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Effect Of An Extract Of Superior Ovarian Nerve On Steroidogenesis By Porcine Theca Cells In Vitro

Paul Andrew Morley

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EFFECT OF AN EXTRACT OF SUPERIOR OVARIAN NERVE ON STEROIDOGENESIS BY PORCINE THECA CELLS IN VITRO

by

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ABSTRACT

Ovarian steroidogenesis may be controlled not only by pituitary gonadotropins, but also by ovarian nerves. Nerves reach the ovary via the plexus nerve and the superior ovarian nerve (SON), and innervate the theca cells of all follicles. Thecal androgen biosynthesis is primarily controlled by luteinizing hormone (LH); however, the presence of nerves close to these cells has raised the question of whether nerves can modulate their androgen biosynthesis. The purpose of this study was to develop a porcine theca cell culture system, and to use it to investigate the effects of putative ovarian neurotransmitters and ovarian nerve factors on thecal steroidogenesis.

Theca cells from prepubertal gilts cultured under serum-free conditions responded to LH with increased cyclic AMP and androstenedione accumulation. Catecholamines, acting via β-adrenergic receptors, caused a generalized inhibition of thecal steroidogenesis at a site distal to the generation of cyclic AMP.

The direct effects of ovarian nerve factors on thecal steroidogenesis were studied by adding extracts of rat SON to cultured theca cells. Addition of SON extract inhibited androstenedione accumulation. The inhibitory activity of the SON extract was unaffected by chymotrypsin treatment or boiling, but was removed by charcoal treatment. The extract contained immunorea tive estradiol which, in common with the inhibitory activity, was extractable with organic solvents and eluted in the same fractions on Sephadex LH-20 columns. Estradiol, catecholestrogens and the SON extract inhibited the 17α-hydroxylase:$C_{17,20}$ lyase enzyme complex.

The results suggest that the SON contains a factor which inhibits thecal androstenedione production and a factor which cross reacts with an

estradiol antibody. It is not known if these factors are the same or distinct compounds. Therefore, the nervous system may have the potential to modulate follicular steroid biosynthesis via a direct innervation of the ovaries. Ovarian nerves may play important roles in the regulation of follicular development, since thecal androgens are substrates for estradiol biosynthesis and are involved in follicular atresia.

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ALP	alkaline phosphatase
BSA	bovine serum albumin
CCK-8	cholecystokinin-8
CNS	central nervous system
COMT	carboxy-o-methyl transferase
cpm	counts per minute
CST	cervical sympathetic trunk
cyclic AMP	3':5'-cyclic adenosine monophosphate
DHBA	3,4-dihyroxybenzylamine
DHT	5α-dihydrotestosterone or 5α-androstan-17β-ol-3-one
dibutyryl cylic AMP	N^6-2'-O-dibutyryl 3':5'-cylcic adenosine monophosphate
DMEM	Dulbecco's modified Eagle's medium
DPBS	Dulbecco's phosphate buffered saline
E_2	estradiol-17β
EDTA	ethylenediamine tetraacetic acid
EGF	epidermal growth factor
estradiol	estradiol-17β
FGF	fibroblast growth factor
FSH	follicle-stimulating hormone
g	gram
GABA	gamma amino butyric acid
GnRH	gonadotropin releasing hormone
h	hour
HBSS	Hanks' Balanced Salt Solution
hCG	human chorionic gonadotropin

HPLC	high-pressure liquid chromatography
IBMX	1-isobutyl methyl xanthine
IGF-I	insulin-like growth factor I or Somatomedin C
IU	international units
l	liter
LH	luteinizing hormone
MAO	monoamine oxidase
2-Me-E_1	2-methoxyestrone
2-Me-E_2	2-methoxyestradiol
mg	milligram
ml	milliliter
ng	nanogram
NPY	neuropeptide Y
2-OH-E_1	2-hydroxyestrone
2-OH-E_2	2-hydroxyestradiol
4-OH-E_2	4-hydroxyestradiol
PGE_2	prostaglandin E_2
PMSG	pregnant mares' serum gonadotropin
RIA	radioimmunoassay
saline	0.9% sodium chloride
SCN	sciatic nerve
S.E.M.	standard error of the mean
SON	superior ovarian nerve
3β-HSD	3β-hydroxysteroid dehydrogenase
TSH	thyroid stimulating hormone
μg	microgram
μl	microliter

μM	micromolar
VIP	vasoactive intestinal peptide

CHAPTER 1 - INTRODUCTION

Over the last fifty years, the role of the pituitary gonadotropins as the primary controllers of ovarian function has been investigated extensively; however, over the same time period the existence and possible function of the ovarian innervation has been relatively neglected. While the role of the central nervous system in the control of secretion of anterior pituitary gonadotropins and thus indirectly in the regulation of the ovary has become well established, increasing evidence for an additional neural influence on ovarian function via direct innervation of the ovaries has developed.

The first description of the innervation of the mammalian ovary was in 1867 by Frankenhauser. The nerve supply to the ovary arises from the lower thoracic segments of the spinal cord and travels to the ovaries via two separate routes: (1) the ovarian plexus nerve, which runs alongside the ovarian artery, and (2) the superior ovarian nerve (SON) which runs in the suspensory ligament (Lawrence and Burden, 1980). Histochemical and immunocytochemical studies have shown the presence of noradrenergic (Jacobowitz and Wallach, 1967; Owman et al., 1967; Lawrence and Burden, 1976) and cholinergic (Bulmer, 1965; Jacobowitz and Wallach, 1967) fibers innervating the ovary. In addition, nerve fibers immunoreactive for vasoactive intestinal peptide (VIP; Larsson et al., 1977), substance P (Dees et al., 1985; Papka et al., 1985), cholecystokinin-8 (CCK-8; McNeill and Burden, 1986), neuropeptide Y (NPY; McDonald et al., 1987) and calcitonin gene-related peptide (Calka et al., 1988) have also been observed in the rat ovary. Gamma amino butyric acid (GABA) and somatostatin have also been identified in the mammalian ovary (Schaeffer

and Hsueh, 1982; McNeill and Burden, 1986). Although the degree of innervation varies, the general pattern of distribution is the same in all species. Generally, nerves enter the ovary at the hilus initially following the branching of the ovarian blood vessels. The nerves innervate blood vessels and the theca layer of all sizes of developing follicles. The nerves do not penetrate the follicular basement membrane and consequently neither the granulosa cell layer of the follicle, nor the corpora lutea, are innervated (Jacobowitz and Wallach, 1967; Burden, 1972; Papka et al., 1985). Nerves also travel directly to the ovarian interstitial cells which, together with the theca interna of antral follicles, are responsible for the production of androgens by the ovary.

Even though anatomists have studied ovarian nerves for well over 100 years, physiologists have only recently taken an interest in their role in regulating ovarian function. Many physiological roles have been suggested based on the results of studies using a variety of techniques including denervation, autotransplantation and the administration of agonists and antagonists. These include roles in follicular development (Brink and Grob, 1973), compensatory ovarian hypertrophy following the removal of one ovary (Burden and Lawrence, 1977a), ovulation (Bahr et al., 1974), ovarian blood flow (Gibson and Roche, 1986) and steroidogenesis.

In vivo experiments have shown that stimulation of specific brain areas in hypophysectomized, adrenalectomized rats causes changes in ovarian venous estrogen and progesterone concentrations without affecting ovarian blood flow (Kawakami et al., 1981). Also, chronic stimulation of the SON in rats leads to changes in ovarian progesterone output (Weiss et al., 1982). There have also been numerous in vitro studies which have shown

that in response to catecholamines, granulosa (Kliachko and Zor, 1981) and luteal cells (Condon and Black, 1976) increase their production of progesterone, and rat theca-interstitial cells increase their production of androgen (Dyer and Erickson, 1985). The ovarian androgen producing cells are the only ovarian endocrine cells that are directly innervated. The presence of nerves in close proximity to these cells has raised the question of whether ovarian nerve factors can modulate their steroidogenesis. If so, then ovarian nerves may play a role in physiological ovarian responses such as follicular growth and atresia, in which androgens are known to play important roles.

The purpose of the present research was: to develop a porcine theca cell culture model to investigate the effects of ovarian nerve factors and putative ovarian neurotransmitters on theca cell steroidogenesis without the effects of changes in blood flow that confound in vivo studies. The potential for a regulatory role of ovarian nerves on thecal androgen production will also be studied by adding extracts of the superior ovarian nerve of rats to cultured porcine follicular theca cells.

CHAPTER 2-LITERATURE REVIEW

The aim of this review is to introduce the ovarian androgen producing cells. I will first discuss the process of steroid biosynthesis in the mammalian ovary, the historical perspectives of the discovery that the ovary produces androgenic hormones and their physiological functions. I will then review the classification of the ovarian androgen producing cells; the function of these cells; their primary control by the pituitary gonadotropin luteinizing hormone (LH); and the circulatory, follicular fluid and neural factors which can modulate their responsiveness to LH.

2.1 OVARIAN STEROIDOGENESIS

The pathways of gonadal steroid biosynthesis are well established and have recently been reviewed in detail (Gore-Langton and Armstrong, 1988). Within the ovarian follicle, the theca interna and granulosa cells are the major steroidogenic cell types which secrete the three major classes of ovarian steroids: progestins (C_{21} steroids), androgens (C_{19}) and estrogens (C_{18}). The biosynthetic pathways for the production of these compounds are shown in Figure 1. In all steroid producing tissues cholesterol is the major precursor for steroidogenesis. The first step in the conversion of cholesterol to steroids is the formation of the C_{21} steroid pregnenolone by the mitochondrial cholesterol side chain cleavage enzyme complex. Pregnenolone is the key intermediate for the formation of all classes of steroid hormones. It is converted to progesterone by a microsomal enzyme complex: Δ^5-3β-hydroxysteroid dehydrogenase (3β-HSD):$\Delta^{5,4}$ isomerase. Similar, but perhaps not identical enzymes convert 17α-hydroxypregnenolone to 17α-hydroxyprogesterone, dehydroepiandrosterone (DHEA) to androstenedione and androst-5-ene-3β,17β-diol to testosterone.

Figure 1. Pathways of ovarian steroid biosynthesis.

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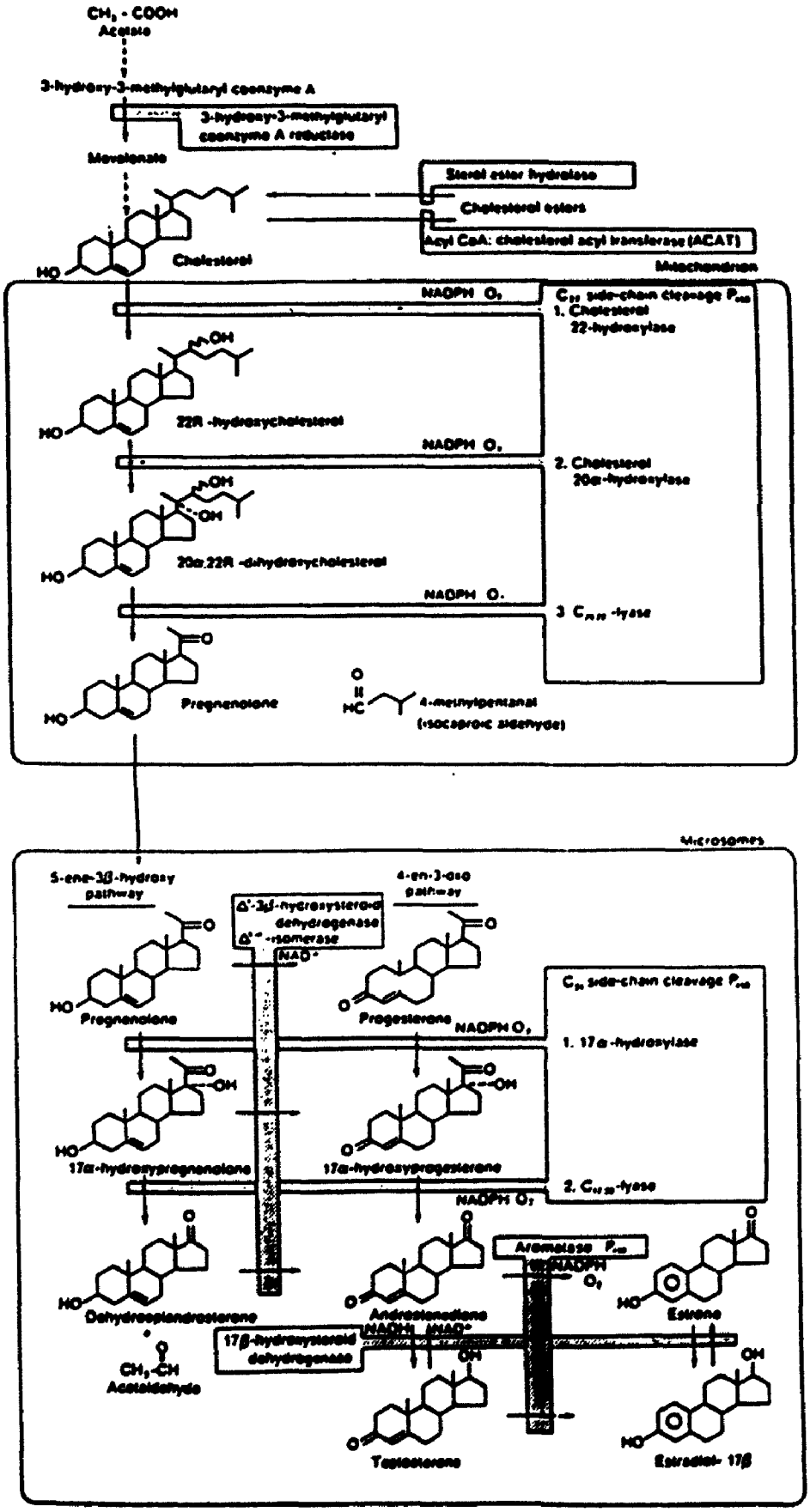
CH3 - COOH
Acetate
3-hydroxy-3-methylglutaryl coenzyme A
3-hydroxy-3-methylglutaryl coenzyme A reductase
Mevalonate
Sterol ester hydrolase
Cholesterol esters
Acyl CoA: cholesterol acyl transferase (ACAT)
Cholesterol
HO
Mitochondrion
NADPH O2
C27 side-chain cleavage P450
1. Cholesterol 22-hydroxylase
22R -hydroxycholesterol
NADPH O2
2. Cholesterol 20α-hydroxylase
20α,22R -dihydroxycholesterol
NADPH O2
3 C20,22 -lyase
Pregnenolone
4-methylpentanal
(isocaproic aldehyde)
Microsomes
5-ene-3β-hydroxy pathway
Δ5-3β-hydroxysteroid dehydrogenase
Δ5,4-isomerase
NAD+
4-en-3-one pathway
Pregnenolone
Progesterone
C21 side-chain cleavage P450
NADPH O2
1. 17α-hydroxylase
17α-hydroxypregnenolone
17α-hydroxyprogesterone
2. C17,20-lyase
NADPH O2
Aromatase P450
NADPH
O2
Dehydroepiandrosterone
Androstenedione
Estrone
17β-hydroxysteroid dehydrogenase
NADPH NAD+
CH3-CH
Acetaldehyde
Testosterone
Estradiol- 17β

The rate-limiting step in the biosynthesis of androgens is the 17α-hydroxylase:C_{17-20} lyase enzyme complex which causes 17α-hydroxylation and cleavage of the C_{17-20} bond. The reaction uses either pregnenolone or progesterone as substrates to produce DHEA or androstenedione, respectively. This enzymatic step is one of the main sites at which physiological regulation of follicular steroidogenesis occurs. The resulting androgens, androstenedione and testosterone, are converted to the estrogens estrone and estradiol-17β by an enzyme complex referred to as aromatase, or undergo A ring substitution by the 4-ene-5α-reductase enzyme to yield the non-aromatizable androgens 5α-androstane-3,17-dione and 5α-dihydrotestosterone (DHT).

Luteinizing hormone stimulates theca cells to convert steroid precursors to progesterone and progesterone to androgens. The ability of theca cells to aromatize androgens to estrogens depends on the species. Granulosa cells on the other hand are unable to convert C_{21} steroids to androgens, but can synthesize progesterone. Granulosa cells can also aromatize androgens to estrogens under the stimulation of follicle-stimulating hormone (FSH). Androgens of thecal origin diffuse into the granulosa cells where they are used as substrates for aromatization to estrogen. This cooperation between theca and granulosa in the production of estrogen is called the "two-cell, two-gonadotropin" model for ovarian follicular steroid biosynthesis (Armstrong and Dorrington, 1979).

2.2 HISTORICAL PERSPECTIVES OF OVARIAN ANDROGEN PRODUCTION

The concept that the mammalian ovary can produce androgens began in the 1930's with descriptions of the effects of human pregnancy urine or

placental extracts on the ovary of immature or hypophysectomized female rats. It is now known that these extracts contained large amounts of human chorionic gonadotropin (hCG). When rats were injected with pregnancy urine, ovarian thecal cells proliferated, hypertrophied and stored large amounts of fat. Although no true corpora lutea were formed, the ovary became filled with pseudo or thecal corpora lutea (Collip et al., 1933a; Collip et al., 1933b; Selye et al., 1933). Under conditions in which excessive luteinization of the ovary occurred, masculinization of the female was also observed. The masculinizing effects were determined by a hypertrophy of the clitoris, prepuce and preputial glands and by a "muscularizing" effect on the skeletal musculature, effects comparable to testosterone treatment (Papanicolaou and Falk, 1938). Papanicolau and Falk (1934) believed that the theca corpora lutea were causally linked with t masculinization of the female, since the masculinizing effects were not observed in ovariectomized animals. Bradbury and Gaensbauer (1939) reported masculinization of infantile female rats treated with gonadotropic extracts; however, these effects were not observed in ovariectomized animals. Greene and Burrill (1939) showed that treatment of immature female rats, containing a transplanted ventral prostate of a male rat, with placental extracts caused a typical androgenic stimulation and maintenance of the ventral prostate tissue. In the absence of the ovaries there was no effect and the ovaries of the masculinized animals contained an excessive amount of theca and interstitial tissue, suggesting the hypertrophied interstitial tissue produces an androgenic substance.

Hill (Hill and Gardner, 1936; Hill, 1937) reported that grafting ovaries to castrated male mice restored the atrophied seminal vesicles and prostates to their normal state of growth and secretion, suggesting

that the ovary secretes a male hormone into the circulation. These observations were repeated by Deanesly (1938a; 1938b), who found similar effects of grafting ovaries to immature male rats. Luteinization of the theca interna in transplanted ovaries was described by both Hill and Deanesly and the androgenic output was found to be directly related to the amount of thecal luteinization in the grafted ovaries (Hill, 1937; Deanesly, 1938b). These observations were supported by the work of Burrill and Greene (1941) who showed that the masculinizing effect was mediated through the ovary since it did not occur in rats which had been ovariectomized before the treatment was started.

Domm (1937) reported masculinization of the newly hatched chick by treatment with anterior pituitary extracts. The ovarian reponsiveness was characterized by a marked hypertrophy of the medullary portion of the ovary and an absence of follicular stimulation. It suggested the medullary portion of the ovary was the site of production of androgenic hormones.

In the 1960's Short (1960) identified androstenedione and testosterone in the follicular fluid of the mare. At the same time, Falck et al. (1962), grafted ventral prostate tissue together with a variety of ovarian tissues in the anterior chamber of the eye of castrated rats, and observed that only pieces of follicle wall (containing theca and granulosa cells) and the interstitial gland cells were capable of producing androgen activity to sustain the ventral prostate tissue. In some of the follicle wall fragments in which the granulosa cells did not survive and only theca interna cells were found at the end of the experiment, the androgen effect was still recorded. In the presence of corpus luteum cells or granulosa cells alone, the ventral prostate tissue maintained the morphology of a

typical castrate appearance indicating that neither of these cell types produced androgens (Falck, 1959).

Confirmation that the ovary produced androgens came in 1966 when Ryan and Petro, using a technique to separate granulosa and theca cells, showed that isolated human theca interna cells could convert radiolabelled pregnenolone or progesterone to androstenedione. At the same time, Rice and Savard (1966) reported that slices of human ovarian stromal tissue incubated in the presence of 1-^{14}C-acetate formed the radioactive androgens androstenedione and testosterone. These authors concluded that the ovarian stroma forms a distinct endocrine gland within the ovary, where the major steroid product is androgens.

2.3 FUNCTION OF OVARIAN ANDROGENS

In the ovary, the androgen producing cells are physiologically important because the androgens they produce play important roles in follicle maturation, estrogen biosynthesis, follicular atresia, progesterone biosynthesis and oocyte maturation. The next section will briefly review experimental evidence to support the contention that androgens are important intraovarian regulators of ovarian function.

