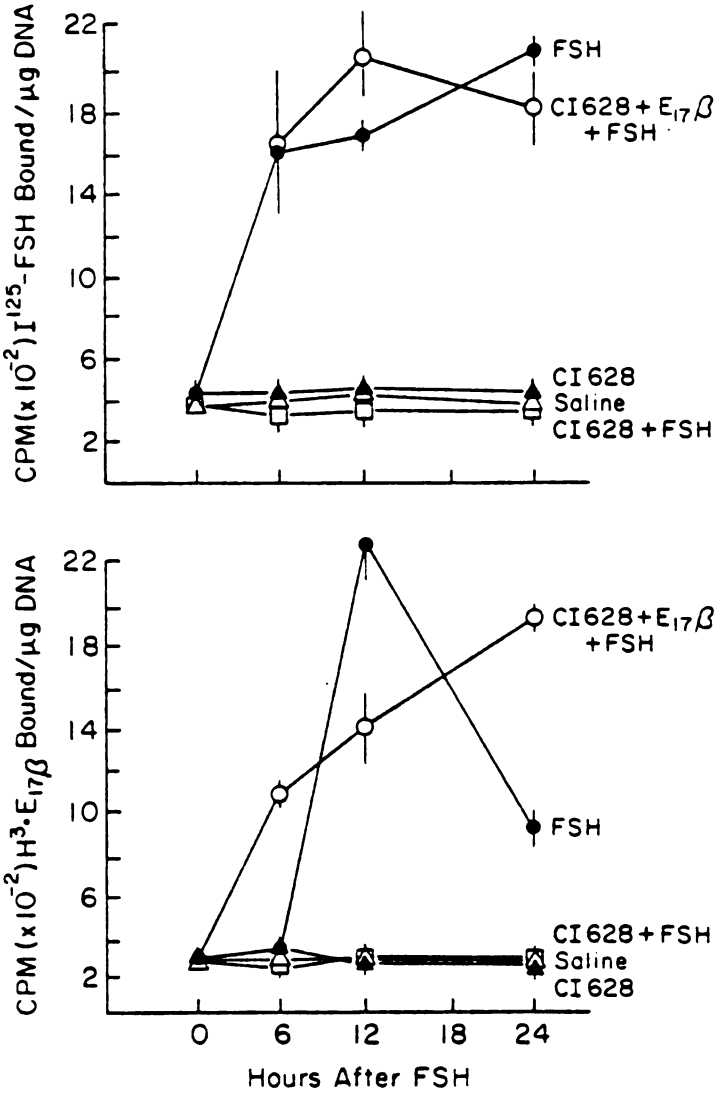

Figure 14. EFFECT OF CI628 ON FSH INDUCTION OF FSH AND NUCLEAR ESTRADIOL BINDING SITES ON GRANULOSA CELLS. Hypophysectomized rats (20/group) were divided into 5 groups: 1) saline-treated, 2) CI628-treated (1 mg), 3) hFSH-treated (2 ug), 4) CI628-treated with hFSH 6 h later, and 5) CI628 plus E_{17B} (2 mg) with hFSH 6 h later. Rats were killed at 0, 6, and 12 h. Dots represent means $\pm$ SEM. (n = 20 rats/group).

elevated by 12 h after FSH alone but decreased ($p<0.01$) by 24 h. CI628 treatment prior to FSH prevented the FSH- induced increase in estradiol receptors. However, levels of estradiol receptors increased ($p<0.01$) over controls beginning 6 h after treatment with hFSH.

Levels of receptors for LH (hCG) were unchanged unless estradiol administration preceeded an FSH injection. In this group (CI628+E_{17B}+ FSH), LH receptor numbers increased 3 to 4-fold at 6, 12, and 24 h (Table 11).

To insure that increases in FSH and E_{17B} receptors after FSH injection were due to an increase in numbers of receptors and not a change in affinity, Scatchard analyses were performed. At 0 h, affinities and binding capacity for FSH or estrogen receptors were similar for saline-treated and CI628-treated animals (Figure 15, Table 12). At 6 h, there were no changes in numbers of receptors for estrogen in all groups. However, number of FSH receptors were increased after FSH administration. By 12 h, numbers of FSH and estrogen receptors were increased further after FSH administration while levels of FSH receptors in other treatment groups were similar to controls. Affinity and number of estradiol or FSH receptors for CI628 or CI628+FSH groups 6 and 12 h after treatment were similar to values at 0 h (Figure 15, Table 12).