2.3.1 Follicle Maturation

There have been reports that androgens have no physiological function, other than as substrates for aromatase, in follicle maturation. In support of this concept, the antiandrogen hydroxyflutamide had no effect on the FSH induction of aromatase activity or LH receptors in diethylstilbesterol-primed, hypophysectomized, immature rats, suggesting that androgens are not required for FSH to initiate development of antral

follicles (Zeleznik et al., 1979). In addition, the antiandrogen cyproterone acetate did not disrupt estrous cycles or ovulation in adult rats (Neumann et al., 1970). In addition, normal reproductive cycles, follicular maturation, conception and pregnancy occur in tfm/o mice, in which the females are genetically androgen resistant (Lyon and Glemister, 1974). This evidence suggests that androgens are not directly involved in follicle maturation.

2.3.2 Estrogen Bioynthesis

In most species follicular estrogen biosynthesis requires both granulosa and theca cells (Falck, 1959; Makris and Ryan, 1975; Moor, 1977) and both LH and FSH (Fevold, 1941; Greep et al., 1942; Lostroh and Johnson, 1966). These observations led to the 2-cell, 2-gonadotropin theory of follicular estrogen production, namely, that LH induces androgen production by the ovarian theca (and perhaps also interstitial cells), which can then be aromatized to estrogens in the granulosa cells under the stimulation of FSH (reviewed in Armstrong and Dorrington, 1979). Much evidence has been collected to support this theory using both *in vitro* (Fortune and Armstrong, 1977; Makris and Ryan, 1975; Fortune and Armstrong, 1978; McNatty et al., 1980b) and *in vivo* (Lostroh and Johnson, 1966; Armstrong and Papkoff, 1976) techniques.

Androgens of theca cell origin are therefore essential intermediates in estrogen biosynthesis. Treatment of immature hypophysectomized rats with LH caused marked increases in ovarian androgen content, but only if FSH was administered concomitantly, were estradiol levels increased. If aromatizable androgen was substituted for LH, in the presence of FSH, an increased estrogen production occured (Armstrong and Papkoff, 1976),

indicating that LH stimulates androgen production which is then used as substrate for conversion to estrogen by the aromatase enzymes. Many studies have shown that androgens act as substrates for aromatization by granulosa cells, whole follicles and ovaries in vitro (Dorrington et al., 1975; Erickson and Ryan, 1976; Erickson and Hsueh, 1978; Fortune and Armstrong, 1978; Moon et al., 1978). Androgens also enhance FSH-induction of aromatase activity (Daniel and Armstrong, 1980), and this is blocked by antiandrogens (Hillier and deZwart, 1982; Daniel and Armstrong, 1984).

An inhibitory action of LH on estrogen secretion is suggested by the close temporal association between the preovulatory LH surge and the termination of estrogen synthesis in the preovulatory follicle (Moor, 1974). After the LH surge, androgen levels in ovarian tissue, follicular fluid, and ovarian venous blood initially rise and then fall several hours later (Bahr, 1978; Goff and Henderson, 1979). The LH surge initially stimulates these cells to produce androgens which are aromatized to estrogens by the granulosa cells. The estrogens inhibit the thecal 17α-hydroxylase:C_{17-20} lyase enzyme complex, thereby inhibiting further androgen synthesis and their subsequent use as substrates for aromatization (Lieberman et al., 1975; Hillensjo et al., 1976). Therefore the theca cell switches from an androgen to a progesterone producing tissue and when this occurs the follicle switches from an estrogen-secreting to a progesterone-secreting structure.

2.3.3 Follicular Atresia

At the opposite extreme from the constructive role that thecal androgens have on the follicle selection process are the destructive effects they exert on both non-growing and growing follicles at all stages

of development. These destructive processes are called atresia. The mechanisms that initiate and control atresia are not understood.

Follicular atresia is characterized by degenerative changes in the granulosa cell layer including pyknotic nuclei, the loss of gap junctions and LH and estrogen receptors, and lipid accumulation (Byskov, 1979). Ultrastructural examination of theca cells in atretic rat follicles revealed lipid droplets, an agranular endoplasmic reticulum and mitochondria with tubular cristae typical of steroid secreting cells (Guraya and Greenwald, 1965; Schwall and Erickson, 1984). These theca cells are the progenitors of the secondary interstitial cells. These thecal cells undergo condensation, fragmentation and ultimate phagocytosis by still healthy theca cells. The degenerating cellular material accumulates and blocks the thecal capillaries of the atretic follicle and the follicle collapses.

The involvement of endocrine mechansims in atresia has been recognized for a long time. During atresia the pattern of follicular steroid production is altered. In some species follicular androgen synthesis declines (Braw and Tsafriri, 1980). In other species, including the sheep, pig and human, atretic follicles secrete increased amounts of androgens while a reduction in estrogen synthesis occurs (Moor et al., 1978; Mori et al., 1982). The decreased estrogen and increased androgen output by atretic porcine ovaries are due to a loss of aromatase activity (McNatty et al., 1979b). Hypophysectomy resulted in atresia of antral follicles (Ingram, 1953) and the addition of estrogen or gonadotropins to hypophysectomized animals reduced the rate of atresia (Pencharz, 1940; Williams, 1944; Ingram, 1959; Goldenberg et al., 1972; Harman et al., 1975). Estrogens have long been known to have direct anti-atretic effects

(Richards, 1975; Louvet et al., 1975b; Hillier and Ross, 1979). Further studies showed that androgens (Payne and Runser, 1958; Hillier and Ross, 1979) or exposure to LH/hCG, which stimulates ovarian androgen synthesis by theca cells, (Louvet et al., 1975a,b; Hillier and Ross, 1979) induced atresia in the rat ovary. By contrast, pregnant mares' serum gonadotropin (PMSG) and FSH prevented atresia (Peters et al., 1975; Byskov, 1979). It is not known if the high levels of androgen are the cause of atresia or a result of the process. Treatment with antiandrogens or an antiserum against androgen attenuated the atretic effects of LH/hCG (Louvet et al., 1975a,b). Treatment of immature, hypophysectomized, PMSG-treated rats with the non-aromatizable androgen, DHT, induced atresia (Bagnell et al., 1982). This effect of DHT could be partially prevented by coadministration of estradiol (Bagnell et al., 1982). Although there is a lot of evidence implicating androgens, the mechanism of androgen action in the process of follicular atresia is not known.

2.3.4 Follicular Progesterone Biosynthesis

The literature on the regulation of ovarian progesterone biosynthesis by androgens is somewhat equivocal; androgens have been reported to both stimulate and inhibit progesterone production depending on the species concerned. Androgens inhibited progesterone production by granulosa cells from humans (Channing et al., 1978) and pigs (Thanki and Channing, 1978; Evans et al., 1984; Lischinsky et al., 1983). Aromatizable androgens were considerably more effective inhibitors of progesterone synthesis than non-aromatizable androgens. Since androgens did not affect progesterone metabolism, the decreased progesterone accumulation must be due to an inhibition of progesterone synthesis (Evans et al., 1984).

Aromatization of androgen to estrogen is not required for androgen action (Evans et al., 1984) and androgens appear to act at a site distal to cyclic AMP formation (Lischinsky et al., 1983). In addition, in cultures containing aromatizable androgens, pregnenolone production in FSH-stimulated granulosa cell cultures was significantly elevated, suggesting androgens suppress 3β-HSD/Δ^{5-4} isomerase enzyme activity (Lischinsky et al., 1983).

The inhibitory effects of androgens on porcine granulosa cell progesterone secretion are in contrast to their effects on granulosa cells from other species. Androgens increase progesterone production by granulosa cells of sheep (Moor et al., 1975), pigs (Schomberg et al., 1976; Haney and Schomberg, 1978; Schomberg et al., 1978), rats (Armstrong and Dorrington, 1976; Nimrod and Lindner, 1976; Hillier et al., 1977; Lucky et al., 1977) and mice (Corredor and Flickinger, 1983). Stimulation of granulosa cell progesterone production by androgens in vitro has only been observed under conditions of culture where media was supplemented with serum (Schomberg et al., 1976; Nimrod and Lindner, 1976; Lucky et al., 1977) or FSH (Armstrong and Dorrington, 1976). This response appears to be androgenic and not dependent on aromatization to estrogens, since both aromatizable and non-aromatizable andr- ns were effective and treatment in vivo or in vitro with antiandrogens decreased progesterone secretion by cultured porcine, ovine and rat granulosa cells (Hillier et al., 1977; Schomberg et al., 1978).

Androgens, in addition to having their own stimulatory effects, enhance FSH-stimulated progesterone synthesis (Nimrod and Lindner, 1976; Armstrong and Dorrington, 1976; Welsh et al., 1982). This effect was blocked by the antiandrogen hydroxyflutamide, indicating the effect is mediated via

specific androgen receptors (Hillier and deZwart, 1982). The mechanism of the interaction between FSH and androgens is not fully understood. The increase in progesterone production stimulated by FSH and androgen is not due to androgen-stimulated increases in the conversion of progesterone to 5α reduced metabolites (Nimrod, 1977a). The addition of androstenedione to cultured medium did not affect the number of FSH binding sites, the coupling of the FSH-receptor to adenylate cyclase, or phosphodiesterase activity, leading to the conclusion that androgens act at a step in the regulation of progesterone biosynthesis distal to cyclic AMP production (Nimrod, 1977b). In support of this, androgens enhanced dibutyryl cyclic AMP-stimulated granulosa cell progesterone production (Nimrod, 1977b). Androgens and FSH both increased the activity of the cholesterol side chain cleavage enzyme (Welsh et al., 1982). Androgens may (Welsh et al., 1982) or may not stimulate the 3β-HSD/Δ^{5-4} isomerase enzyme in granulosa cells (Dorrington and Armstrong, 1979).

When granulosa cells have acquired LH receptors, LH stimulates granulosa cell progesterone production (Goff et al., 1979; Sheela Rani et al., 1981). Treatment of immature rats with testosterone (Leung et al., 1979), but not DHT (Goff et al., 1979), resulted in an enhancement of LH-stimulated progesterone production by granulosa cell in vitro (Leung et al., 1979). These data suggest that a pituitary factor (probably FSH) is involved in the mechanism by which androgens enhance LH-stimulated progesterone synthesis in vivo. In support of these observations, the combined treatment with androgen and FSH in vitro resulted in increased LH-responsive progesterone synthesizing capability in cultured rat granulosa cells (Goff et al., 1979).

2.3.5 Oocyte Maturation

Androgens may also be involved in the maturation of mammalian oocytes. Testosterone inhibits the maturation of cumulus-enclosed mouse oocytes (Downs and Eppig, 1984) *in vitro* and enhances the inhibitory effect of dibutyryl cyclic AMP in oocytes of mice (Downs and Eppig, 1984; Schultz et al., 1983) and pigs (McGaughey, 1977; Daniel et al., 1986).

The mechanism by which androgens enhance cyclic AMP-inhibition of meiotic maturation is not fully understood. Androgens have been shown to abolish the production of an oocyte maturation inhibitor-like factor by porcine granulosa cells *in vitro* (Anderson and Stone, 1980). Androgens appear to act via the cumulus cells since when cumulus cells were removed from pig oocytes, testosterone was ineffective in enhancing the inhibitory effects of dibutyryl cyclic AMP (Daniel et al., 1986). Hydroxyflutamide also suppressed the inhibitory actions of testosterone suggesting that the inhibitory action of androgens in porcine oocyte maturation *in vitro* is receptor-mediated (Daniel et al., 1986).

2.4 THE OVARIAN ANDROGEN PRODUCING CELLS

The ovarian androgen producing cells are found in the connective tissue of the ovarian cortex and medulla. Erickson and coworkers (1985) have recently described four groups of ovarian androgen producing cells: 1) the primary interstitial cells, 2) the hilar interstitial cells, 3) the theca interna cells and 4) the secondary interstitial cells. The location of the ovarian androgen producing cells is shown in Figure 2.

2.4.1 The Primary Interstitial Cells

A transient population of cells called the primary interstitial cells

Figure 2. Location of the ovarian androgen producing cells.

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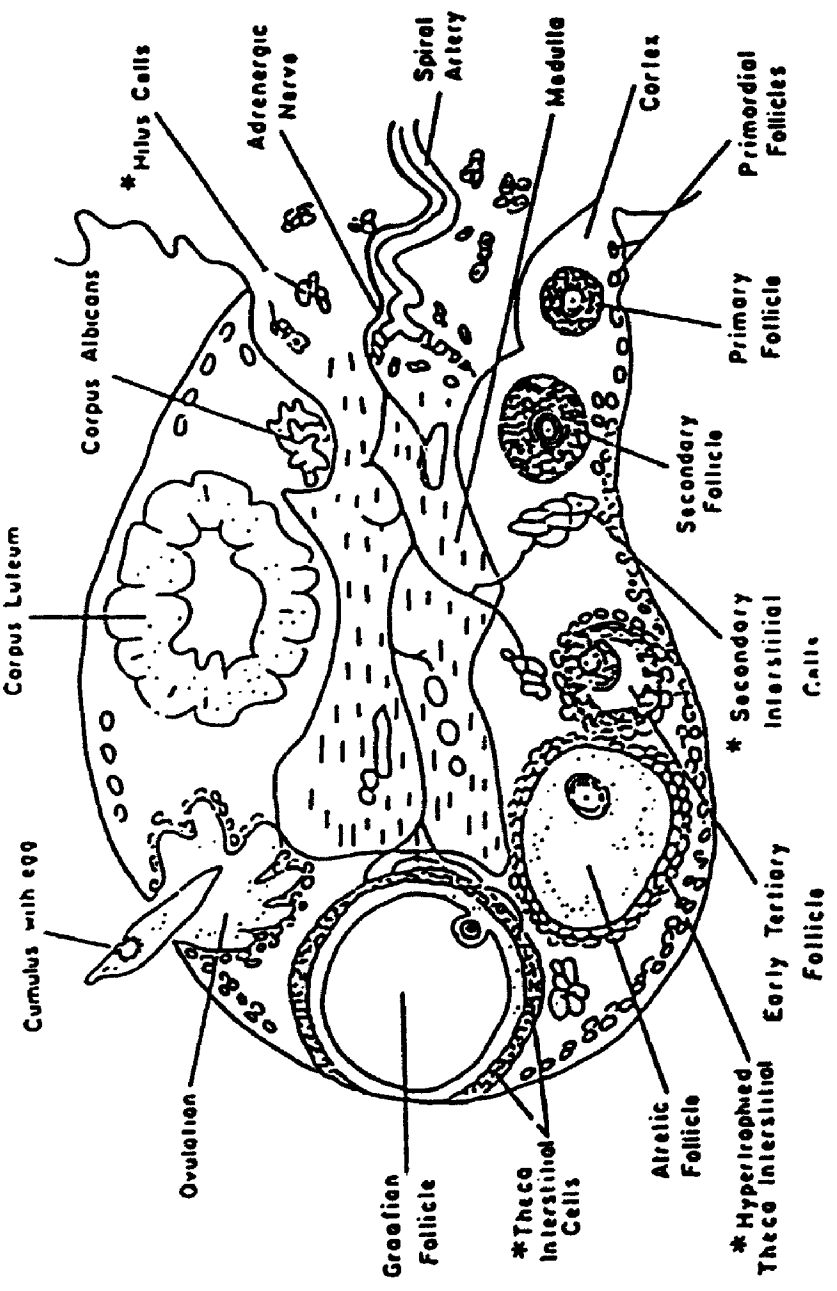
LOCATION OF OVARIAN INTERSTITIAL CELLS



appear between 12 and 20 weeks gestation in the medullary regions of fetal human ovaries (Gondos and Hobel, 1973). The cells contain abundant smooth endoplasmic reticulum, large spherical mitochondria and well developed Golgi complexes, features typical of active steroidogenic cells (Gondos and Hobel, 1973). However, these cells are not able to produce de novo steroids in vitro and are unable to convert exogenous steroid precursors to androgens (Jungmann and Schweppe, 1968; Bloch, 1964; Resko, 1970). These observations are in agreement with results of Abramowich and Rowe (1973) who found very low testosterone levels in cord blood of female fetuses near mid-term.

Human primary interstitial cells can, however, convert pregnenolone sulfate to pregnenolone, 17α-hydroxypregnenolone, DHEA and androstenedione via the Δ^5 pathway (Payne and Jaffe, 1974; Schindler and Friedrich, 1975). This is an important observation since the blood of human fetuses of 10-20 weeks gestation has been shown to contain very high concentrations of pregnenolone sulfate (Huhtaniemi and Vihko, 1970), suggesting that the primary interstitial cells of the human fetal ovary may synthesize and secrete androgens. These cells are unresponsive to stimulation by pituitary gonadotropins (Wilson and Jawad, 1979). The physiological function and regulation of steroid production in the primary interstitial cells is unknown.

2.4.2 The Hilus Interstitial Cells

The hilar region of the mammalian ovary contains clusters of large steroidogenic cells (Sternberg, 1949). These cells contain a large amount of smooth endoplasmic reticulum, fat droplets and ribosomes (Sternberg, 1949; Schnoy, 1982), and crystals called the crystals of Reinke (Jefficate

and Prunty, 1968; Schnoy, 1982). The chemical nature of these crystals and their relationship to the endocrine activity of the cells are not known. The presence of large amounts of these crystals is seen in ovarian hilar cell tumors. Incubations of ovarian hilar tumor cells demonstrated that these cells synthesize and secrete androgens in response to LH stimulation (Sternberg, 1949; Sternberg et al., 1953; Jeffcoate and Prunty, 1968). Ovarian tumors of the hilus cells are always associated with masculinization (Sternberg, 1949; Jeffcoate and Prunty, 1968). In humans, the hilus cells are always closely associated with unmyelinated sympathetic nerve fibres, blood vessels and lymphatics (Sternberg, 1949); however, the relationship between the nerve fibres and these cells is not clear. The function of these cells is not known.

2.4.3 The Theca-Interstitial Cells

This section will discuss the development of the ovarian follicle from the primordial to the Graafian stage with special reference to changes in the theca cell layer. Figure 3 outlines the changes that occur in the follicle during the various stages of ovarian follicle development.

Early in folliculogenesis, undifferentiated primordial follicles, consisting of a single layer of granulosa cells condensed around a primordial germ cell, are selected from the non-growing pool of follicles and growth is initiated. In the initial phases of growth, the primordial follicles form primary follicles in which the oocyte is surrounded by a unilaminar layer of cuboidal granulosa cells. The granulosa cell layer develops as a stratified epithelium limited peripherally by a basal lamina (Priedkhalns et al., 1968) and the oocyte increases in size.

Following the growth of the granulosa cells to 2-3 layers thick and the

Figure 3. Classification of developing follicles.

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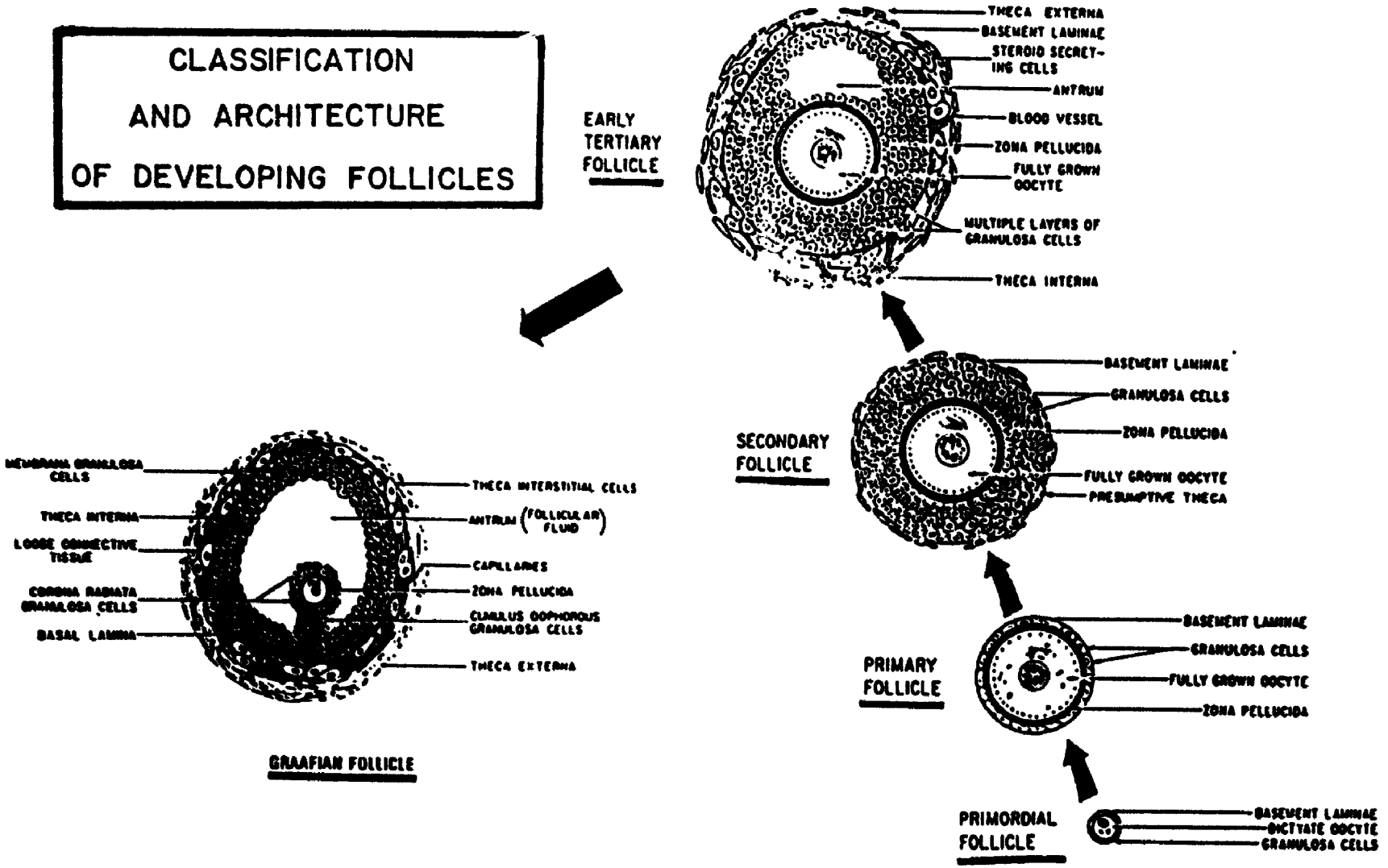
CLASSIFICATION AND ARCHITECTURE OF DEVELOPING FOLLICLES



full growth of the oocyte, the follicle is termed a secondary follicle. On the other side of the basal lamina, ovarian stromal cells condense on the basal lamina to form a loose matrix of cells called the theca. Initially this layer consists of spindle-shaped cells which are indistinguishable from fibroblasts and epithelioid cells (Hiura and Fujita, 1977). At this stage the cells have a moderate amount of granular endoplasmic reticulum, a small Golgi, but no smooth endoplasmic reticulum or lipid droplets (Fawcett et al., 1969).

The origin of the thecal cells is unknown but there is some evidence that they may arise from a population of unspecialized mesenchymal cells with migratory capabilities located in the ovarian stroma (Weakley, 1966; Hoage and Cameron, 1976). The mesenchymal cells are thought to be pluripotent stem cells which are capable of both proliferation and differentiation. This is important since the primary follicles do not contain theca cells, theca cells must arise continually throughout reproductive life. Based on ultrastructural evidence, the theca cells may originate from cells which form the follicular granulosa cells, but become separated from the oocyte when the follicle is enclosed (Mori and Matsumoto, 1970; Quattropani, 1973; Hoyer and Byskov, 1981).

The signal for the migration of cells to the outer surface of the follicle, their organization and ultimate differentiation is not known. However, interactions between granulosa and theca are undoubtedly very important in the development of these cells.

When the migratory stromal cells reach the basal lamina they align in a parallel manner, forming a ring of fibroblast-like cells around the follicle. The thecal cells divide into two distinct layers, the vascularized theca interna and the fibrous theca externa. The theca

externa consists of densely packed fibrous connective tissue with abundant collagen fibres and it is traversed by blood and lymph vessels. The theca externa develops from fibroblasts and smooth muscle cells (O'Shea, 1970). Immunofluorescent studies have shown staining for actin and myosin in the theca externa (Amsterdam et al., 1977).

The secondary follicle also acquires a blood supply. With the development of the theca, one or two arterioles are seen terminating in a wreath-like network of arterioles and capillaries in the inner portion of the theca adjacent to the basal lamina (Basset, 1943; O'Shea et al., 1978a). At the time of vascularization, the fibroblast-like cells develop LH receptors (Midgley, 1973) and the enzyme 3β-hydroxysteroid dehydrogenase (Magoffin and Erickson, 1982c). The stimulus for this transformation is not known. Gap junctions link thecal cells in multilaminar follicles in the hypophysectomized rat and hCG treatment increases the size and frequency of the gap junctions and leads to thecal hypertrophy (Burghardt and Anderson, 1981). In the theca interna of sheep follicles, as the size of the follicle increases, from small differentiating follicles of less than 3 mm to the largest follicles on day 15, the number of cells showing ultrastructural features of steroidogenically active cells increases from 20% to 50%. Other cell types include fibroblasts and a large number of undifferentiated cells (O'Shea et al., 1978a). As development of the follicle continues, the theca stem cells continue to divide by mitosis, thus increasing their number (Lane and Davis, 1939).

The final phase of development is the conversion of the secondary follicle to a tertiary follicle. At this stage granulosa cells develop specific membrane receptors for FSH, and under FSH stimulation, fluid

appears between the dividing granulosa cells which eventually coalesce to form the fluid filled cavity known as the follicular antrum.

During the antral growth stage, follicles show a steady increase in estrogen production under the stimulation of FSH and LH (Armstrong and Papkoff, 1976; Erickson and Hsueh, 1978). FSH acts on the granulosa cells of antral follicles to induce the aromatase enzyme(s) that convert thecal androgens to estrogens. The estrogen produced is secreted into the circulation and also acts locally in the follicle by binding to receptors in the granulosa cells resulting in stimulation of granulosa cell proliferation (Goldenberg et al., 1972). FSH also induces the formation of LH (Richards et al., 1976; Richards and Williams, 1976), prolactin (Richards and Williams, 1976; Wang et al., 1979), and catecholamine (Coleman et al., 1979) receptors in granulosa cells during the antral stage.

In the sheep follicle, the maximum differentiation of the theca interna occurs in late estrus, but even then, not all cells contain smooth endoplasmic reticulum (O'Shea et al., 1978b). The LH surge causes ovulation, after which connective tissue cells and blood vessels from the theca interna invade the granulosa cell layer. Formation of the corpus luteum is initiated by a series of morphological and biochemical changes in the cells of the theca interna and membrana granulosa. This process is called luteinization. The granulosa cells are morphologically transformed to granulosa lutein or large luteal cells and the theca interna form the theca lutein or small luteal cells.

2.4.4 The Secondary Interstitial Cells

Secondary interstitial cells found in the ovary from infancy to old age

are derived from theca interna cells of degenerating or atretic follicles in the cat (Kingsbury, 1939), rat (Dawson and McCabe, 1951), human (Mossman et al., 1964), hamster (Guraya and Greenwald, 1968), rabbit (Deanesly, 1972), and guinea pig (Deanesly, 1972). The oocyte and granulosa cells of atretic follicles degenerate and are eliminated; however, the theca cells surrounding the follicle hypertrophy and remain as groups of steroidogenically active cells in the ovarian stroma and give rise to the secondary interstitial cells. Secondary interstitial cells maintain their ultrastructural characteristics of active steroid secreting cells (Mori and Matsumoto, 1973; Al-Mehdi, 1979), and respond to LH with androstenedione production (Rice and Savard, 1966; McNatty et al., 1979a). Unlike the theca cells, androgen production by the secondary interstitial cells continues in aged ovaries (Dennefors et al., 1980). In the pig, dog, and sheep, theca cells regress completely without any contribution to the interstitium (Priedkalns et al., 1968; O'Shea et al., 1978b; Centola, 1982).

Ultrastructural studies in several species have shown that sympathetic adrenergic nerves terminate directly on secondary interstitial cells (Burden, 1972; Svensson et al., 1975; Lawrence and Burden, 1978; Capps et al., 1978). Stimulation of the ovarian sympathetic nerves causes changes in the ultrastructure (Capps et al., 1978) and histochemcial characterisitics (Burden and Lawrence, 1977b) of the secondary interstitial cells. I will discuss the function of the ovarian nerves in greater detail in a later section.

2.5 THE ANDROGEN PRODUCING CELLS: STEROIDOGENIC FUNCTIONS

Despite the importance of the ovarian androgens, there have been few

studies on the physiology of the ovarian androgen producing cells due to difficulty in obtaining large numbers of these cells for in vitro experiments. Little progress was made in understanding the function of these cells until the development of suitable methods for collecting and separating ovarian androgen producing cells from other ovarian cell types. Two cell culture models have been used to investigate the function of the ovarian androgen producing cells: 1) a primary cell culture model for theca-interstitial cells derived from hypophysectomized immature rats as developed by Magoffin and Erickson, (1982c) and 2) the culture of enzymatically dispersed theca cells, obtained by mechanical separation of the theca layer from the ovarian follicular basement membrane, following the removal of the granulosa cells.

2.5.1 The Theca-Interstitial Cell Model From Hypophysectomized Immature Rats

A rat theca-interstitial cell culture system was developed by Erickson using hypophysectomized immature rats. Hypophysectomy results in an increase in follicular atresia, and a subsequent increase in the number of interstitial cells (Dawson and McCabe, 1951). In addition, hypophysectomy causes an accumulation of preantral follicles, of which greater than 90% are atretic (Magoffin and Erickson, 1982c).

In culture, theca-interstitial cells form aggregates of a few to 50 rounded cells and exhibit markers of their differentiated state. Freshly dispersed cells contain a single class of high affinity LH receptors, 3β-HSD activity as determined by histochemistry, and cholesteryl esters as determined by oil-red O staining (Magoffin and Erickson, 1982c; Erickson et al., 1985).

In this system LH or hCG can induce androgen synthesis following a latent period of 3 days, and LH responsiveness is maintained in serum-free medium for up to 30 days. Androgens account for 96% of the total steroids produced (androsterone > 5α-androstane-3α,17β-diol > androstenedione). The 5α-reduction of androgens in culture is consistent with reports showing that androsterone and 5α-androstane-3α,17β-diol are the principal steroids produced by the prepubertal rat ovary (Eckstein et al., 1970; Karakawa et al., 1976), and their production has been shown to be stimulated by LH (Fukuda et al., 1979; Terakawa et al., 1978).

The mechanism by which LH induces theca-interstitial cell differentiation is thought to involve a cyclic AMP-dependent activation of gene expression (Magoffin and Erickson, 1982c). It is not known which genes are activated; however, metabolic studies have shown that LH increases cholesterol side chain cleavage (Bai et al., 1973), 17α-hydroxylase and C_{17-20} lyase (Fukuda et al., 1979; Bogovich and Richards, 1982), and 5α-reductase (Terakawa et al., 1978) activities in vivo.

Initial studies of theca-interstitial cell structure/function relationships used this model; however, the cell population employed was not pure. Contamination by other ovarian cell types such as granulosa cells, oocytes or fibroblasts may have an effect on the differentiation of theca-interstitial cells. Magoffin and Erickson (1988a) have recently reported a method for obtaining highly enriched theca-interstitial cells using a discontinuous Percoll gradient. The purified cells produced more cyclic AMP and androstenedione, and showed greater 3β-HSD activity and ^{125}I-hCG binding than unfractionated cells. The purified cells were estimated to be 93% theca-interstitial cells. The cells were LH responsive

and expressed differentiated functions in response to physiological concentrations of LH.

2.5.2 Theca Interna Cultures From Antral Follicles

Establishing the metabolic capabilities of the various cells of the ovarian follicle required the separation of follicular granulosa and theca cells so the biosynthetic capabilities of each could be studied directly. The first development was by Bjersing and Carstensen (1964) who scraped the granulosa cells from ruptured porcine follicles and cultured the granulosa cells and follicle walls, which were expected to contain mainly theca cells. Using this system these authors demonstrated the conversion of androstenedione to testosterone and testosterone to estrone and estradiol by isolated porcine follicle walls (Bjersing and Carstensen, 1964).

The system was improved such that developing follicles were dissected from surrounding connective and stromal tissue, the follicles ruptured, and the granulosa cells were gently scraped away along with the oocytes, leaving the follicle wall intact. The follicle wall contained both theca interna and externa; however, a procedure was developed to peel the theca interna layer from the follicular basement membrane so that theca interna cells could be cultured separately. Theca interna cells were either cultured as shells or were dispersed into single cell suspensions using trypsin (Stoklosowa et al., 1978) or collagenase-hyaluronidase (Tsang et al., 1985). Stoklosowa et al. (1978) estimated that porcine theca interna cell cultures isolated by this procedure contained 70% theca interna cells, 8% granulosa cells and 23% non-steroidogenic cells, which were mainly fibroblasts. This procedure has now been used to prepare theca cell

cultures from many species, including the pig (Bjersing and Carstensen, 1964; Tsang et al., 1979a), horse (Ryan and Short, 1965; Ryan and Short, 1966), hamster (Makris and Ryan, 1975), rabbit (Younglai, 1972), rat (Fortune and Armstrong, 1978), sheep (Weiss et al., 1978), cow (Lacroix et al., 1974) and human (Ryan and Petro, 1966; Ryan et al., 1968; McNatty et al., 1979a; Tsang et al., 1980).

Using this procedure, it was discovered that theca cells have the capacity for the de novo synthesis of cholesterol (Ryan and Short, 1966), de novo steroid synthesis from acetate (Ryan et al., 1968), the aromatization of androgens to estrogens (Ryan and Short, 1965) as well as side chain cleavage of C_{21} steroids.

Studies from many species have determined that LH is the gonadotropin mainly responsible for androgen production by theca cells; however large amounts of androgen can be produced in the absence of exogenous gonadotropins (Makris and Ryan, 1975; Erickson and Ryan, 1976; Fortune and Armstrong, 1977; Moor, 1977; Tsang et al., 1979b). In porcine theca cell cultures, LH, but not FSH, stimulates a 2-3-fold increase in thecal androstenedione, testosterone, 5α-dihydrotestosterone, 17α-hydroxyprogesterone and progesterone production as well as a 50-200-fold increase in extracellular cyclic AMP accumulation (Tsang et al., 1979a; Evans et al., 1981; Tsang et al., 1987). The greater abundance of androstenedione than testosterone is due to a deficiency in 17β-hydroxysteroid dehydrogenase and has been observed in the rat (Hamberger et al., 1978), hamster (Makris and Ryan, 1975), sheep (Moor, 1977), cow (Lacroix et al., 1974) and human (McNatty et al., 1980a). As the follicle develops and increases in size, thecal steroidogenesis increases (Evans et al., 1981; Tsang et al., 1985). Porcine theca cells in culture also

produce estrogen in the absence of exogenous aromatizable substrate; however, estrogen production is not stimulated by gonadotropins (Tsang et al., 1982). Porcine theca cell cultures have also been shown to produce relaxin (Evans et al., 1983a) and the prostaglandins $PGF_{2\alpha}$ and PGE_2 (Evans et al., 1983b). Thecal prostaglandin production is not functionally coupled to steroidogenesis and is not stimulated by LH or FSH (Evans et al., 1983b).

Following LH/hCG treatment, significant increases in cyclic AMP accumulation occur prior to increases in androgen production. In addition, dibutyryl cyclic AMP can mimic the action of LH/hCG in stimulating androgen production, suggesting that LH/hCG stimulates androgen production through the activation of the adenylate cyclase system (Erickson and Ryan, 1976; Tsang et al., 1979b). In addition, theca cells from these large preovulatory follicles contain lipid droplets as determined by oil red O staining, have alkaline phosphatase and glucose-6-phosphate activity (Stadnicka and Stoklosowa, 1976) and show intense 3β-HSD activity (Stoklosowa et al., 1978). Like the theca-interstitial cell preparation from hypophysectomized immature rats, this system provides a good model for the study of structure/function relationships in the cytodifferentiation of the ovarian theca cells.

2.5.3 Hormonal Control of Androgen Production: Role of LH

LH is the most important stimulator of ovarian androgen production. LH acts via interaction with specific membrane receptors found on theca cells at all stages of follicular development (Zeleznik et al., 1981; Oxberry and Greenwald, 1982) to activate adenylate cyclase (Weiss et al., 1978; Tsang et al., 1979b). The elevated levels of cyclic AMP activate specific

protein kinases which phosphorylate regulatory proteins (Dufau et al., 1977). The addition of physiological concentrations of LH/hCG, cyclic nucleotide analogues or natural activators of adenylate cyclase to theca-interstitial or theca cell cultures causes marked increases in androgen synthesis. Inhibitors of RNA and protein synthesis reversibly block LH action, suggesting that LH, via cyclic AMP, activates genes in interstitial cells which code for cholesterol side chain cleavage, 17α-hydroxylase and the C_{17-20} lyase enzymes (Erickson et al., 1985).

Although LH is the primary regulator of ovarian androgen production, the cells' responsiveness can be modulated in either an inhibitory or stimulatory manner by a variety of circulating, follicular fluid and neural factors. These factors will be looked at in the following sections.

2.5.4 Inhibitors of Ovarian Androgen Production

A. Estrogen

The concept that estrogen could modulate the function of ovarian androgen producing cells, emerged from the demonstration that ^{3}H-estradiol is concentrated in the nuclei of the theca and secondary interstitial cells (Stumpf, 1969). Leung and coworkers (1978) showed that treating intact immature rats with estradiol suppressed ovarian androgen production.

The inhibitory effects of estrogen on ovarian androgen synthesis have also been demonstrated *in vitro*. Whole ovaries from estrogen-treated immature, intact, or hypophysectomized rats responded to LH-stimulation *in vitro* with decreased androgen production when compared to that of ovaries obtained from rats that were not treated with estrogen (Leung and

Armstrong, 1979a). In vivo estradiol treatment of immature rats also suppressed androgen production by isolated thecal tissue (Leung and Armstrong, 1980). In addition, treatment of isolated porcine thecal tissue (Leung and Armstrong, 1980) or dispersed theca cells (Tsang et al., 1979a; Hunter and Armstrong, 1987) with estrogens inhibited LH-stimulated androgen production in a dose-dependent manner, establishing that estrogens act directly on the theca cells.

The mechanism of the inhibitory action of estrogen on androgen production appears to be at a site distal to cyclic AMP production, at the 17α-hydroxylase:C_{17-20} lyase enzyme complex, thereby converting the cells from androgen producing cells to progestin producing cells (Leung and Armstrong, 1979b; Magoffin and Erickson, 1981; Magoffin and Erickson, 1982d). It is not known how estradiol inhibits the action of the enzymes in the ovary; however, in the testes there is evidence that estradiol is a competitive inhibitor of the 17α-hydroxylase:C_{17-20} lyase enzyme complex (Onoda and Hall, 1981). To prove this theory, in vivo estrogen pretreatment of ovaries from intact immature rats inhibited the conversion of radiolabelled progesterone to androgens in vitro (Leung and Armstrong, 1980). However, incorporation of the labelled progesterone into 3α-hydroxy-5α-pregnan-20-one was enhanced, suggesting that estrogen may act by inhibiting the 17α-hydroxylase:C_{17-20} lyase enzyme system, or by diverting C_{21} substrates into an alternate pathway resulting in the formation of 5α-reduced pregnanes (Leung and Armstrong, 1980). Estrogens have no effect on LH receptor number and/or affinity, LH-stimulated cyclic AMP accumulation or the viability of the ovarian steroidogenic cells (Magoffin and Erickson, 1982d).

(Magoffin and Erickson, 1982a). GnRH antagonists block the GnRH responses (Magoffin et al., 1981).

The importance of GnRH in ovarian development is not known. Hypothalamic GnRH is unlikely to act on the ovarian theca cells due to its very low concentrations; the highest concentration in human plasma occurs during the preovulatory period when levels reach 8 x 10^{-12} M (Elkind-Hirsch et al., 1982). These concentrations are too low to cause the inhibitory effects observed.

D. Growth Factors (EGF, FGF)

It is now evident that many different "growth factors" are capable of influencing ovarian cell steroidogenesis. Gospodarowicz et al. (1977) was the first to demonstrate that epidermal growth factor (EGF) and fibroblast growth factor (FGF) are mitogenic agents for granulosa cells. It was shown susequently that growth factors were also capable of influencing granulosa cell steroidogenesis (Mondschein and Schomberg,1981a; Mondschein and Schomberg, 1981b).

In the rat theca-interstitial cell model EGF inhibits LH-induced steroidogenesis at a site between cyclic AMP and the cholesterol side-chain cleavage enzyme (Erickson and Case, 1983). In testicular Leydig cells, EGF acts by decreasing the number of LH receptors on the cells' membrane (Ascoli, 1981). It is not know if EGF affects LH receptors on the ovarian androgen producing cells.

The physiological role of growth factors in follicular development is not known. The possibility that growth factors may be produced locally by ovarian cells and have autocrine actions affecting follicular function has to be considered.