Effect of CI628 on FSH-induced changes in ovarian and uterine weights and plasma levels of progesterone and testosterone

Ovarian and uterine weights were similar in saline-treated, CI628-treated, and CI628 plus FSH-treated animals (Table 13). Rats injected with hFSH had ovarian and uterine weights similar to saline-treated controls at 6 and 12 h, but by 24 h ovarian and uterine weights had

TABLE 11

EFFECT OF CI628 ON FSH INDUCTION OF LH BINDING SITES ON GRANULOSA CELLS

Treatment	LH RECEPTOR (cpm/ug DNA) 0 h	6 h	12 h	24 h
Control	206.8 ± 19.6	189.3 ± 9.2	190.7 ± 30.3	237.5 ± 6.4
hFSH	--------	245.1 ± 29.2	188.5 ± 17.3	192.8 ± 11.9
CI628	247.0 ± 10.2	279.4 ± 16.3	258.4 ± 7.8	282.4 ± 17.8
CI628+hFSH	--------	204.1 ± 13.1	185.0 ± 24.9	207.8 ± 15.2
CI628+E_{17B}+ hFSH	--------	244.0 ± 24.1	695.0 ± 48.0*	770.5 ± 18.4*

Experiment conducted as described in legend of Figure 14.
* Statistically significant at the 0.01 level compared with controls.