B. Prolactin

Prolactin has also been reported to have an inhibitory influence on ovarian androgen secretion. Treatment of adult rats with prolactin caused decreased levels of androstenedione in preovulatory follicles (Tsai-Morris et al., 1983). Injection of prolactin into LH primed rats arrested androgen production by the interstitial cells (Magoffin and Erickson, 1982b). On the other hand, bromocryptine induced hypoprolactinemia in vivo was accompanied by marked increases in androgen secretion by preovulatory rat ovaries in response to hCG stimulation (Advis et al., 1981).

Ovarian theca and interstitial cells of antral and preantral follicles possess prolactin binding sites (Magoffin and Erickson, 1982b; Oxberry and Greenwald, 1982). The addition of physiological concentrations of prolactin to cultured rat theca-interstitial cells caused an irreversible, dose-dependent inhibition of LH-stimulated androgen formation at a step distal to adenylate cyclase (Magoffin and Erickson, 1982b).

C. Gonadotropin Releasing Hormone (GnRH)

In 1976, Rippel and Johnson reported that a GnRH agonist blocked the ability of hCG to stimulate ovarian weight gain in immature hypophysectomized rats, suggesting a direct effect of GnRH on the ovary.

Specific high affinity binding sites for GnRH have been demonstrated in ovarian theca cells (Pelletier et al., 1982) at all stages of differentiation. The absence of GnRH binding sites in the porcine, ovine and bovine ovaries suggests these species may not share the GnRH responsiveness seen in the rat (Brown and Reeves, 1983). GnRH inhibits LH-stimulated differentiation of rat theca-interstitial cells via a selective inhibition of the 17α-hydroxylase:$C_{17,20}$ lyase enzyme complex

2.5.5 Amplifiers of Ovarian Androgen Production

A. Lipoproteins

Ovarian steroids are produced from cholesterol which can be obtained from three sources: 1) de novo cholesterol synthesis from acetate, 2) preformed cholesterol stored within the ovarian cell, either as free cholesterol, a constituent of cell membranes or liberated from cholesterol stored in cytoplasmic lipid droplets, or 3) preformed cholesterol taken up from the blood, principally in the form of lipoproteins, either low density lipoproteins in the human, or high density lipoproteins in the rat. Lipoprotein cholesterol is the most important source of steroidogenic cholesterol (Strauss et al., 1981). Theca interna cells receive a rich vascular supply and would therefore be exposed to this cholesterol source.

The addition of lipoproteins to LH-stimulated theca-interstitial cells, significantly increased androgen production over that stimulated by LH alone (Dyer et al., 1985; Magoffin and Erickson, 1988b). LH increases the capacity of cholesterol side chain cleavage to convert cholesterol to pregnenolone by increasing the amount of cholesterol bound to the cytochrome P-450 side chain cleavage (Hall and Young, 1968; Leaver and Boyd, 1981).

B. Insulin and Insulin-Like Growth Factors

Insulin has been shown to augment LH-stimulated androgen production by cultured porcine theca tissue (Barbieri et al., 1983) and cultured rat theca-interstitial cells (Erickson and Case, 1983; Magoffin and Erickson, 1988b). In addition, the insulin-like growth factor-I (IGF-I; Somatomedin C), has also been shown to enhance thecal androgen production in vitro

(Caubo and Tonetta, 1988; Hernandez et al., 1988a).

Studies using cultured porcine theca cells have shown that insulin acts, at least in part, via an enhancement of the generation of cyclic AMP leading to increased activity of the steroidogenic enzymes cholesterol side chain cleavage and 3β-HSD (Morley et al., 1989). Insulin has a general permissive effect on the synthesis of all steroids by porcine theca cells and rat theca-interstitial cells (Magoffin and Erickson, 1988b; Morley et al., 1989). Insulin is known to increase glucose transport into cells raising the possibility that the increased steroid synthesis results from increased intracellular glucose, increased de novo synthesis of cholesterol or a generalized enhancement of the theca cells metabolic capacity.

The observation that supraphysiological concentrations of insulin are required for insulin effects suggests the possibility that insulin is acting via IGF-I receptors rather than through high affinity insulin receptors. IGF-I mimics the effects of insulin on rat theca-interstitial cells, but at 500-fold lower concentration (Magoffin and Erickson, 1988b) suggesting that IGF-I, and not insulin, may be the more important physiological regulator of ovarian androgen production. Each peptide binds with high affinity to its own receptor and with lower affinity to the receptors for the related peptides. This occurs due to structural similarities between the peptides (Zapf et al., 1981) and their receptors (Czech et al., 1983). The presence of insulin and/or insulin-like growth factor receptors have been characterized in rat theca-interstitial cells (Hernandez et al., 1988a), but not in dispersed porcine theca cells.

The possibility that the insulin-like growth factors are produced locally in the theca cells, as they are in the granulosa cells (Hammond

et al., 1985; Hsu and Hammond, 1987), is an interesting possibility that has not yet been investigated.

The control of ovarian androgen producing cells is affected by a dynamic balance between stimulatory and inhibitory factors. The mammalian ovary receives sympathetic innervation which may also play a role in controlling a variety of ovarian functions, including steroidogenesis. The following section will review the literature on the anatomy and possible functions of the ovarian nerves.

2.6 OVARIAN NERVES

Over the past 50 years the hormonal control of ovarian function has been studied extensively. Anatomists have been studying the innervation of the ovary for over 100 years, however few physiologists regard this subject as important to the understanding of ovarian function, as witnessed by the lack of reference to the innervation in most literature pretaining to ovarian function. Recently the study of the physiological role of ovarian nerves in ovarian function has increased dramatically. We now realize that ovarian nerves, in addition to the peptide and steroid hormones, are involved in ovarian regulation.

2.6.1 Innervation of the Ovary

The anatomical study of ovarian nerves began in 1867 when Frankenhauser first described the nerve supply to the female reproductive tract. Since that time the innervation of the mammalian ovary has been well documented in many species. The perikarya of preganglionic sympathetic nerves destined for the ovary arise from the lower thoracic (T_{10} and T_{11}) segments

Figure 4. Innervation of the mammalian ovary.

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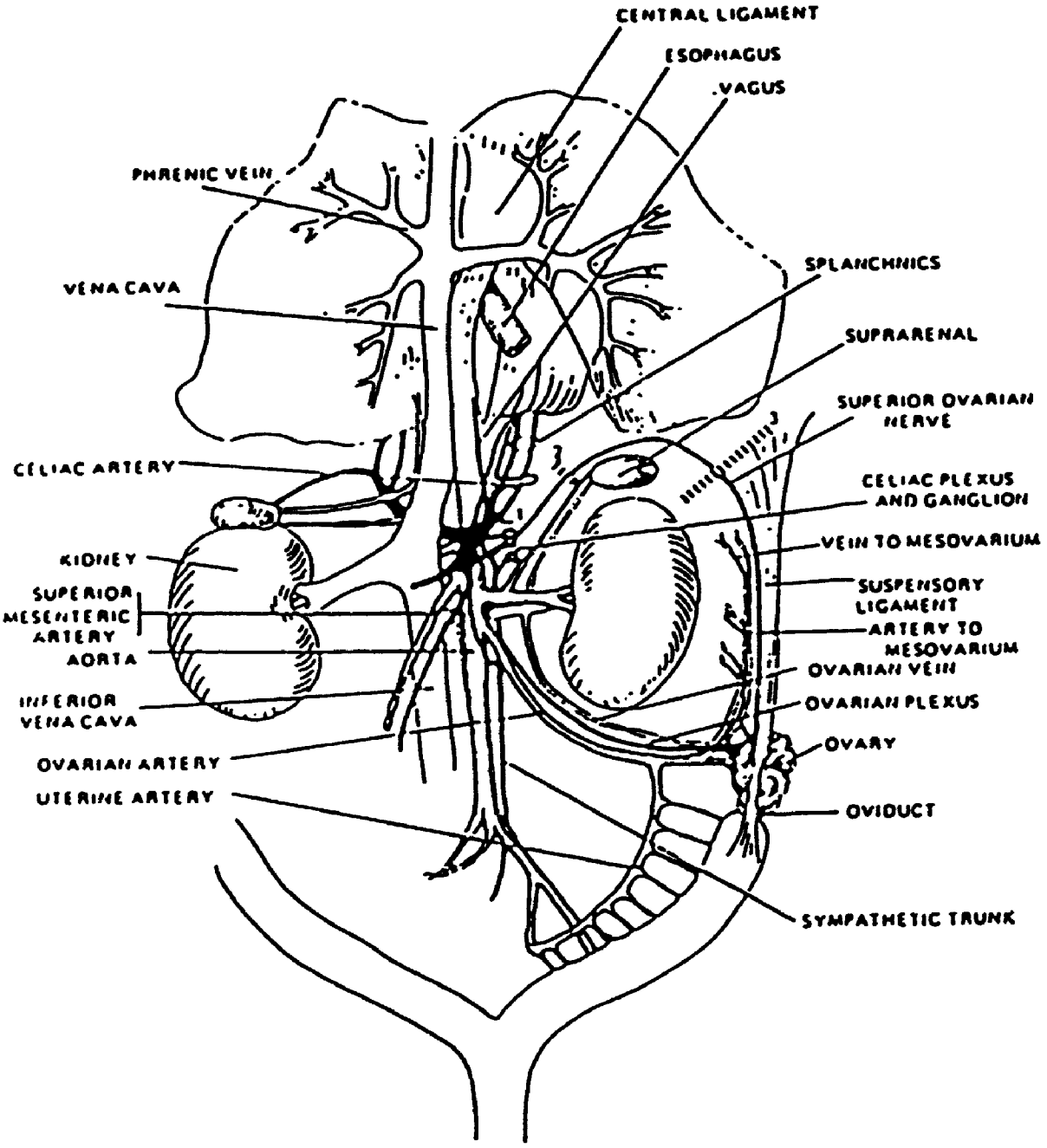

CENTRAL LIGAMENT
ESOPHAGUS
VAGUS
PHRENIC VEIN
VENA CAVA
SPLANCHNICS
SUPRARENAL
SUPERIOR OVARIAN NERVE
CELIAC ARTERY
CELIAC PLEXUS AND GANGLION
KIDNEY
VEIN TO MESOVARIUM
SUPERIOR MESENTERIC ARTERY
SUSPENSORY LIGAMENT
ARTERY TO MESOVARIUM
AORTA
OVARIAN VEIN
INFERIOR VENA CAVA
OVARIAN PLEXUS
OVARY
OVARIAN ARTERY
UTERINE ARTERY
OVIDUCT
SYMPATHETIC TRUNK

of the spinal cord (Gillilan, 1954). The postganglionic fibers arise in the cells of the ovarian ganglion, which is located at the origin of the ovarian artery, or from the cell bodies of the celiac or renal plexuses (Crosby et al., 1962). In the rat, the noradrenergic innervation reaches the ovary by two separate routes (Figure 4): via the traditionally described ovarian plexus along the ovarian artery and via a large nerve in the suspensory ligament, called the superior ovarian nerve (SON) (Lawrence and Burden, 1980).

The mammalian ovarian plexus contains both afferents and efferents, the latter believed to be composed of both sympathetic and parasympathetic fibers (Crosby et al., 1962; Bahr et al., 1974). In the rat, the parasympathetic innervation is derived from the vagus nerve (Fink and Schofield, 1971; Burden, 1978; Burden and Lawrence, 1978). The parasympathetic innervation may also arise from S-2 to S-4 cord levels via the hypogastric plexus (Mitchell, 1938). Most of the vagal innervation is probably composed of sensory fibers, since in the rabbit only 10% of the abdominal vagal fibers are motor neurons (Gabella and Pease, 1973). These fibers have been shown to project via the nucleus of the solitary tract to the preoptic area, the arcuate, dorsal medial and paraventricular nuclei of the hypothalamus (Ricardo and Koh, 1978).

The cell bodies of the afferent nerves of the rat ovary have been demonstrated by horseradish peroxidase injections into the ovary. Peroxidase was localized in the perikarya of nodose ganglia of the vagus nerve and in the ipsilateral dorsal root ganglia from levels T_{10}-L_2 (Burden et al., 1983). These observations suggest there are visceral afferent fibers from the ovary which may mediate visceral reflexes to control ovarian function.

The other source of the ovarian innervation, the SON, is found in the suspensory ligament. The suspensory ligament is a muscular ligament which attaches to the lower edge of the thoracic cage, extends caudally, and is continuous with the longitudinal musculature of the uterus (Gabella, 1976). As the suspensory ligament passes the ovary and oviduct it attaches to these structures and nerve fibers enter the ovarian hilus and bursa and also continue to innervate the top third of the uterus (Melton and Saldivar, 1970; Thorbert et al., 1977; Musgrove et al., 1978). The suspensory ligament has been shown with fluorescence microscopy to contain bundles of noradrenergic and acetylcholinesterase-positive nerve fibres (Musgrove et al., 1978; Lawrence and Burden, 1980). The majority of the nerves derived from the plexus nerve are perivascular, while those derived from the SON innervate blood vessels, follicles and the interstitial tissue (Lawrence and Burden, 1980). Myelinated and nonmyelinated nerves were observed in the SON and in the plexus nerve (Payer, 1978). The functions of the myelinated nerves are unknown; however, since myelinated autonomic postganglionic afferents are rare, they are probably efferents. It is reasonable that a major nerve supply to the ovary is by way of the suspensory ligament since both the ovary and the suspensory ligament are derivatives of the same primordium, the genital ridge (Franchi et al., 1962).

2.6.2 Distribution of Ovarian Nerves

Classical studies on the distribution of nerve fibers in the ovary produced conflicting information because the silver impregnation technique used could not clearly distinguish between reticular fibers, elastic fibers and nerve fibers (Bahr et al., 1974). It was also impossible to

distinguish between noradrenergic and other types of nerve fibers. Contemporary studies have made use of the highly-specific Falck-Hillarp fluorescence histochemical technique for the localization of noradrenaline in nerve tissues (Falck and Owman, 1965). The general pattern of distribution of ovarian noradrenergic nerves is similar in all species studied, although the degree of innervation varies among species. Ovarian nerves enter the ovary at the hilar perivascular plexus and initially follow the branching of the ovarian artery. From this perivascular plexus, nerve branches extend into the ovarian parenchyma and innervate blood vessels (for reviews see Burden, 1978; Mohsin and Pennefather, 1979). Bundles of non-myelinated nerves in the stroma innervate smooth muscle cells as well as the smooth muscle of blood vessels (Neilson et al., 1970; Unsicker, 1970; Burden, 1972; Svensson et al., 1975). The naked nerve terminals lie in close proximity (50 nm) to the smooth muscle cells and endocrine cells, a distance short enough for the diffusion of neurotransmitters to provide innervation (Dahl, 1970; Unsicker, 1970).

Many investigators have demonstrated the presence of noradrenergic nerve terminals in close proximity to blood vessels and follicles of all stages of development (Jacobowitz and Wallach, 1967; Owman et al., 1967; Stefenson et al., 1981). A dense noradrenergic innervation of the perifollicular region without any relation to blood vessels has been described for several species, although the nerves do not penetrate the basement membrane of the follicle, so there is no direct innervation of the granulosa cell layer (for reviews see Burden, 1978; Mohsin and Pennefather, 1979). The perifollicular nerves innervate the theca cells of mature follicles (Jacobowitz and Wallach, 1967; Burden, 1972; Svensson et al., 1975; Stefenson et al., 1981). Like the follicles, the corpora

lutea are not innervated (Jacobowitz and Wallach, 1967; Unsicker, 1974). Several investigators have observed noradrenergic innervation of the interstitial cells (Unsicker, 1970; Burden, 1972; Svensson et al., 1975; Lawrence and Burden, 1976). The number of noradrenergic nerves and the intensity of their fluorescence in the interstitial gland increases during pregnancy (Lawrence and Burden, 1976).

The development of the ovarian innervation from fetal to adult life has been reported by Burden et al., (1985). In the guinea pig, before birth, there are few ovarian noradrenergic nerves and correspondingly low noradrenaline levels. After birth and through day 20, nerves gradually grow into the ovarian stroma; this is reflected by increased ovarian levels of noradrenaline (Burden et al.,1985).

2.6.3 Ovarian Catecholamines

There is a good correlation between the density of fluorescent nerve fibres and the concentration of noradrenaline in the ovary. Noradrenaline has been measured in the ovary of many species and its concentration varies in different species, ranging from 0.2 $\mu g/g$ in the rabbit to 4.9 $\mu g/g$ in the cat (Jacobowitz and Wallach, 1967; Stefenson et al., 1981). There is no information on the compartmentalization of catecholamines within the mammalian ovary. In the ovary of the domestic hen noradrenaline is found in significantly greater concentrations in the theca layer than in the granulosa cell layer (Bahr et al., 1986). Species with a rich ovarian innervation include the cow, sheep, cat and guinea pig (Stefenson et al., 1981). Species with an intermediate degree of ovarian innervation include the human, pig, dog and rat, while the rabbit and mouse have a sparse innervation (Stefenson et al., 1981). The concentration of

noradrenaline in porcine follicular fluid from small follicles reaches 34 nM (Veldhuis et al., 1980). This intrafollicular concentration of noradrenaline is 10-fold greater than in the general circulation, suggesting a local site of production may exist. Denervation of the rat ovary by sectioning the suspensory ligament or ovarian plexus nerve greatly reduces or virtually abolishes both the catecholamine-staining nerve fibers and the amount of chemically determined noradrenaline in the ovary (Lawrence and Burden, 1980). In some species with both the SON and plexus nerve cut, ovarian noradrenaline could still be measured even though nerve fibres could not be demonstrated histologically, suggesting the ovary has intrinsic cells capable of noradrenaline synthesis (Lawrence and Burden, 1980). Alternatively, the noradrenaline measured could be contained in the blood in the vascular system.

The concentrations of ovarian noradrenaline during various reproductive states have been studied. Changes in the levels of ovarian steroids occurring during the estrous cycle or following hormone administration have no effect on ovarian noradrenaline concentration in the rabbit or guinea pig (Farrar et al., 1980). Others have shown an increase in ovarian noradrenaline concentration in the rat ovary on days 14 and 18 as compared with day 4 of pregnancy (Lawrence and Burden, 1976). The concentration of noradrenaline in porcine follicular fluid increases during the preovulatory period (Bahr and Ben-Jonathan, 1985). The functional significance of the increased ovarian noradrenaline is unknown. Recent studies by Bahr and Ben-Jonathan (1981) have shown that stimulation of the rat ovary with gonadotropins depletes the ovary of noradrenaline and dopamine. Pregnant mares' serum gonadotropin (PMSG)-treated prepubertal rats showed a 40% reduction in ovarian noradrenaline concentration 12 h

after PMSG, followed by a further 40% drop 4 h after the preovulatory LH surge. If the LH surge is blocked with barbituates there is no decrease in noradrenaline concentration (Ben-Jonathan et al., 1982). The noradrenaline depletion in response to endogenous and exogenous gonadotropin was also demonstrated in Graafian follicles, and was found to be due to FSH rather than LH or prolactin (Ben-Jonathan et al., 1982). The significance of these findings is not known.

2.6.4 Cholinergic Innervation of the Ovary

Little information is available concerning the presence and distribution of cholinergic fibres supplying the ovary. The presence of cholinergic nerves in the ovary has been suggested based on the demonstration of acetylcholinesterase in ovarian nerves. However, problems have arisen because of the presence of non-specific pseudocholinesterases giving false positive results, and because acetylcholinesterases are not specifically localized in cholinergic nerves. Acetylcholinesterase activity is also present in sensory neurons, noradrenergic nerves and in postganglionic sympathetic nerves (Koelle, 1955).

Acetylcholinesterase-positive nerves have been observed in the rat, rabbit and guinea pig ovary (Bulmer, 1965; Owman and Sjoberg, 1966; Chaffee, 1974). On the other hand, Jordan (1971) was unable to show acetylcholinesterase-positive nerves in the rabbit, rat or guinea pig ovary. The authors that have observed acetylcholinesterase-positive nerves in the ovary have found dense networks of these nerves associated with the ovarian blood vessels, the ovarian stroma and the follicles (Jacobowitz and Wallach, 1967; Chaffee, 1974; Walles et al., 1976; Burden and Lawrence, 1978). These nerves formed patterns that corresponded with the

perivascular pattern of noradrenerigc nerves although acetylcholinesterase-positive nerves were fewer (Burden and Lawrence, 1978). Based on evidence that most of the acetylcholinesterase-positive nerves were only visible if the noradrenergic nerve supply was intact suggested that the cholinesterase was located in the noradrenergic nerves (Burden and Lawrence, 1978).