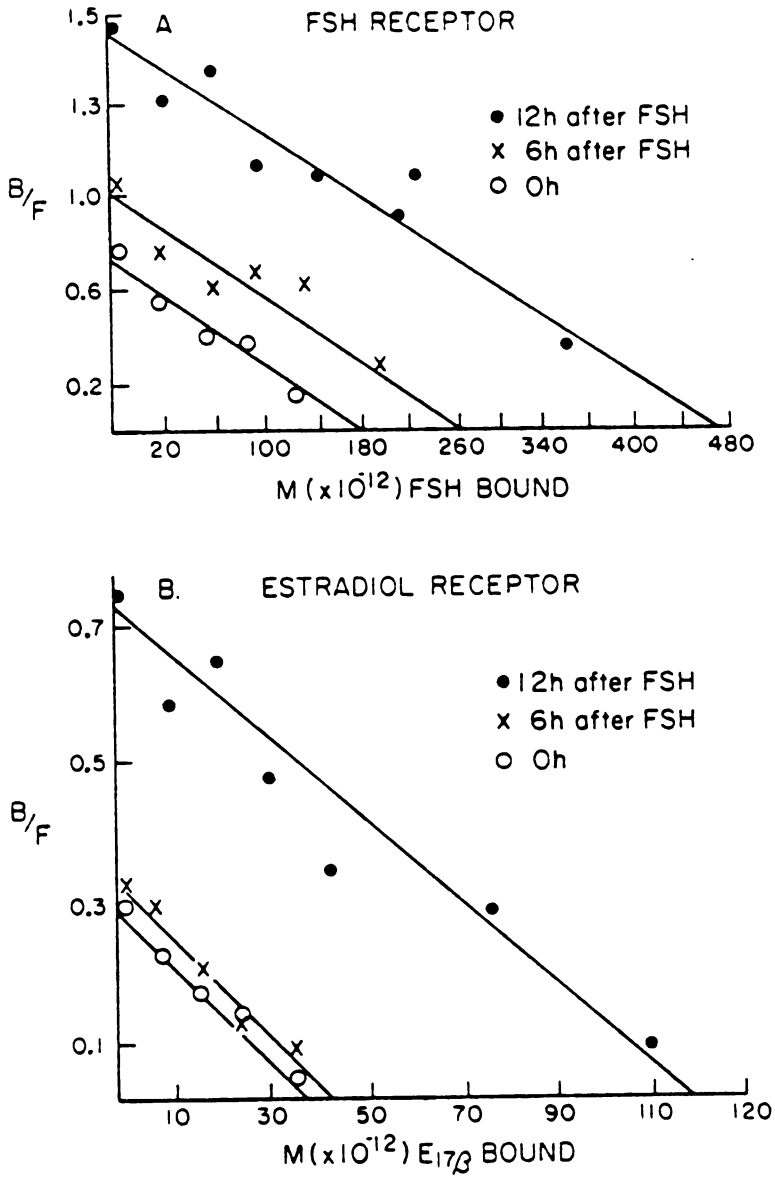

Figure 15. EFFECTS OF CI628 ON hFSH INDUCTION OF FSH AND NUCLEAR ESTRADIOL RECEPTORS. Rats were hypophysectomized on day 24 and divided into five treatment groups. Pools of granulosa cells were obtained at 0, 6, and 12 h after administration of hFSH. Only Scatchard plots for data from the saline-treated group at 0 h (O) and FSH-treated groups at 6 (X) and 12 h (o) are shown since binding affinities of FSH (panel A) or estradiol (panel B) for their receptors and number of FSH or estradiol receptors in the saline, CI628, and CI628+FSH groups were similar at 0, 6, and 12 h.

TABLE 12

EFFECT OF CI628 ON FSH INDUCTION OF FSH AND NUCLEAR ESTRADIOL RECEPTORS IN GRANULOSA CELLS OF RATS

Treatments		FSH Receptors		Nuclear Estradiol Receptor	
		K_a ($\times 10^{-9}$)	R_o (pM)	K_d ($\times 10^{-10}$)	R_o (pM)
Controls	0 h	4.28	153	1.5	46
	6 h	3.50	199	1.1	40
	12 h	4.14	181	1.6	43
CI628	0 h	4.66	173	1.5	42
	6 h	5.36	166	1.5	38
	12 h	3.37	190	1.6	42
hFSH	6 h	3.70	261	1.4	45
	12 h	3.29	470	1.5	120
CI628 + hFSH	6 h	3.90	197	1.5	43
	12 h	4.18	191	1.5	45

Experiment was conducted as described in legend of Figure 15.

TABLE 13

EFFECT OF CI628 AND hFSH ON OVARIAN AND UTERINE WEIGHTS AND LEVELS OF PROGESTERONE AND TESTOSTERONE IN SERUM

Treatments		Ovarian Weight (mg)	Uterine Weight (mg)	Progesterone (ng/ml)	Testosterone (ng/ml)
Saline, CI628, or FSH + CI628 [a]		10.0 ± 0.5	92.0 ± 3.5	0.33 ± 0.02	0.22 ± 0.02
hFSH	6 h	10.6 ± 0.4	88.6 ± 5.8	0.34 ± 0.01	0.17 ± 0.01
	12 h	10.1 ± 0.5	90.7 ± 4.6	0.35 ± 0.01	0.26 ± 0.01
	24 h	29.9 ± 0.9*	218.6 ± 4.9*	0.60 ± 0.02*	0.23 ± 0.02
FSH+E_{17B}+ CI628	6 h	18.4 ± 0.7*	170.6 ± 10.2*	0.48 ± 0.01*	0.29 ± 0.02
	12 h	23.3 ± 0.7*	211.9 ± 11.0*	0.60 ± 0.01*	0.29 ± 0.02
	24 h	28.2 ± 0.5*	265.4 ± 11.7*	0.67 ± 0.02*	0.23 ± 0.02

[a] Experiment was conducted as described in legend for Figure 15. As results for the saline, CI628, and FSH+CI628-treated groups were not statistically different at all times, this data was pooled.

*Statistically different from controls at the 0.05 level of significance. Data are expressed as mean ± standard error.

increased significantly ($p<0.05$). Ovarian and uterine weights in rats injected with estradiol and CI628 prior to FSH were heavier ($p<0.05$) than controls at all times sampled.

Testosterone levels were similar in all groups across all times studied and averaged 0.24 ng/ml (n=15). FSH caused a 2-fold ($p<0.05$) increase in serum progesterone levels at 24 h compared to controls, but pre-treatment with estradiol concomitant with CI628 prior to FSH significantly increased ($p<0.05$) progesterone levels over controls at 6, 12, and 24 h. Levels of progesterone were unchanged in all other treatment groups.

Effect of CI628 on FSH stimulation of cAMP

Levels of cAMP were similar in saline- and CI628-treated rats (Table 14). FSH treatment significantly increased ($p<0.01$) cAMP levels over controls; whether or not rats were pre-treated with CI628.