2.6.5 Other Putative Neurotransmitters in the Ovary

In addition to noradrenaline and acetylcholine other putative neurotransmitters have been identified in the ovary, although few physiological studies on their actions have been reported. Dopamine has been measured in the rat and guinea pig ovary and in both species the ovarian dopamine concentration is about 10% that of noradrenaline (Farrar et al., 1980; Bahr and Ben-Jonathan, 1981). Adrenaline is present in porcine follicular fluid at levels 6- to 10-fold lower than noradrenaline (Bahr and Ben-Jonathan, 1985) but was not measurable in the rat ovary (Bahr and Ben-Jonathan, 1981). The ovaries of many species have been shown by immunofluorescnce to contain nerve fibers immunoreactive for VIP (Larsson et al., 1977; Papka et al., 1985), substance P (Alm et al., 1978; Dees et al., 1985; Papka et al., 1985), cholecystokinin-8 (CCK-8; McNeill and Burden, 1986), neuropeptide Y (NPY; McNeill and Burden, 1986; Owman et al., 1986; McDonald et al., 1987) and calcitonin gene-related peptide (Calka et al., 1988). Although 25% of the ovarian efferent perikarya are immunoreactive for somatostatin, nerve fibers containing this peptide have not been demonstrated in the ovary (McNeill and Burden, 1986). McNeill and Burden (1986) have shown VIP, substance P and CCK-8 to be present in ovarian primary afferent perikarya. VIP-encoding mRNA has been

demonstrated in the rat ovary which may suggest the presence of VIP synthesizing cells within the ovary (Gozes and Tsafriri, 1986).

Studies in which the SON, or plexus nerve, or both, are sectioned prior to immunohistochemistry, have shown VIP-immunoreactive nerve fibers reach the ovary via the SON while NPY-, CCK-8- and substance P-immunoreactive nerve fibers reach the ovary via the plexus nerve (Dees et al., 1985; McNeill and Burden, 1986; McDonald et al., 1987). The density of the peptidergic nerve fibers in the ovary is sparse but they are distributed in the same manner as the noradrenergic nerves.

The functions of these peptides in the ovary remains unknown. VIP stimulates progestin and estrogen production by rat granulosa cell cultures (Davoren and Hsueh, 1985); however, substance P and NPY could not be shown to have a direct role in modulating ovarian steroidogenesis in vitro (Ojeda et al., 1985; McDonald et al., 1987). These peptides may be involved in the regulation of ovarian blood flow or in the processing of sensory information from the ovary (Dees et al., 1985; Ojeda et al., 1985; Papka et al., 1985; McNeill and Burden, 1986; McDonald et al., 1987). The rat ovary also contains high concentrations of the inhibitory neurotransmitter GABA and glutamate decarboxylase, the synthetic enzyme for GABA production (Schaeffer and Hsueh, 1982). Isolated granulosa cells do not contain the synthetic enzyme but have large numbers of GABA binding sites as determined by the binding of the GABA agonist [^{3}H]muscimol (Schaeffer and Hsueh, 1982). Ovarian denervation decreases the levels of glutamate decarboxylase and GABA, and increases the number of GABA binding sites (Apud et al., 1983). There have been no reports demonstrating a physiological role for GABA in ovarian function.

2.6.6 Function of Ovarian Nerves

Studies on the physiology of ovarian nerves have made use of several techniques to denervate the ovary while leaving the vascular and hormonal conditions intact. Some of the denervation procedures used have been autotransplantation, abdominal vagotomy, mesovarium sectioning, spinal cord transection, vascular stripping and chemical denervation. There are potential problems with the interpretation of the denervation and autotransplantation studies. The autotransplantation studies, in which the ovary is removed and relocated to another part of the body have the inherent problem of the ovary undergoing total ischemia for a time and histological evidence of necrosis is always present. Reinnervation accompanies the revascularization of the transplant, usually within one week of transplantation (Jacobowitz and Laties, 1970).

In the mesovarium sectioning procedure the ovarian plexus is cut thereby severing the plexus nerve and the ovarian artery, and resulting in the complete loss of noradrenergic nerve fluorescence in the ovary (Lawrence and Burden, 1976). When the mesovarium is sectioned adequate vascular anastomoses exist to maintain the ovary despite the removal of the major arterial supply. A better procedure has been used: the nerves along the ovarian artery have been isolated and stripped off the artery or destroyed by painting with ethanol (LePere et al., 1966; Weiner et al., 1975). No histological evidence of damage to the ovary was detected and there was a complete absence of fluorescent noradrenrgic nerves and a significant reduction in ovarian noradrenaline concentration (LePere et al., 1966; Weiner et al., 1975).

Abdominal vagotomy has also been used as a means of ovarian denervation. This technique does not eliminate the fluorescent

noradrenergic nerves (Burden and Lawrence, 1978) and, in addition, vagotomy is known to have many other physiological consequences such as distension of the stomach, decreased gastric emptying (Mordes et al., 1977) and decreased food intake (Mordes et al., 1977), all of which may affect gonadotropin release (Howland, 1971) and ovarian cyclicity (Mulinos et al., 1939). Similar problems develop in studies in which the ovaries are denervated by spinal cord transection.

Chemical denervation has been achieved using 6-hydroxydopamine or reserpine which deplete noradrenergic nerve terminals (Tranzer and Thoenen, 1968). Both drugs have been shown to remove all fluorescent noradrenergic nerves from the ovary (Burden and Lawrence, 1978; Curry et al., 1984a).

Ovarian nerves have been implicated as having roles in the control of follicle growth and development, ovarian cyclicity, ovulation, ovarian blood flow, compensatory ovarian hypertrophy, puberty and steroidogenesis. These functions have been investigated using the denervation procedures described above. The experimental evidence suggesting a role for ovarian nerves in these physiological processes will be briefly reviewed.

A) <u>Follicle Growth and Development</u>

The studies of Grob (1972) in the immature mouse have shown that compared to intact contralateral ovaries or unoperated controls, denervation of the ovary by cutting the mesovarium decreased the number of maturing follicles. The author also noted that bilateral denervation in prepubertal mice resulted in delayed vaginal opening and the absence of normal estrous activity (Grob, 1972). Normal cyclic activity could be restored by administration of noradrenaline. These data suggested that

normal follicle maturation requires the local release of noradrenaline prior to gonadotropin stimulation. Further experiments from the same laboratory support this idea. Brink and Grob (1973) noted that denervated ovaries did not respond with increased follicle growth like control ovaries when stimulated with PMSG and hCG. A later study by the same authors found that hypophysectomized mice showed a decreased incorporation of [^{3}H]thymidine in denervated, compared to contralateral intact ovaries (Grob, 1974). Subcutaneous administration of noradrenaline 3 h prior to thymidine administration reversed the decreased uptake in the denervated ovaries (Grob, 1974). These findings were interpreted as indicating a direct effect of noradrenaline on the growth and maturation of ovarian follicles. Similarly, Curry et al., (1984a) have shown that chemical denervation of the guinea pig ovary results in a decrease in the number of healthy preovulatory follicles although they were unable to show differences in the total number of follicles per ovary or number of follicles in any size distribution. On the other hand, other authors were not able to show changes in the number or type of maturing follicles following ovarian denervation by vascular stripping (Weiner et al., 1975), spinal cord transection (Gelderd and Peppler, 1979) or autotransplantation (Chilal et al., 1976).

B) <u>Ovarian Cyclicity</u>

Several authors have shown that surgical or chemical interruption of the nerve supply to the ovary has little or no effect on the estrous cycle of the rat, cat, mouse or baboon (Sweet and Thorp, 1929; Bacq, 1932; Carlson and DeFeo, 1965; LePere et al., 1966; Castren et al., 1973, MacDonald and Airaksinen, 1973; Burden and Lawrence, 1977b). However, in

studies which have used abdominal vagotomy, bilateral cutting of the mesovarium or intrabursal injection of 6-hydroxydopamine to denervate the ovary, marked alterations were seen in the estrous cycle of the rat, hamster and mouse (Hill, 1962; Grob, 1972; Burden and Lawrence, 1977b; Gelderd and Peppler, 1979; Burden et al., 1981; Curry et al., 1984a). The mechanism by which vagotomy alters ovarian cyclicity is unknown; however, studies show gonadotropin and/or steroid levels may be altered following interuption of the vagus (Burden and Lawrence, 1977b).

The effect of denervation on other reproductive functions such as pregnancy have also been investigated. Weiner and coworkers (1975) were unable to show alterations in ovulation, oocyte pickup, tubal transport, or implantation, in rabbits denervated using the vascular stripping procedure. Similarly, Roche and coworkers (1985) have shown that freezing the nerve supply to the rat ovary did not affect the ovulation rate or the numbers or spacing of conceptuses. Conversely, others have reported that chemical denervation decreases litter size in mice (Castren et al., 1973) and increases the number of resorbed and stillborn fetuses in rats (MacDonald and Airaksinen, 1973).

Despite its many shortcomings, abdominal vagotomy has been extensively used in the study of ovarian nerve function. Various studies have shown that vagotomy alters gonadotropin (Lawrence et al., 1978) and prolactin secretion (Burden et al., 1981), compensatory ovarian hypertrophy (Burden and Lawrence, 1977b), steroid secretion (Lawrence et al., 1978), delays the onset of puberty (Ojeda et al., 1983), blocks the induction of pseudopregnancy in the rat (Burden et al., 1981) and decreases the number of live fetuses and increases fetal resorption during pregnancy in the rat (Lawrence et al., 1978). Vagotomy in rats during late pregnancy also

decreases 3β-HSD activity in the interstitial gland and corpora lutea and plasma LH and progesterone concentrations compared to sham-operated controls (Burden and Lawrence, 1977a; Lawrence et al., 1978). These authors suggested a direct inhibitory action of vagotomy on LH secretion, which in turn lowered 3β-HSD activity and progesterone levels in ovarian tissue and plasma, leading to fetal resorption. Therefore the vagus may participate in the control of LH secretion during pregnancy via a neural feedback to the hypothalamus. This hypothesis is supported by the observation that vagotomy inhibits the LH and FSH surge normally seen 5 hours after unilateral ovariectomy (Burden and Lawrence, 1977b). Alternatively, vagotomy may stimulate the production of ovarian steroids which may inhibit LH release.

C) Ovulation

Most of the research attempting to define a functional role for ovarian nerves has focused on the presence of smooth muscle in the theca externa and the possibility that its contraction is involved in ovulation. A review of the positive and negative evidence for a role of nerves in ovulation has been presented by Bahr et al. (1974). In support of this theory one can demonstrate the presence of α- and β-adrenergic receptors in the follicle wall (Walles et al., 1975) and the presence of spontaneous rhythmic contractions of the isolated ovaries of many species. Catecholamines can increase the frequency of these contractions (Rocerto et al., 1969). However, following denervation (LePere et al., 1966; Weiner et al., 1975), transplantation (Jacobowitz and Wallach, 1967) or the use of adrenergic drugs (Bahr et al., 1974), normal ovulation occurs. Transplanted ovaries may be reinnervated and the presence or absence of

nerves in transplanted ovaries which have ovulated has not been confirmed (Jacobowitz and Laties, 1970). The distended Graafian follicle may also stimulate sensory nerve endings in the theca which initiate the ovulatory process by causing the reflex release of pituitary gonadotropins (Barjaktarovitsch, 1959). Recently it has been shown that bilateral section of the vagus nerve in rats significantly increases ovulation rate (Chavez et al., 1987). When unilateral vagotomies were performed the effects observed depended upon which ovary was left in situ, suggesting the information carried by the left and right vagus is different. On the basis of what we know from all of these studies we cannot exclude the possibility that ovarian nerves play no role in ovulation.

D) Ovarian Blood Flow

The neural regulation of ovarian blood flow, while never precisely elucidated, remains unquestioned. When studying the role of ovarian nerves in the control of steroid secretion concomitant effects on ovarian blood flow are very important since changes in blood flow could lead to changes in the levels of steroids secreted or in the delivery of steroidogenic precursors to the cells. Studies by Varga and coworkers (1979) have shown in the perfused human ovary that α-adrenergic stimulation causes vasodilation and β-adrenergic stimulation elicits vasoconstriction of the ovarian blood vessels. On the other hand Gibson and Roche (1986) were unable to show changes in ovarian blood flow following denervation of the rat ovary, suggesting that sympathetic nerves do not exert a tonic vasoconstrictor control of the ovarian vasculature. Whether the ovarian nerves play no role in ovarian blood flow or play a role in the

redistribution of blood flow to structures such as developing follicles and corpora lutea remains to be determined.

E) Compensatory Ovarian Hypertrophy (COH)

Removal of one ovary causes the remaining ovary to increase in size and secretory activity. The remaining ovary also shows an increase in follicular development and number of ovulations. Recent studies suggest a neural pathway between the ovaries and CNS may be involved in the mechanism of COH. Gerendai et al., (1978) have shown that chemical denervation of the ovarian noradrenergic nerves in one ovary results in hypertrophy of the contralateral ovary. Also, in rats with one ovary removed, local application of 6-hydroxydopamine to the ovary or abdominal vagotomy blocked the COH response (Burden and Lawrence, 1977b; Gerendai et al., 1978). These data suggest the participation of a neural noradrenergic mechanism in both the afferent signal for and in the efferent expression of COH. Nevertheless, in guinea pigs, a species with dense ovarian noradrenergic innervation, chemical sympathectomy did not cause hypertrophy in the contralateral ovary, nor did 6-hydroxydopamine prevent hypertrophy of the remaining ovary (Curry et al., 1984b).

F) Puberty

In the immature rat, there is a significant increase in ovarian noradrenaline concentration between days 28 and 31, more than one week before the onset of puberty (Ben-Jonathan et al., 1984). The increase in ovarian noradrenaline concentration preceded the elevation in circulating gonadotropin levels and the onset of puberty by 5-10 days, suggesting the increased noradrenaline in the ovary plays a role in ovarian

responsiveness to gonadotropins and the onset of puberty. Aguado et al. (1982) found that a steroidogenic response to β_2-adrenergic agonists developed during the first proestrus of puberty and that both the β-receptor content and the progesterone response increase after the first ovulation, suggesting the involvement of an adrenergic mechanism in the initiation of adult ovarian function. Abdominal vagotomy in immature rats delayed the onset of puberty and suppressed the responsiveness of the juvenile ovary to gonadotropins by mechanisms which do not involve alterations in ovarian gonadotropin or β-adrenergic receptors, changes in serum levels of pituitary hormones or availability of precursors for steroid synthesis (Ojeda et al., 1983). Aguado and Ojeda (1984a) however, found that sectioning of the SON in juvenile rats did not alter puberty onset even though ovarian noradrenaline was decreased by greater than 60%, 7 days after SON section. An increase in β-adrenergic receptor number also occurred, suggesting that hypersensitivity developed as a result of the denervation. This hypersensitivity could maintain normal ovarian function by increasing ovarian responsiveness to noradrenaline.

G) Ovarian Steroidogenesis

A direct neural control of hormone secretion has been described for several endocrine cell systems (Wurtman, 1973). Studies on the role of ovarian nerves in the control of ovarian steroidogenesis have looked at catecholamine-stimulated progesterone production by ovarian cells and more recently at interstitial androgen production.

In Vivo Studies

Kawakami and coworkers (1981) have done a series of experiments in rats

which suggest an efferent neural system from the brain to the ovaries supplements the brain-pituitary-ovarian hormonal mechanisms in the regulation of ovarian steroid secretion. These authors found that stimulation of selective hypothalamic nuclei in hypophysectomized, adrenalectomized rats led to changes in ovarian venous estrogen and progesterone concentrations (Kawakami et al., 1981). Weiss et al. (1982) provided additional evidence for a direct neural control of ovarian steroidogenesis in rats. Stimulation of the SON in ovariectomized diestrous animals reduced ovarian progesterone levels even when the β receptor antagonist propranolol was administered. Administration of the α receptor blocker phentolamine reversed this effect. The authors concluded that neural control of ovarian steroidogenesis may be either excitatory through the stimulation of β receptors or inhibitory through the stimulation of α receptors. Other studies showed that cutting the SON during estrus had no effect on progesterone or estrogen secretion (Aguado and Ojeda, 1984b). However, if the nerve was cut during proestrus a significant decline in ovarian venous progesterone and estrogen concentration was measured. The authors report that cutting the nerve had no effect on blood flow. These observations support the idea that the ovary is under direct neural control which is complimentary to its hormonal regulation.

Adrenergic Receptors in the Ovary

Many studies have shown both the immature and adult ovary to contain a single class of specific, high-affinity β-adrenergic receptors (Coleman et al., 1979; Harwood et al., 1980; Ratner et al., 1980; Jordan, 1981; Aguado et al., 1982) which have been determined by both biochemical and

functional criteria to be of the β_2 subtype (Ratner et al., 1980; Veldhuis et al., 1980; Adashi and Hsueh, 1981; Aguado et al., 1982). Receptor concentrations vary during the estrous cycle, with the highest levels during proestrus, falling to 50% of proestrus levels in estrus, then increasing to near proestrus levels during metestrus and diestrus (Jordan, 1981). The majority of the β-adrenergic binding sites are found in the granulosa compartment; however the presence of β-binding sites in the residual ovary (tissue remaining after removal of most granulosa cells), and the capacity of the β-agonist zinterol to induce androstenedione release, suggest the thecal cells are also under noradrenergic influence (Aguado et al., 1982).

When granulosa cells are isolated and put into culture, a marked increase in β-receptor density occurs (Kliachko and Zor, 1981; Aguado and Ojeda, 1984a). This increase is due at least in part to the loss of noradrenergic inputs since noradrenaline down-regulated the receptors and desensitized the adenylate cyclase system coupled to the receptors (Aguado and Ojeda, 1984a). The next sections will discuss the coupling of β-adrenergic receptors to ovarian steroidogenesis.

Catecholamine-Stimulated Progesterone Production

Many studies have shown β-receptor mediated effects of catecholamines on progesterone production. Intrafollicular injections of adrenergic agonists to the rabbit ovary have shown that β_2-adrenergic stimulation, but not β_1- or α-adrenergic stimulation, result in increased progesterone but not estrogen production (Bahr et al., 1974). In vitro studies have shown catecholamine-stimulated progesterone production by corpus luteum slices and by dispersed luteal cells (Bahr et al., 1974; Condon and Black, 1976;

Godkin et al., 1977; Jordan et al., 1978; Harwood et al., 1980; Veldhuis et al., 1980; Norjavaara et al., 1982; Sheela Rani et al., 1983), granulosa cells (Adashi and Hsueh, 1981; Kliachko and Zor, 1981), whole preovulatory ovaries in vitro (Aguado et al., 1982; Ratner et al., 1980) or ovaries in situ (Zsolnai et al., 1982). These effects are readily blocked by the β-adrenergic antagonist propranolol, but not by α-antagonists (Condon and Black, 1976; Godkin et al., 1977; Jordan et al., 1978; Adashi and Hsueh, 1981). If granulosa cells are treated with FSH for 48 h, in vivo or in vitro, they aquire noradrenergic responsiveness and show a dose-dependent stimulation of progesterone production when stimulated with β-adrenergic agonists, indicating that FSH plays a role in the aquisition of noradrenergic responsiveness (Adashi and Hsueh, 1981). The catecholamine-stimulated increase in progesterone synthesis is due to the inhibition of progesterone metabolism (Jones and Hsueh, 1981) and to increased synthesis as a result of the stimulation of pregnenolone production and 3β-HSD activity (Jones et al., 1983). The interpretation of these studies is difficult due to the fact that neither the granulosa nor luteal cells are directly innervated in vivo (Unsicker, 1970; Burden, 1972). Physiologically, catecholamines could reach the luteal cells via the bloodstream.

Theca-Interstitial Androgen Production

Functional relationships between the ovarian nerves and the secondary interstitial cells have been reported in in vivo studies. Capps et al., (1978) found that following mesovarium sectioning in rats there was an increase in interstitial cell regression. Electrical stimulation of the

ovarian plexus caused the regressed cells to assume the structural features of active steroidogenic cells.

Catecholamines have also been shown to act on cultured rat theca-interstitial cells to amplify gonadotropin-stimulated androgen production (Dyer and Erickson, 1985). The addition of catecholamines with LH or hCG to theca-interstitial cell cultures stimulated the secretion of 3-4 times more androgen than occurs in the presence of LH alone. The catecholamine effect was mediated by β_2 and possibly β_1 receptors with no involvement of the α receptors. Steroid hormone metabolic studies showed that the catecholamines stimulated the production of androstenedione as opposed to the 5α-reduced androgens, suggesting that catecholamines inhibit the activity of the 5α-reductase enzyme. Although catecholamines alone stimulate cyclic AMP secretion, catecholamines alone cannot induce the theca-interstitial cells to secrete steroids (Erickson et al., 1985). LH may be necessary to induce and/or maintain the functional capacity of the β-adrenergic receptors on the cells. It has been shown that LH can induce catecholamine receptors in rat granulosa cells (Kliachko and Zor, 1981; Sheela Rani et al., 1983).

Therefore, the ovarian androgen producing cells are innervated and are targets of catecholamines. Since interstitial androgens are the precursors for follicular estrogen synthesis and are involved in atresia, neural transmission in the control of ovarian follicular development may be important.

From the data available we can conclude that neural pathways exist between the brain and the ovary. This may include both an afferent pathway from the ovary to the hypothalamus or to other brain areas, and also an efferent pathway originating in the central nervous system and terminating

in the ovary. Nerves may alter ovarian function, however it is doubtful that nerves exclusively control ovarian function. Rather, nerves probably act directly or indirectly by modulating the sensitivity of ovarian components to hormonal factors. Neilson and colleagues have postulated the existence of a dual control system in the ovary, and proposed that the final secretory state of a given cell depends on the balance of neural and gonadotropin stimuli (Neilson et al., 1970).

Although it is clear that the ovary can respond to noradrenergic stimulation, whether the ovarian nerves are a functionally important source of these inputs remains controversial. Studies to date have failed to demonstrate that neural mechanisms are essential for the control of ovarian reproductive functions. Conflicting data and paradoxical observations make it very difficult at this time to draw conclusions on the physiological role of the ovarian innervation in ovarian function.

2.7 LITERATURE REVIEW-CONCLUSION

The ovarian androgen producing cells development and function depends on complex and interrelated events under the control of many physiological hormones and the nervous system. Under normal conditions androgen production is controlled by a balance between inhibitory and stimulatory influences.

CHAPTER 3-RATIONALE AND OBJECTIVES

Androgens are important intraovarian regulators. They play important roles in controlling ovarian progesterone biosynthesis, follicle growth, atresia and oocyte maturation. The influence of androgens is shown by the severe reproductive abnormalities which occur when ovarian androgen production is abnormal. Therefore it is important to understand the normal physiological control of theca cells in order to understand the disturbances that occur under pathological conditions. Compared to the ovarian granulosa cells, little is known about the control of androgen biosynthesis by the theca and interstitial cells of the ovary. Studies to date have focused on the role of the pituitary gonadotropins in controlling androgen biosynthesis and on the ability of a variety of follicular fluid and circulatory factors to modulate the responsiveness of these cells to gonadotropins.

Anatomists have shown that the ovarian androgen producing cells are directly innervated by noradrenergic, cholinergic and a variety of peptidergic nerve fibers. The androgen producing theca and interstitial cells are the only ovarian endocrine cells which are innervated. The long range goal of this research project was to elucidate the possible regulatory role of ovarian nerves in controlling ovarian androgen production and to determine the extent and mechanisms by which nerves from the central nervous system to the ovary interact with the hypothalamic-pituitary-ovarian hormonal system in regulating ovarian androgen biosynthesis.

To achieve this objective, the following specific objectives were proposed:

(1) The development of an *in vitro* culture system for porcine theca cells in which the direct effects of various neural agents can be tested for effects on ovarian androgen production.

(2) The study of the direct effects of catecholamines and a variety of putative ovarian neurotransmitters, that have been reported to be present in the ovary, on theca cell androgen production.

(3) The study of the direct effects of factors present in ovarian nerves on porcine theca cell androgen production by adding an extract of rat superior ovarian nerves to cultured porcine theca cells.

(4) When it was observed that the rat superior ovarian nerve extracts had significant inhibitory effects on porcine theca cell androgen production, studies were undertaken to determine the mechanism of action and the biochemical properties of the inhibitory factor(s).

(5) The inhibitory effects, biochemical properties and site of action of the rat superior ovarian nerve inhibitory factor(s) were compared to other compounds known to inhibit theca cell androgen production.

CHAPTER 4-GENERAL MATERIALS AND METHODS

The methods used in these studies vary considerably among experiments; therefore details of each are given in subsequent sections. There are, however, some general procedures that were used in many of these experiments which are described here.

4.1 Porcine Theca Cell Preparations

Theca cells were obtained from the ovaries of prepubertal gilts as described by Tsang et al. (1979a) and modified by Hunter and Armstrong (1987). Ovaries were obtained from local abattoirs (Thorndale Abattoir, Thorndale, Ontario; Lobo Abattoir, Lobo, Ontario; Schneider's, Kitchener, Ontario) and transported to the laboratory on ice. Ovaries were thoroughly washed in sterile 0.9% (w/v) saline containing 75 U penicillin/ml, 75 μg streptomycin/ml and 0.94 μg Fungizone/ml. The macroscopic criteria of Moor et al. (1978) described for sheep ovaries, were used to distinguish between healthy and atretic follicles and only apparently healthy porcine follicles were used. Medium-sized (3-6 mm) antral follicles were dissected and pooled in a Petri dish containing Hanks' Balanced Salt Solution (HBSS; Gibco Laboratories, Grand Island, NY, USA) without magnesium and calcium and containing 50 U penicillin/ml, 50 μg streptomycin/ml and 0.625 μg Fungizone/ml. Follicles were bisected and the granulosa cells were removed by gentle scraping with a heat-bent Pasteur pipette. Theca interna cells were prepared by mechanically peeling the theca interna from the theca externa and follicle walls devoid of granulosa cells. The theca interna were then dispersed enzymatically in HBSS containing 0.25% collagenase (Type II Sigma Chemical Co., St. Louis, MO, USA), 0.05% hyaluronidase (Type I, Sigma) and 0.05% protease (Type XIV, Sigma) at 37°C in a shaking

water bath. The dispersion of the cells was aided by gentle pipetting of the suspension with a Pasteur pipette. Following dispersion, the cells were washed in HBSS and recovered by centrifugation at 270g for 5 min. The cell pellet was resuspended in Dulbecco's Modified Eagle's medium (DMEM; Gibco) containing 1.2 g/l $NaHCO_3$ and the same antibiotic supplements as HBSS. Cultures of theca cells consisted of 500,000 cells in 1 ml of DMEM plated into each well in 24-well tissue culture plates (Falcon Plastics, Los Angeles, CA). Cultures were incubated at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. Treatments were normally added to the cultures at the time of plating and cells were cultured for 48 h, the medium was removed and stored at -20°C until extraction. Cell viability was assessed by incubating the cells with trypan blue (0.05%). Viability was always > 85%.

4.2 Hormones

Ovine FSH (NIAMDD-oFSH-14: 9X NIH-FSH-S1 by the hCG augmentation bioassay of Steelman-Pohley; 0.02X NIH-LH-S1 by the ovarian ascorbic acid depletion assay) was provided by the Pituitary Hormone Distribution Program, NIAMDD. LH used in these studies was USDA-bovine LH B5 (2.1X NIH-LH-S1 by the ovarian ascorbic acid depletion assay).

4.3 Collection of Nerves

All nerves used in the preparation of nerve extracts were obtained from adult female Sprague-Dawley rats (Charles River Canada Inc., St. Constant, Quebec, Canada). Superior ovarian nerve extracts were prepared from the portion of the suspensory ligament between the kidney and ovary and sciatic nerves (SCN) were removed from the upper thigh region. Segments

of suspensory ligaments weighed approximately 2 mg and segments of sciatic nerves weighed approximately 6 mg. Nerves were stored at -20°C until processed.

4.4 Preparation of Nerve Extracts

Suspensory ligaments and sciatic nerves were weighed and homogenized for 4 min in ice-cold Dulbecco's phosphate-buffered saline (DPBS; Gibco; 0.2 mg tissue/μl) using a hand-held ground glass Ten Broeck tissue grinder. The homogenate was centrifuged at 1,600g for 15 min at °C and the supernatant was collected and used as the nerve extract.

4.5 Radioimmunoassays

Radioimmunoassays were used to determine steroid concentrations in the nerve extracts and in culture media. In all studies except the time course studies, culture media were removed after 48 h of incubation and stored at -20°C until assayed, without extraction, for secreted pregnenolone (Morley et al., 1989), progesterone (Leung and Armstrong, 1979a), 17α-hydroxyprogesterone (Engelhardt et al., 1989), androstenedione (Leung and Armstrong, 1979a), testosterone (Leung and Armstrong, 1979a), estradiol-17β (Daniel and Armstrong, 1984) and estrone (Daniel and Armstrong, 1984) using radioimmunoassays which have been previously described and validated for direct measurements in culture media.

For the determination of cyclic 3',5'-adenosine monophosphate (cyclic AMP), 0.2 ml of medium was withdrawn from cultures, heated in a boiling water bath for 20 min, and stored at -20°C until assayed by a previously validated radioimmunoassay (Reddoch and Armstrong, 1984).

4.6 Protein and DNA Assays

Protein contents were determined in nerve extracts and in some cell cultures at the end of the incubation periods. Attached cells were solubilized by sonication in 1.0 ml of 1% sodium dodecyl sulfate and 1 mM ethylenediamine tetraacetic acid (EDTA) and measured by the method of Lowry et al. (1951), using bovine serum albumin (BSA) as a standard.

DNA was assayed by the fluorometric technique of Karsten and Wallenberger (1977) with the modifications of Louis and Fritz (1979).

4.7 Alkaline Phosphatase Assay

Alkaline phosphatase activity was measured according to the method described by Lowry (1957). The alkaline phosphatase activity is expressed as unit activity (nmol substrate hydrolyzed/500,000 cells plated/30 min).

4.8 Measurement of Noradrenaline in Ovaries

Ovarian noradrenaline concentrations were measured by a previously described procedure for measuring noradrenaline levels in kidneys (Kline, 1983). Briefly, ovaries were removed, weighed and homogenized in cold perchloric acid. Homogenates were centrifuged at 27,000 *g* and the supernatants frozen at -75°C. Noradrenaline was extracted on alumina and measured using high-pressure liquid chromatography (HPLC) and electrochemical detection. External standards of noradrenaline and 3,4-dihydroxybenzylamine (DHBA; Sigma) were carried through the extraction procedure to enable calibration of the system. DHBA was also added to each sample to serve as an internal standard; thus by using the ratio of the responses obtained for the external standards, the internal standard allowed quantitation of noradrenaline and correction for recovery in each

individual sample. Preliminary experiments showed the noradrenaline and DHBA were extracted and detected in the same ratio when added to homogenizing solution or tissue homogenates. The samples were injected onto an HPLC column (Waters Scientific, Corasil 18); the mobile phase was 0.055 M phosphate buffer with 15% methanol, pH 4.0, and containing 0.0016 M octyl sodium sulfate (Eastman Kodak Co.). Noradrenaline was detected with glassy carbon electrodes (Bioanalytical Systems) at a potential of 0.8 V and quantitation of chromatograms was done with an integrator (Hewlett Packard 3390).

4.9 Statistical Analyses

Statistical analyses were made by analysis of variance. Where required, data were transformed logarithmically prior to statistical analyses to remove heterogeneity of variance as determined by Bartlett's test. When significant effects were observed, Duncan's new multiple range test was used for multiple comparisons. Treatments were replicated in quadruplicate within an experiment and each experiment was performed 2-3 times. All tests were performed as described by Steel and Torrie (1980) and statistical significance was inferred at $p < 0.05$.

CHAPTER 5- THE PORCINE THECA CELL CULTURE SYSTEM

5.1 Introduction

Despite the importance of ovarian androgens, very little is known about the regulation of androgen synthesis by the ovarian androgen producing cells. In order to study the mechanisms and regulation of androgen production it is necessary to have a suitable tissue culture system in which androgen production can be investigated under defined conditions. This is particularly important when studying the role of ovarian nerves in the control of androgen production. The tissue culture system allows the study of the direct effects of ovarian nerve factors on the ovarian androgen producing cells. This eliminates effects the nerves may have on ovarian blood flow which could directly affect androgen secretion or indirectly affect it through changes in the delivery of steroidogenic precursors to the cells.

Methods for the culture of theca interna cells from antral follicles were first described in the 1960's (reviewed in Literature Review-Section 2.5.2). These initial studies used pieces of theca interna. Some difficulty was encountered maintaining the viability of the cells which were not in contact with the medium, and in the diffusion of exogenous hormones through the tissue. To eliminate these problems, enzymes were used to disperse porcine theca interna cells to provide cell suspensions (Stoklosowa et al., 1982; Tsang et al., 1985).

In this chapter, a primary serum-free cell culture system for porcine theca cells in which theca cell differentiation is specifically stimulated by luteinizing hormone, is described. This is the culture system that was

used to investigate the direct effect of ovarian nerve factors on androgen production, to be described in subsequent sections.

5.2 Methods and Results

5.2.1 Gonadotropin-Stimulation of Thecal Androgen and Cyclic AMP Accumulation

To investigate the dose-dependence of gonadotropin effects on theca cell extracellular cyclic AMP, androstenedione and testosterone accumulation, theca cells were cultured in DMEM, without serum or other source of protein, in the absence or presence of LH (0.1-1000 ng/ml) or FSH (0.1-1000 ng/ml) for 48 h. Significant amounts of cyclic AMP, androstenedione and testosterone accumulated in unstimulated cultures (Table 1). Treatment with increasing concentrations of LH resulted in significant ($p < 0.05$) dose-dependent increments in cyclic AMP, androstenedione and testosterone accumulation above control values at all concentrations above and including 100 ng/ml. A maximally stimulating dose of LH (250 ng/ml) consistently increased androgen accumulation to 2-4 times basal levels. In contrast, theca cells exposed to FSH showed no significant changes in testosterone accumulation compared to basal levels. Extracellular cyclic AMP and androstenedione accumulation in FSH-stimulated cultures were significantly ($p < 0.05$) greater than controls only at the highest dose of FSH (1000 ng/ml) employed.

Quantitatively, androstenedione is the major androgen produced by the porcine theca cell culture system. Testosterone levels were typically 10-20% of the levels of androstenedione. In initial experiments it was determined that the 5α-reduced androgen 3α-hydroxy-5α-androstan-17-

Table 1. Gonadotropin-stimulation of porcine theca cell androgen and cyclic AMP accumulation.

Treatment* (ng/ml)	Androstenedione (ng/500,000 cells)	Testosterone (ng/500,000 cells)	Cyclic AMP (pmol/500,000 cells)
LH 0	1.44 ± 0.03^{a}	0.22 ± 0.04^{a}	0.69 ± 0.23^{abc}
0.1	1.61 ± 0.07^{a}	0.20 ± 0.02^{a}	0.32 ± 0.01^{ab}
1.0	1.50 ± 0.03^{a}	0.27 ± 0.04^{a}	0.53 ± 0.05^{abc}
10	1.78 ± 0.07^{a}	0.42 ± 0.04^{b}	0.79 ± 0.18^{bc}
100	3.29 ± 0.17^{c}	0.51 ± 0.05^{bc}	3.64 ± 0.23^{d}
500	3.34 ± 0.26^{cd}	0.62 ± 0.11^{c}	5.78 ± 0.[illegible]^{e}
1000	3.88 ± 0.31^{d}	0.61 ± 0.07^{c}	6.01 ± 0.30^{e}
FSH 0	1.55 ± 0.03^{a}	0.28 ± 0.03^{a}	0.40 ± 0.06^{abc}
0.1	1.64 ± 0.06^{a}	0.25 ± 0.02^{a}	0.17 ± 0.17^{a}
1.0	1.53 ± 0.04^{a}	0.25 ± 0.02^{a}	0.27 ± 0.14^{a}
10	1.54 ± 0.05^{a}	0.25 ± 0.02^{a}	0.37 ± 0.04^{ab}
100	1.50 ± 0.04^{a}	0.26 ± 0.01^{a}	0.51 ± 0.14^{abc}
500	1.70 ± 0.07^{a}	0.25 ± 0.02^{a}	0.67 ± 0.07^{abc}
1000	2.20 ± 0.04^{b}	0.26 ± 0.02^{a}	0.92 ± 0.12^{c}

* Porcine theca cells were incubated in the absence or presence of various concentrations of LH and FSH for 48 h. Data are the mean ± S.E.M. of quadruplicate cultures in a single experiment which was replicated two further times. Values with different superscripts are significantly different ($p < 0.05$) within each column. LH caused dose-dependent increases in theca cell androgen and cyclic AMP accumulation.

one (androsterone) was not detectable by radioimmunoassay in the culture medium and 5α-androstane-3β,17β-diol levels were typically 30-40% the levels of androstenedione (data not shown). Since the levels of androstenedione, testosterone and 5α-androstane-3β,17β-diol changed in a similar manner in all experimental manipulations, the data shown throughout this thesis are for the quantitatively more important androgen, androstenedione.

To study the accumulation of extracellular cyclic AMP in basal and LH-stimulated cultures, theca cells were cultured in the absence or presence of a maximally stimulating dose of LH (250 ng/ml) and the accumulation of extracellular cyclic AMP and androstenedione were measured after 48 h of culture. Significant amounts of cyclic AMP were produced in unstimulated cultures and the addition of LH caused a 17-fold increase in extracellular cyclic AMP accumulation over control levels (Table 2). Addition of the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (IBMX; 400 μM; Sigma) to the culture medium enhanced the accumulation of cyclic AMP in both basal and LH-stimulated cultures. Androstenedione levels measured in the same cultures showed LH caused a significant ($p < 0.05$) enhancement of androstenedione production over control levels, which was further increased in both treatment groups in the presence of IBMX.

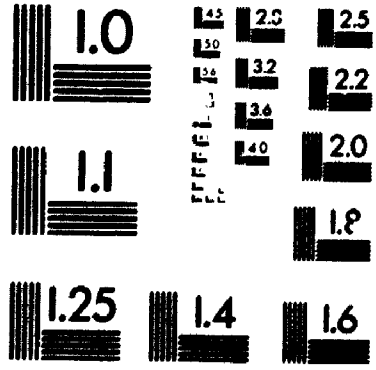
To study the responsiveness of theca cells to other physiological and pharmacological agonists, for which cyclic AMP is a second messenger, theca cells were cultured in the absence or prescence of LH (250 ng/ml), PGE_2 (10 μg/ml; The Upjohn Co., Kalamazoo, MI), cholera toxin (1 μg/ml; Sigma), forskolin (10^{-4} M; Calbiochem-Behring, LaJolla, CA) or N^6-2'-o-dibutyryl adenosine-3',5'-cyclic monophosphate (dibutyryl cyclic AMP; 0.5 μg/ml; 1mM; Sigma). Dibutyryl cyclic AMP-stimulated cultures

Table 2. Porcine theca cell cyclic AMP and androstenedione accumulation in the absence and presence of a phosphodiesterase inhibitor (IBMX).

Treatments*	Extracellular cyclic AMP** (pmol/500,000 cells)	Androstenedione** (ng/500,000 cells)
Control	1.35 ± 0.11	6.23 ± 0.62
IBMX	5.09 ± 0.37	13.80 ± 0.40
LH	23.51 ± 1.35	14.16 ± 0.51
LH + IBMX	107.02 ± 5.17	24.47 ± 2.03

* Theca cells were cultured in the absence (Control) or presence (LH) of LH (250 ng/ml), with or without IBMX (400 μM) for 48 h.

** Values represent the mean ± S.E.M. of quadruplicate cultures from a single experiment which was replicated two further times. Analysis of variance revealed a significant ($p < 0.05$) interaction between the effects of LH and MIX. LH increased both extracellular cyclic AMP and androstenedione accumulation. IBMX enhanced the accumulation of cyclic AMP and androstenedione in both treatment groups, but to a greater extent in the presence of LH.


1.0
1.1
1.25
1.4
1.6
1.8
2.0
2.2
2.5
2.8
3.2
3.6
4.0
4.5
5.0
5.6

contained IBMX (400 μM). Each of these agents significantly ($p < 0.05$) increased androstenedione accumulation over control levels, in a similar manner as LH (Figure 5). LH, PGE_2, cholera toxin, forskolin and dibutyryl cyclic AMP stimulated androgen production by 2.0-, 2.3-, 2.2-, 1.7- and 1.8-fold, respectively, over control levels.

To investigate the time course of theca cell androstenedione accumulation, theca cells were cultured in the absence or presence of LH (250 ng/ml) for varying incubation times (6-48 h). Time-dependent increments in androstenedione accumulation were observed in both basal and LH-stimulated cultures (Figure 6). The accumulation of androstenedione was found to increase significantly ($p < 0.05$) up to 24 h of culture. Only small further increases in androstenedione accumulation were observed during the 24-48 h time interval. The addition of LH (250 ng/ml) caused a significant ($p < 0.05$) stimulation of androstenedione accumulation at all time points.