TABLE 14

EFFECT OF CI628 ON hFSH STIMULATION OF cAMP IN GRANULOSA CELLS[a]

Treatment	cAMP (pmoles/ug DNA)
Saline-treated	0.96 ± 0.09
CI628	0.95 ± 0.13
hFSH	6.10 ± 0.45*
CI628 + hFSH	6.70 ± 0.54*

[a] The experiment was conducted as described in Materials and Methods (Chapter III). Data are expressed as mean ± SEM, n = 10 rats/group. The experiment was repeated twice with similar results.

* Statistically significant at the 0.01 level compared with controls.

Discussion

Growth and differentiation of ovarian follicles are promoted by pituitary gonadotropins and gonadal steroids (Pencharz, 1940; Williams, 1945; Zeleznik et al., 1974; Moon et al., 1975). Administration of the antiestrogen cis-clomiphene can prevent increased ovarian weight and follicular growth induced by FSH (Nakano et al., 1982). Furthermore, FSH plus clomiphene prevents an increase in tertiary follicle development and suppresses mitosis of granulosa cells induced by FSH. Tamoxifen, another antiestrogen, can inhibit FSH-stimulated follicle growth and estrogen synthesis (Watson and Howson, 1977). This suggests that FSH-induced follicle growth and estrogen synthesis might be mediated by estrogens.

In hypophysectomized rats, estradiol (administered for 1-4 days) has little effect on numbers of FSH receptors per granulosa cell, although it enhances the increase in FSH receptors after administration of FSH (Louvet and Vaitukaitus, 1976; Richards et al., 1976). Estradiol enhancement of FSH stimulation of FSH receptor is time dependent (Ireland and Richards, 1978). Estradiol-priming for 12 to 24 h, but not 6 h, enhances the FSH-stimulated increase in its own receptor. From the data in this chapter, hFSH can increase its own receptor by 6 h with no increase in estradiol receptors (Figures 14 and 15) until 12 h. Thus, changes in the nuclear estradiol receptor may be independent of changes in the FSH receptor. Alternatively, the possible explanation for this increase in FSH receptor without a change

in $E_{17\beta}$ receptor is that the amount of nuclear estradiol receptor in granulosa cells prior to FSH may have been sufficient to enable a synergism of estradiol with FSH. In turn, this could result in FSH induction of its own receptor. Also, an increase in nuclear estradiol receptor could have occurred but may have been undetected in our assay system. However, since administration of CI628 prior to hFSH blocked the increase in FSH receptor at 6 h (Figure 14), it appears that the estrogen receptor is linked to FSH induction of its own receptor. As there was no increase in LH receptor or changes in testosterone levels at this time, significant contamination of the FSH preparation with LH, and thus synergism of FSH and LH was unlikely.

Although the mechanism by which FSH and estradiol interact in granulosa cells is unknown, the effects do not seem to be at the level of cAMP stimulation. CI628 does not block the ability of FSH to increase levels of cAMP. From my receptor studies, the number of receptors for FSH in the FSH-treated animals was approximately 3 to 6-fold higher (Figures 13 and 14) than FSH receptors present in the CI628+FSH- treated rats 12 h after an injection of hFSH, yet the amount of cAMP stimulated after FSH administration was similar in both groups. Therefore, the amount of cAMP produced could be independent of the amount of receptors present. After a critical number of receptors are bound with hormone, maximum cAMP response appears to occur. This phenomena of "spare receptors" occurs in other tissues (Catt and Dufau, 1973; Mendelson et al., 1975; Naor et al., 1980). Also, increases in FSH-responsive adenylate cyclase in granulosa cells of preovulatory follicles are not directly related to increases in FSH binding sites

per granulosa cell (Jonassen et al., 1982). However, though the cAMP response was similar in the hFSH- and CI628+hFSH-treated groups, the stimulation of progesterone and increases in ovarian and uterine weights were significantly different in these groups. This indicates that the interaction of FSH and estradiol appears to occur distal to the receptor-adenylate cyclase-cAMP response system. And, though cAMP may increase in the absence of changes in numbers of FSH or E_{17B} receptors, the actions of FSH and estradiol may not be expressed unless concomitant increases occur in number of receptors for each of these hormones. For example, estradiol induction of proteins which are later phosphorylated may be required for FSH induction of its own receptor, progesterone synthesis, and ovarian and uterine weight gains.

My study indicates that FSH induction of its own receptor requires estradiol, although I cannot categorically rule out involvement of other steroids. The exact mechanism by which estradiol interacts with FSH remains to be determined. However, estradiol appears to be required for FSH action, and estradiol appears to exert its effects at some point after the FSH receptor-adenylate cyclase-cAMP interaction.

GENERAL DISCUSSION

The main objective of this dissertation was to determine if estradiol is required for FSH to increase the number of FSH receptors in granulosa cells. As the preparations of granulosa cells used in my studies probably had some contamination of thecal cells, it is possible that some of my results are due to FSH action on thecal cells. However, FSH binding appears to be only to granulosa cells in the rat (Richards and Midgley, 1976). Thus, FSH action should be limited to granulosa cells.

Based on my results, estradiol appears to be required for FSH to maximally increase the level of FSH receptors. The ability of progesterone and testosterone to partially reverse the inhibitory action of cyanoketone is in part due to conversion of these steroids to estradiol as the number of receptors for estradiol was increased after progesterone or testosterone addition. However, as the non-metabolizable forms of these steroids had some effect on reversing the inhibitory effects of cyanoketone prior to FSH, progesterone and testosterone may also have some direct effects. To determine if progesterone and testosterone have direct effects, CI628 can be injected into hypophysectomized rats to block estrogen receptor action. Progesterone and testosterone could then be administered followed by FSH and numbers of receptors for FSH and estradiol determined. If

progesterone and testosterone are involved in FSH action independent of the estradiol receptor, there would be an increase in numbers of receptors for FSH without any effect on numbers of receptors for estradiol. In other experiments, anti-progestins and anti-androgens along with an aromatase inhibitor could be used to further determine the role of these steroids in FSH action.

The second objective of the dissertation was to determine if the interaction of estradiol and FSH occurred at the level of cAMP production. As CI628 had no effect on FSH stimulation of cAMP production, the interaction of estradiol and FSH appears to be distal to the cAMP-adenylate cyclase system perhaps involving protein kinases or other intracellular proteins.

The effects of estradiol alone on protein kinase activity remain controversial (DeAngelo et al., 1975; Richards et al., 1979). However, after concomitant administration of estradiol and FSH, there is a 10-fold increase in granulosa cell content of RII, the regulatory subunit of type II cAMP-dependent protein kinase (Richards and Rolfes, 1980). Richards et al. (1983) have also demonstrated that after administration of FSH for 3 days, five proteins were induced in antral follicles that are then phosphorylated by protein kinase. Since FSH stimulates production of estradiol (Moon et al., 1975), it is possible that estradiol induces synthesis of these five follicular proteins. As receptors for progesterone and testosterone also interact with DNA, these steroids may play a role in protein synthesis.

Both calcium and the calcium-dependent regulatory protein, calmodulin, appear to be required for regulation of steroidogenesis in

granulosa cells by LH and FSH (Carnegie and Tsang, 1983; Tsang and Carnegie, 1983). This participation in gonadotropic regulation of steroidogenesis is independent of stage of follicular maturation and cellular differentiation (Carnegie and Tsang, 1984). Whether estradiol has any effects on the calcium-calmodulin system is unknown, however, estradiol could induce calmodulin or proteins that affect this system.

Although the exact mechanism of interaction of estradiol and FSH in follicular development is unknown, a model speculating their interaction in granulosa cells follows.

Even though initiation of follicular growth may not require hormones (Pederson, 1970; Pederson and Peters, 1968; 1971), basal levels of gonadotropins are required for continuation of growth (Richards and Midgley, 1976). Granulosa cells of all follicles appear to posses receptors for FSH whereas receptors for LH are present on theca cells (Eshkol and Lunenfeld, 1968; Richards and Midgley, 1976; Richards et al., 1976). Only granulosa cells of large, preovulatory follicles have receptors for LH (Channing and Kammerman, 1974; Zeleznik et al., 1974; Amsterdam et al., 1975; Richards and Midgley, 1976; Nimrod et al., 1977). As synthesis of estradiol requires the interaction of LH and FSH with their receptors in follicles, the basal levels of gonadotropins present in early follicular development are important for estradiol synthesis.