5.2.2 Steroid Accumulation Profile

The steroidogenic profile of porcine theca cells in the absence and presence of LH (250 ng/ml) was investigated by measuring steroid levels in culture medium at the end of 48 h of incubation. As shown in Table 3 cultured porcine theca cells secreted significant amounts of progesterone and 17α-hydroxyprogesterone in addition to androstenedione and testosterone. Porcine theca cells also possess the aromatase enzymes that convert androgens to estrogens, and therefore small amounts of estradiol-17β and estrone could also be measured in theca cell cultures. After 48 h of treatment with LH, the levels of each of these steroids, except pregnenolone, was significantly ($p < 0.05$) increased over the

Figure 5. Stimulation of porcine theca cell androgen accumulation by LH, PGE_2, cholera toxin, forskolin and dibutyryl cyclic AMP. Theca cells were cultured in the absence (CON) or presence of LH (250 ng/ml), PGE_2 (10 μg/ml), dibutyryl cyclic AMP (1 mM) and IBMX (400 μM), cholera toxin (1 μg/ml) or forskolin (10^{-4} M) for 48 h. Values represent the mean $\pm$ S.E.M. of quadruplicate cultures from a single experiment which was replicated one further time. Values with different superscripts are significantly different ($p < 0.05$). Each of these agents stimulated androstenedione accumulation in a similar manner as LH.

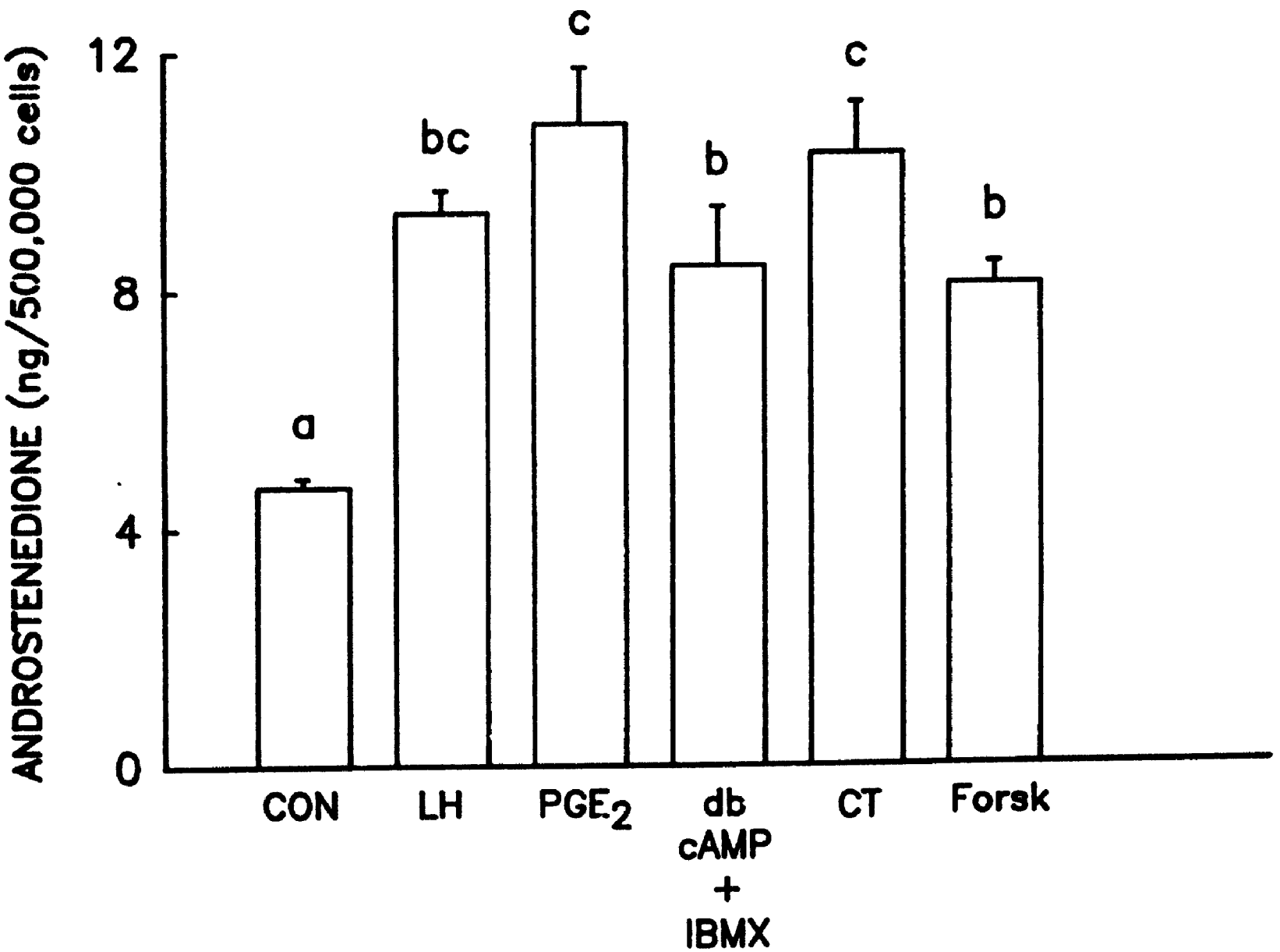

ANDROSTENEDIONE (ng/500,000 cells)
12
8
4
0
a
bc
c
b
c
b
CON
LH
PGE2
db cAMP + IBMX
CT
Forsk

Figure 6. Time course of porcine theca cell androstenedione accumulation. Theca cells were cultured in the absence (CONTROL) or presence of LH (LH; 250 ng/ml) and the medium was collected at various times (6-48 h) after plating. Values represent the mean ± S.E.M. of quadruplicate cultures from a single experiment which was replicated one further time. Where no error bar is shown, the S.E.M. is within the limit of the symbol. Analysis of variance revealed a significant ($p < 0.05$) interaction between the effects of LH and time. Control and LH-stimulated cultures showed time-dependent increments in androstenedione accumulation.

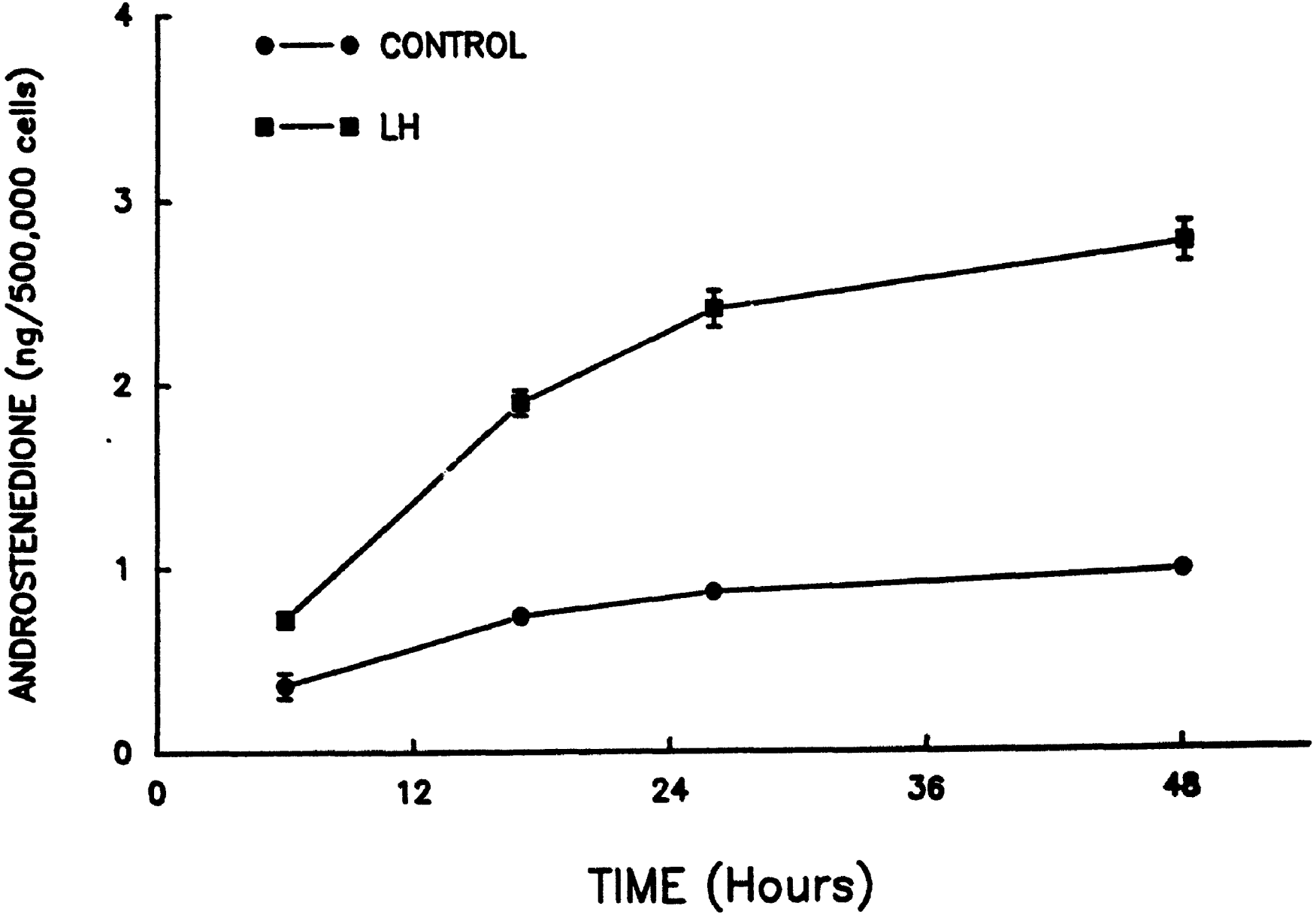

CONTROL
LH
ANDROSTENEDIONE (ng/500,000 cells)
4
3
2
1
0
0
12
24
36
48
TIME (Hours)

Table 3. Steroidogenic profile of control and LH-stimulated porcine theca cells.

Steroid (ng/500,000 cells)	Treatment*	
	Control	LH
Pregnenolone	0 ± 0[a]	0 ± 0[a]
Progesterone	2.90 ± 1.41[a]	5.88 ± 1.02[b]
17α-Hydroxyprogesterone	5.03 ± 0.72[a]	8.73 ± 0.88[b]
Androstenedione	5.75 ± 0.47[a]	13.38 ± 1.16[b]
Testosterone	0.17 ± 0.03[a]	0.96 ± 0.05[b]
Estradiol-17β	0.56 ± 0.01[a]	0.78 ± 0.03[b]
Estrone	0.12 ± 0.01[a]	0.23 ± 0.01[b]

* Theca cells were cultured with (LH; 250 ng/ml) or without (Control) LH. After 48 h of culture the medium was removed for radioimmunoassay of secreted steroids. Values represent the mean ± S.E.M. of quadruplicate cultures from a single experiment which was replicated two further times. Values with different superscripts are significantly different ($p < 0.05$) for each steroid.

levels in control cultures. The level of pregnenolone in both control and unstimulated cultures was very low, usually at or below the sensitivity of the assay.

5.3 Discussion

These initial studies have established that freshly isolated porcine theca cells secrete large amounts of androgens in the absence or presence of gonadotropins in vitro and this secretion was stimulated by LH. The system described differs from the previously described system of Tsang et al. (1985) and Hunter and Armstrong (1987) in that the cells were cultured in plates rather than in suspensions. These findings are consistent with observations that thecal cells from many species are the major source of follicular androgens and that their secretion is regulated by LH (Makris and Ryan, 1975; Erickson and Ryan, 1976; Fortune and Armstrong, 1977; Tsang et al., 1979b). The effect of LH on thecal androgen secretion was dose-dependent and the majority of the androgens were secreted in the first 24 h of culture. It is not known why androgen secretion ceases by 24 h of culture. These observations are also consistent with findings in other species that theca cells secrete more androstenedione than testosterone (Evans et al., 1981). This is, however, inconsistent with reports that the levels of androstenedione and testosterone in porcine follicular fluid are virtually the same irrespective of the stage of the estrous cycle (Ainsworth et al., 1980). Follicular fluid androstenedione and testosterone levels probably reflect the activity of granulosa cell 17β-hydroxysteroid dehydrogenase and therefore need not be consistent with the relative production rates by these cells.

These results are consistent with the well accepted hypothesis that androgens are produced by a cyclic AMP-mediated stimulation of theca cells by LH. LH-stimulation of porcine theca cells causes increases in both extracellular cyclic AMP and androgen production. This finding along with the observation that the LH effect on androgen production was mimicked by exogenously added cyclic AMP, inhibitors of the phosphodiesterase enzyme, or a variety of physiological and pharmacological activators of adenylate cyclase, supports the idea that LH increases androgen production by activating the adenylate cyclase system (Erickson and Ryan, 1976; Tsang et al., 1979b). This finding is consistent with many previous studies showing LH action in ovarian (Sala et al., 1979) and testicular (Dufau et al., 1977) steroid synthesis is mediated by cyclic AMP. The responsiveness of theca cells to PGE_2 suggests theca cells are physiological targets for prostaglandins as well as gonadotropins.

The validity of measuring cyclic AMP secreted into the incubation medium as a reflection of intracellular cyclic AMP levels was not tested in this system. Knecht et al. (1981) have shown that in both basal and FSH-stimulated rat granulosa cell cultures, medium or extracellular cyclic AMP levels reflected those of the intracellular levels. When rat granulosa cell progesterone accumulation was inhibited by GnRH, a similar decline in cyclic AMP in both compartments was observed. Schindler et al. (1982) found similar intracellular and extracellular levels of cyclic AMP in gonadotropin stimulated rat ovarian cells. Addition of the xanthine phophodiesterase inhibitor theophylline increased overall cyclic AMP accumulation identically in both compartments. Extracellular cyclic AMP determinations appear to be useful indicies of intracellular levels, however it is difficult to assess the biological importance of

extracellular cyclic AMP, since it is not known if cyclic AMP secretion occurs in vivo. It is possible that various agents could act by altering the intracellular and extracellular cyclic AMP distribution to alter hormone secretion.

Typically, pregnenolone levels in control and LH-stimulated cultures were very low, indicating that all of the pregnenolone produced is further metabolized and does not accumulate in the culture medium. In agreement with Tsang et al. (1987) progesterone and 17α-hydroxyprogesterone are produced in significant amounts and are responsive to LH. LH stimulation of androgen production increases the availability of substrates for estrogen biosynthesis. In porcine theca cell cultures estrogens can be produced in the absence of exogenous substrates (Tsang et al., 1982).

Each experiment began with a unique population of theca cells due to variability in the stage of sexual development of the ovaries of the pigs provided by the abattoir on any given day. Therefore, while similar trends were observed in replicate experiments, the absolute values of steroid production varied between experiments. Because our experiments used ovarian tissue obtained from the abattoir we could not experimentally induce follicular maturation with exogenous hormones as is done in studies using the gonadotropin-primed rat model and therefore we could not be sure of the amount of gonadotropin to which our cells had been exposed in vivo. This could account for the high levels of unstimulated androgen secretion. In addition, the stage of follicular development is known to affect the cells' responsiveness (Evans et al., 1983).

A major difficulty in the attempt to study theca cell steroidogenesis has been the difficulty of obtaining pure theca cell preparations. The cell population used was not absolutely pure, but within the limitations

of the technique the cells were predominantly theca cells. Stoklosowa et al. (1978) estimated that porcine theca interna cell cultures isolated by a similar procedure contained 70% theca interna cells, 8% granulosa cells and 23% non-steroidogenic cells, which were mainly fibroblasts. It is unlikely that there is significant contamination of the theca cells with granulosa cells, since the accumulation of thecal cyclic AMP, which is stimulated by FSH only in granulosa cells, was stimulated by FSH only at the highest dose tested (1 μg/ml). This stimulation can be accounted for by contamination of the FSH preparation with LH. Androstenedione secretion was also stimulated only by the maximal dose of FSH, and it has been demonstrated that only theca cells synthesize androgens (Bjersing and Carstensen, 1967). Porcine granulosa cells, like those of other species, are unable to produce androgens (Evans et al., 1981; Tsang et al., 1985). The lack of androgen production by granulosa cells is due to a lack of the 17α-hydroxylase and/or the C_{17-20} lyase enzymes in granulosa cells (Bjersing and Carstensen, 1967). In addition, theca cells possess LH and not FSH receptors (Nakano et al., 1977).

These results show that LH induces the functional differentiation of porcine theca cells in serum-free medium in a manner comparable to that observed in vivo. The theca cell culture model provides a good system for the in vitro study of the direct influence of ovarian nerve factors and putative neurotransmitters on theca cell steroidogenesis, without effects on blood flow which confound the interpretation of in vivo studies. Moreover, these studies will be done with theca cells which are known to be innervated (Owman et al., 1967; Burden, 1972; Stefenson et al., 1981; Papka et al., 1985), whereas previous reports of the effects of putative neurotransmitters on ovarian steroidogenesis have used granulosa (Adashi

and Hsueh, 1981; Kliachko and Zor, 1981) and luteal cells (Condon and Black, 1976; Godkin et al., 1977; Jordan et al., 1978; Veldhuis et al., 1980), cell types which are not innervated (Jacobowitz and Wallach, 1967).

CHAPTER 6-EFFECT OF CATECHOLAMINES AND PUTATIVE OVARIAN NEUROTRANSMITTERS ON PORCINE THECA CELL STEROIDOGENESIS

6.1 Introduction

As described in the Literature Review noradrenergic, cholinergic and peptidergic nerves supply the ovarian interstitial and theca cells of all sizes of developing follicles, but do not enter the follicles or corpora lutea. Although there have been many reports of the actions of catecholamines on ovarian cells, few physiological studies on the actions of the other putative neurotransmitters have been reported.

The noradrenergic stimulation of progesterone production was first suggested by Fylling (1971) who demonstrated that noradrenaline increased plasma progesterone levels during early pregnancy in women. Since then, several authors have reported that ovarian tissue contains β-adrenergic receptors (Coleman et al., 1979; Harwood et al., 1980; Ratner et al., 1980; Jordan, 1981; Aguado et al., 1982; Hernandez et al., 1988b). Based on these observations it has been proposed that catecholamines may control progesterone production. Studies with noradrenergic agonists and antagonists have shown that β-adrenergic stimulation increases progesterone production by corpus luteum slices and dispersed luteal cells (Bahr et al., 1974; Condon and Black, 1976; Godkin et al., 1977; Jordan et al., 1978; Harwood et al., 1980; Veldhuis et al., 1980; Norjavaara et al., 1982; Sheela Rani et al., 1983) and granulosa cells (Adashi and Hsueh, 1981; Kliachko and Zor, 1981), even though neither of these cell types is directly innervated *in vivo*. The catecholamine stimulation of steroidogenesis was accompanied by a concomitant increase in cyclic AMP production (Godkin et al., 1977; Jordan et al., 1978; Harwood et al.,

1980; Ratner et al., 1980; Kliachko and Zor, 1981), implying that β-adrenergic agonists act by stimulating adenylate cyclase as has been determined for other catecholamine sensitive tissues (Lefkowitz, 1976). The effects of catecholamines were specifically abolished by the β-adrenergic antagonist propranolol (Condon and Black, 1976; Jordan, et al., 1978).

The presence of noradrenergic nerves in close proximity to the ovarian androgen producing cells suggests that catecholamines may modulate the physiological responses of these cells. Stimulation of ovarian nerves causes the interstitial cells of rats to assume the fine structural features of active steroidogenic cells (Capps et al., 1978). The addition of catecholamines with LH or hCG to cultured rat theca-interstitial cells caused the cells to secrete 3-4 fold more androsterone than occurred in the presence of LH or hCG alone (Dyer and Erickson, 1985; Hernandez et al., 1988b). Pharmacological studies showed the catecholamine effect was mediated by β_2 and possibly β_1 receptors with no involvement of α receptors (Dyer and Erickson, 1985; Hernandez et al., 1988b). Hernandez et al. (1988b) also reported no effect of carbamylcholine (a cholinergic agonist) or GABA on rat theca-interstitial androgen production. There have been no other studies on the effects of putative ovarian neurotransmitters on the ovarian androgen producing cells.

In addition to noradrenaline, there have been reports of the chemical demonstration of dopamine (Farrar et al., 1980; Bahr and Ben-Jonathan, 1981) and adrenaline (Bahr and Ben-Jonathan, 1985) in the ovary, as well as the localization of nerve fibers immunoreactive for VIP (Larsson et al., 1977; Papka et al., 1985), substance P (Alm et al., 1978; Dees et al., 1985; Papka et al., 1985), CCK-8 (McNeill and Burden, 1986) and

neuropeptide Y (McNeill and Burden, 1986; Owman et al., 1986; McDonald et al., 1987). However, there have been few physiological studies of the effects of these agents, and therefore their functions in the ovary remain unknown. An exception is that VIP has been shown to stimulate progesterone and estrogen production by cultured rat granulosa cells (Davoren and Hsueh, 1985).

The objective of the present studies was to examine the effect of catecholamines and some of the other putative ovarian neurotransmitters on androgen production by dispersed porcine theca cells.