Thus, in early follicular development (Figure 16), LH binds to its receptor in theca cells to stimulate testosterone synthesis. FSH, after binding to FSH receptors on granulosa cells, stimulates cAMP production, increases protein kinase activity and stimulates the

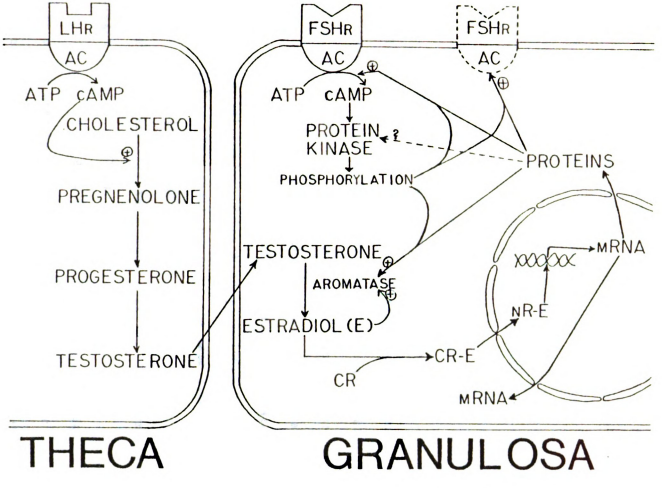

Figure 16. MODEL FOR INTERACTION OF ESTRADIOL AND FSH DURING EARLY FOLLICULAR DEVELOPMENT.

aromatase enzyme system. Testosterone is then converted to estradiol. Estradiol binds to its cytoplasmic receptor and is translocated to the nucleus where it stimulates transcription of specific mRNAs. These mRNAs are translated into specific proteins in the cytosol. These induced proteins (which could be protein kinases, calmodulin or other proteins) can interact with existing proteins, protein kinases or the calcium-calmodulin system triggered by FSH stimulation. There are then further increases in intracellular cAMP, aromatase activity as well as induction of receptors for FSH.

As the follicle develops from preantral through antral stages (Figure 17), theca cells acquire more receptors for LH. Testosterone production increases providing more substrate for estradiol synthesis.

However, most follicles on the ovary do not ovulate, but become atretic (Baker, 1963). Alterations in the functions of gonadotropins and estradiol are involved in this process. In atretic follicles, there is a shift from production of estradiol to progesterone synthesis (Uilenbroek et al., 1980; Hubbard et al., 1981; Braw et al., 1981; Terranova, 1981). This shift occurs at the same time as a decrease in hCG and FSH binding (Carson et al., 1979; Uilenbroek et al., 1980). There are also decreases in aromatase as well as enzymes involved in androgen production (Moor et al., 1978; Uilenbroek et al., 1980; Hubbard et al., 1981; Braw et al., 1981). Administration of estradiol has prevented atresia in hypophysectomized rats, however its exact role in preventing atresia is unknown (Ingram, 1959; Harmon et al., 1975).

Synthesis of estradiol appears to be an important step in control of follicular development. Estradiol is required for FSH to maximally

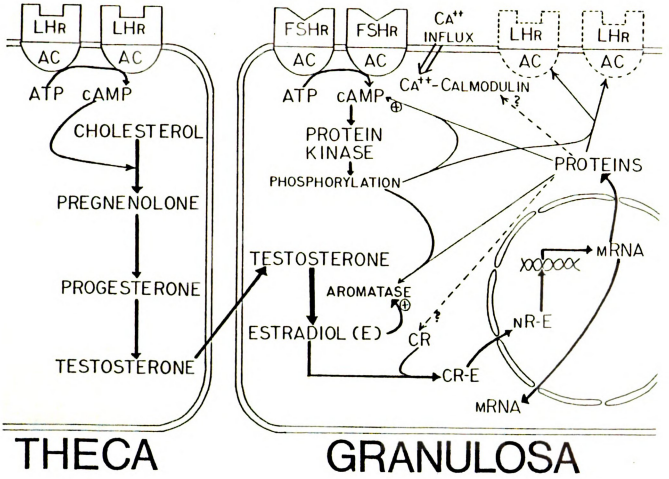

Figure 17. MODEL FOR INTERACTION OF ESTRADIOL AND FSH DURING FOLLICULAR DEVELOPMENT.

increase receptors for FSH as well as increase receptors for LH in granulosa cells. Also, estradiol can enhance FSH-stimulated cAMP production and increase aromatase activity. Therefore, control of estradiol synthesis or action is an important tool in promoting maturation of follicles and preventing atresia.

SUMMARY AND CONCLUSIONS

The studies presented in this dissertation examine the requirement of estradiol for FSH action. It has been shown (Chapter 1) that granulosa cells contain nuclear and cytosolic receptors for estradiol which have properties similar to estrogen receptors in other tissues.

Since blockage of synthesis of estrogen with cyanoketone (Chapter 2) or inhibition of binding of estradiol to its receptor with CI628 (Chapter 3) prevented FSH induction of FSH receptors, estradiol appears to be required for FSH action. Although progesterone and testosterone can partially reverse the inhibitory effects of cyanoketone, non-metabolizable forms of these steroids had less effect. Therefore, although I cannot rule out the importance of other steroids, estradiol is indeed required for FSH to maximally stimulate increases in FSH receptors.

The influence of estradiol on FSH action appears not to be mediated through the adenylate cyclase-cAMP system as CI628 did not block FSH stimulation of cAMP production.

In conclusion, estradiol is required for FSH to increase its own receptor. This interaction, however, occurs distal to the cAMP-adenylate cyclase system. Although the biochemical action of estradiol is unknown, these data support my hypothesis that control of estradiol production is important in the control of FSH action, which,

in turn, promotes follicular development.

APPENDIX

APPENDIX

Estradiol Receptor Assay

Collect tissue, trim of fat, place in TED buffer on ice

Express granulosa cells in TED (1 ml) in a homogenizer (2 ovaries/12 x 75 mm polypropylene tube)

Centrifuge at 800 x g for 20 min ($4^{o}C$)

Wash cells 3 times in TED buffer (2 ml/wash)

Homogenize cells in 2 ml TED with Dounce homogenizer

Centrifuge at 4,000 x g for 20 min, $4^{o}C$

FOR THE NUCLEAR ASSAY

wash pellets 3 times in 2 ml TED for 10 min ($4^{o}C$)

resuspend nuclei in 0.4 ml TED

add TED buffer so final volume is 0.25 ml

for Scatchard plots, add $^{3}H\text{-}E_{17B}$ (1-20 nM) in TED; for saturation analysis, add $^{3}H\text{-}E_{17B}$ (50,000 cpm) in 0.05 ml TED

add 500-fold excess DES in 0.03 ml to NSB tube

add 0.1 ml of granulosa nuclei to 3 tubes plus 0.1 ml to a tube for for DNA determination

incubate on ice for 1 h

FOR THE CYTOSOL ASSAY

supernatant is further centrifuged at 106,000 x g (Type 30 rotor, Beckman) for 1 h ($4^{o}C$)

remove supernatant with Pasteur pipette-exclude fat layer

for Scatchard plots, add $^{3}H\text{-}E_{17B}$ (1-20 nM) in TED; for saturation analysis, add $^{3}H\text{-}E_{17B}$ (50,000 cpm) in 0.05 ml TED

add 500-fold excess DES in 0.03 ml to NSB tube

add 0.5 ml of supernatant

incubate for 3 h at $4^{o}C$

add 1 ml of DCC to each tube (on ice)

add 0.05 ml of TED buffer containing 3 M NaSCN to all tubes

incubate overnight at 4°C

add 0.3 ml DCC to all tubes (on ice)

incubate for 15 min on ice

centrifuge at 8,000 x g for 10 min

decant supernatant into scintillation vials

add 10 ml ACS to vials and count

incubate for 15 min on ice

centrifuge at 8,000 x g for 10 min

decant supernatant into scintillation vials

add 10 ml ACS to vials and count

Definitions:

TED buffer: 10 mM Tris, 1.5 mM EDTA, pH 7.4
DES: diethylstilbestrol disolved in methanol
NSB: non-specific binding
NaSCN: sodium thiocyanate
DCC: 1% charcoal, 0.05% Dextran, in TED buffer
ACS: aqueous counting scintillant

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