6.2 Methods and Results

6.2.1 Effect of Catecholamines on Thecal Androstenedione Accumulation

To examine the effects of α-adrenergic agonists on thecal androgen accumulation, theca cells were cultured in the absence and presence of LH (250 ng/ml) with or without the α_1-adrenergic agonist, phenylephrine (10^{-4}-10^{-8} M; Sigma), or the α_2-adrenergic agonist, clonidine (10^{-4}-10^{-7} M; Sigma). The addition of increasing doses of phenylephrine (Table 4) or clonidine (Table 5) to culture media had no significant ($P > 0.05$) effect on control or LH-stimulated androstenedione accumulation.

To determine if β-adrenergic agonists had an effect on thecal androgen accumulation, LH-stimulated (250 ng/ml) theca cells were cultured in the presence of noradrenaline (10^{-4}-10^{-8} M; Levophed Bitartrate; Sigma) or the non-specific β-adrenergic agonist, isoproterenol (10^{-5}-5×10^{-9} M; Sigma). In the presence of either agonist thecal androstenedione accumulation was significantly ($p < 0.05$) inhibited in a dose-dependent manner (Figures 7A

Table 4. Effect of phenylephrine on theca cell androstenedione accumulation.

	Androstenedione (ng/500,000 cells)[1]	
Phenylephrine (M)	Control	LH
—	5.94 ± 0.30[a]	12.58 ± 0.80[b]
10^{-6}	6.36 ± 0.08[a]	12.20 ± 0.45[b]
10^{-5}	6.39 ± 0.22[a]	13.09 ± 0.23[b]
10^{-4}	5.75 ± 0.35[a]	11.98 ± 0.74[b]

[1]Theca cells were cultured in the absence (Control) and presence of LH (250 ng/ml) with or without increasing concentrations of the α_1-adrenergic agonist phenylephrine (10^{-6}-10^{-4} M) for 48 h. Data are the mean ± S.E.M. of quadruplicate cultures from a single experiment which was replicated one further time. Values with different superscripts are significantly ($p < 0.05$) different. Phenylephrine did not significantly affect thecal androstenedione accumulation.

Table 5. Effect of clonidine on thecal androstenedione accumulation.

Clonidine (M)	Androstenedione (ng/500,000 cells)[1]	
	Control	LH
—	11.87 ± 0.55[a]	25.63 ± 2.37[b]
10^{-7}	10.35 ± 1.05[a]	25.69 ± 0.54[b]
10^{-6}	11.77 ± 0.44[a]	25.36 ± 1.08[b]
10^{-5}	10.01 ± 0.42[a]	25.96 ± 2.22[b]
10^{-4}	10.46 ± 0.42[a]	24.04 ± 0.15[b]

[1]Theca cells were cultured in the absence (Control) and presence of LH (250 ng/ml) with or without the α_2-adrenergic agonist clonidine (10^{-7}-10^{-4} M) for 48 h. Data are the mean ± S.E.M. of quadruplicate cultures from a single experiment which was replicated one further time. Clonidine had no significant effect on thecal androstenedione accumulation.

and 7B). Significant inhibition of androstenedione accumulation by noradrenaline was observed at doses greater than $5x10^{-5}$ M and by isoproterenol at doses greater than 5×10^{-6} M. Isoproterenol and noradrenaline inhibited control androstenedione accumulation in a similar manner (data not shown). Neither isoproterenol nor noradrenaline interfered in the androstenedione radioimmunoassay.

The concentrations of β-adrenergic agonists required to elicit significant inhibitory effects on androstenedione accumulation were very high. This was thought to be due to the rapid metabolism of catecholamines in culture. Therefore, agents which slow the metabolism of catecholamines in culture were tested to see if they could enhance the inhibitory effect of isoproterenol. Catecholamines are metabolized by oxidation and enzymatic degradation by monoamine oxidase (MAO) and catechol-o-methyl transferase (COMT).

The anti-oxidant ascorbic acid is frequently used in cell cultures to prevent catecholamine oxidation. A concentration of 1 μg/ml ascorbic acid was determined to be the maximum concentration in culture medium which itself did not affect thecal androstenedione accumulation. At higher ascorbic acid concentrations, the buffering capacity of the medium was exceeded and the media was too acidic to support the cells. As shown in Table 6 the addition of ascorbic acid (1 μg/ml) to LH-stimulated (250 ng/ml) theca cells with or without isoproterenol (10^{-4} and 10^{-6} M) or noradrenaline (10^{-4} and 10^{-6} M) did not significantly ($p > 0.05$) enhance the inhibitory effect of 10^{-4} M isoproterenol or noradrenaline, nor did it render effective a lower dose of isoproterenol or noradrenaline (10^{-6} M), which did not significantly inhibit androstenedione accumulation alone.

Figure 7. Inhibition of LH-stimulated thecal androstenedione accumulation by isoproterenol (A) and noradrenaline (B). LH-stimulated (250 ng/ml) theca cells were cultured in the presence of increasing concentrations of noradrenaline (10^{-8}-10^{-4} M) and isoproterenol ($5x10^{-8}$-10^{-5} M) for 48 h. Data are the mean ± S.E.M. of quadruplicate cultures from a single experiment which was replicated two further times. Values with different superscripts are significantly ($p < 0.05$) different. Noradrenaline and isoproterenol caused a dose-dependent inhibition of thecal androstenedione accumulation.

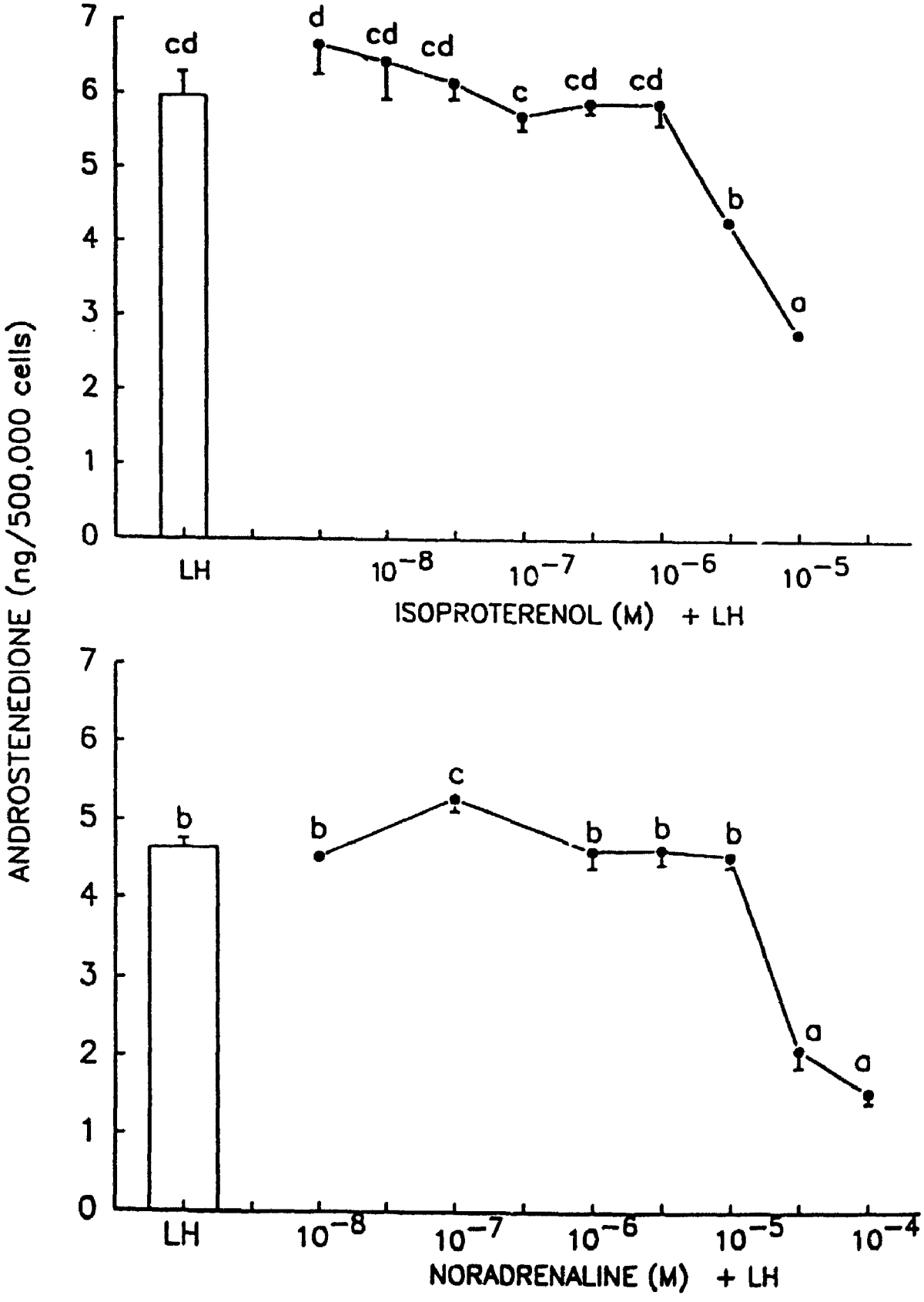

ANDROSTENEDIONE (ng/500,000 cells)
7
6
5
4
3
2
1
0
cd
d
cd
cd
c
cd
cd
b
a
LH
10⁻⁸
10⁻⁷
10⁻⁶
10⁻⁵
ISOPROTERENOL (M) + LH
b
b
c
b
b
b
a
a
LH
10⁻⁸
10⁻⁷
10⁻⁶
10⁻⁵
10⁻⁴
NORADRENALINE (M) + LH

Table 6. Effect of ascorbic acid on the inhibition of thecal androstenedione accumulation by isoproterenol and noradrenaline.

	Androstenedione (ng/500,000 cells)[1]	
Treatment[1]	Control	Ascorbic Acid
—	22.86 ± 1.50^{c}	22.00 ± 0.73^{c}
ISO 10^{-4} M	13.28 ± 1.22^{a}	13.68 ± 0.98^{ab}
ISO 10^{-6} M	21.19 ± 0.63^{c}	21.13 ± 1.78^{c}
NOR 10^{-4} M	16.82 ± 0.38^{b}	15.99 ± 0.87^{ab}
NOR 10^{-6} M	21.67 ± 0.64^{c}	22.30 ± 1.34^{c}

[1]LH-stimulated (250 ng/ml) theca cells were cultured in the absence (Control) and presence of ascorbic acid (1 μg/ml) with or without isoproterenol (ISO; 10^{-6}-10^{-4} M) or noradrenaline (NOR; 10^{-6}-10^{-4} M) for 48 h. Data are the mean ± S.E.M. of quadruplicate cultures from a single experiment which was replicated three further times. Values with different superscripts are significantly ($p < 0.05$) different. Ascorbic acid did not enhance the inhibitory effects of isoproterenol or noradrenaline on thecal androstenedione accumulation.

An inhibitor of MAO, nialamide, was used to attempt to decrease the rate of catecholamine metabolism in culture and therefore shift the inhibition to lower catecholamine concentrations. Preliminary studies showed concentrations of nialamide up to 10^{-6} M could be added to cell cultures without significantly affecting thecal androstenedione accumulation. Nialamide (10^{-6} M) and ascorbic acid (1 μg/ml) were added to LH-stimulated (250 ng/ml) theca cells in the presence of increasing concentrations of isoproterenol (10^{-8}-10^{-4} M). The addition of the inhibitors did not significantly affect LH-stimulated androstenedione accumulation (Table 7). The inhibitory effect of 10^{-4} M isoproterenol was not significantly ($p > 0.05$) increased in the presence of the inhibitors, and lower doses of isoproterenol (10^{-8} and 10^{-6} M) which were not inhibitory, were not rendered effective by the addition of nialamide and ascorbic acid to the culture medium.

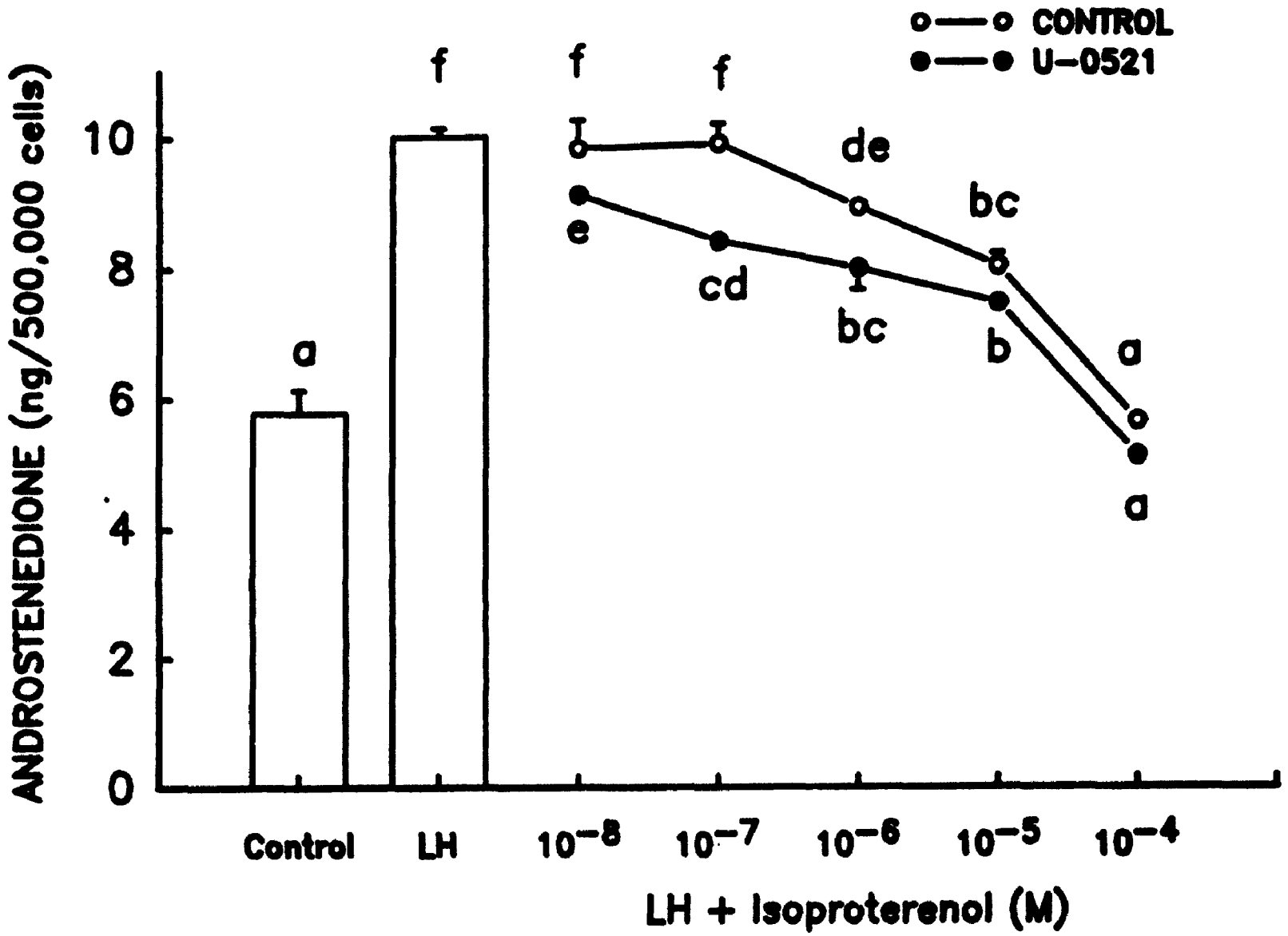
An inhibitor of the COMT enzyme, U-0521 (propiophenone; Upjohn), was used to protect catecholamines from enzymatic metabolism. To determine if the addition of U-0521 would enhance the inhibitory effect of isoproterenol on thecal androgen accumulation, theca cells were cultured in the absence and presence of LH (250 ng/ml) and increasing concentrations of isoproterenol (10^{-8}-10^{-4} M) with or without U-0521. A concentration of 3.6 μg/ml (10 μM) was determined to be the maximum concentration of U-0521 in culture media which itself did not affect thecal androstenedione accumulation. In the absence of U-0521, thecal androstenedione accumulation was significantly ($p < 0.05$) inhibited by isoproterenol at concentrations of 10^{-4}-10^{-8} M (Figure 8). In the presence of U-0521 the effects of isoproterenol (10^{-6}-10^{-8} M) were significantly ($p < 0.05$) enhanced so that concentrations of isoproterenol up to 10^{-8} M caused

Table 7. Effect of the monoamine oxidase (MAO) inhibitor nialamide and ascorbic acid on the inhibition of thecal androstenedione by isoproterenol.

	Androstenedione (ng/500,000 cells)	
Isoproterenol (M)[1]	Control	NIAL+ASC
—	8.15 ± 0.27[bc]	8.48 ± 0.26[bc]
10^{-4}	5.12 ± 0.32[a]	4.15 ± 0.14[a]
10^{-6}	8.40 ± 0.39[bc]	7.90 ± 0.18[b]
10^{-8}	8.94 ± 0.49[c]	8.47 ± 0.35[bc]

[1]LH-stimulated (250 ng/ml) theca cells were cultured in the absence (Control) and presence of the MAO inhibitor nialamide (NIAL; 10^{-6} M) and ascorbic acid (ASC; 1 μg/ml) with or without isoproterenol (10^{-8}-10^{-4} M) for 48 h. Data are the mean ± S.E.M. of quadruplicate cultures from a single experiment which was replicated one further time. Values with different superscripts are significantly ($p < 0.05$) different. Nialamide and ascorbic acid did not enhance the inhibitory effect of isoproterenol an thecal androstenedione accumulation.

Figure 8. Effect of the catechol-o-methyl transferase (COMT) inhibitor U-0521 on the inhibition of thecal androstenedione accumulation by isoproterenol. Thecal cells were incubated in the absence (Control) and presence of LH (250 ng/ml) with increasing concentrations of isoproterenol (10^{-8}-10^{-4} M), with or without U-0521 (10 μM) for 48 h. Values represent the mean $\pm$ S.E.M. of quadruplicate cultures in a single experiment which was replicated one further time. Where no error bar is shown the S.E.M. is within the limit of the symbol. Values with different superscripts are significantly ($p < 0.05$) different. U-0521 enhanced the inhibitory effect of isoproterenol on thecal androstenedione accumulation.


o—o CONTROL
●—● U-0521
ANDROSTENEDIONE (ng/500,000 cells)
0
2
4
6
8
10
a
f
f
f
e
cd
bc
de
bc
b
a
a
Control
LH
10⁻⁸
10⁻⁷
10⁻⁶
10⁻⁵
10⁻⁴
LH + Isoproterenol (M)

significant ($p < 0.05$) inhibitory effects on androgen accumulation. At high concentrations of isoproterenol (10^{-4} or 10^{-5} M) there was no significant effect of the addition of U-0521. In addition, concentrations of isoproterenol (10^{-7} and 10^{-6} M) which normally had no significant ($p > 0.05$) effect on thecal androgen accumulation, significantly ($p < 0.05$) inhibited androgen accumulation in the presence of U-0521.

6.2.2 Isoproterenol Inhibition of Androstenedione Accumulation

To examine the ability of isoproterenol to inhibit androstenedione accumulation in the presence of increasing concentrations of LH, theca cells were cultured in the absence or presence of increasing concentrations of LH (1-1000 ng/ml) with or without isoproterenol (10^{-4} M). Treatment with increasing concentrations of LH caused dose-dependent increases in androstenedione accumulation in the absence and presence of isoproterenol (Figure 9). However, at all doses of LH studied, androstenedione accumulation in the presence of isoproterenol was significantly ($p < 0.05$) less than in cells cultured in the absence of isoproterenol.

To investigate the time course of isoproterenol inhibition of LH-stimulated androstenedione accumulation, LH-stimulated (250 ng/ml) theca cells were cultured with or without isoproterenol (10^{-4} M) for various incubation times (1-48 h). Time-dependent increments in androstenedione accumulation were observed in LH-stimulated cultures in the absence and presence of isoproterenol (Figure 10). Treatment of LH-stimulated cultures with isoproterenol (10^{-4} M) caused a significant ($p < 0.05$) inhibition of androstenedione accumulation between 5 and 17 h of incubation and at all subsequent times studied.

Figure 9. Isoproterenol inhibition of thecal androstenedione accumulation: LH dose-dependence. Theca cells were cultured in the absence and presence of increasing concentrations of LH (1-1000 ng/ml) with or without isoproterenol (10^{-4} M) for 48 h. Data are the mean $\pm$ S.E.M. of quadruplicate cultures from a single experiment which was replicated one further time. Analysis of variance revealed a significant ($p < 0.05$) interaction between the effects of LH and isoproterenol. Asterisks indicate significant ($p < 0.05$) differences between cultures with and without isoproterenol. Isoproterenol significantly inhibited androstenedione accumulation at all doses of LH tested.

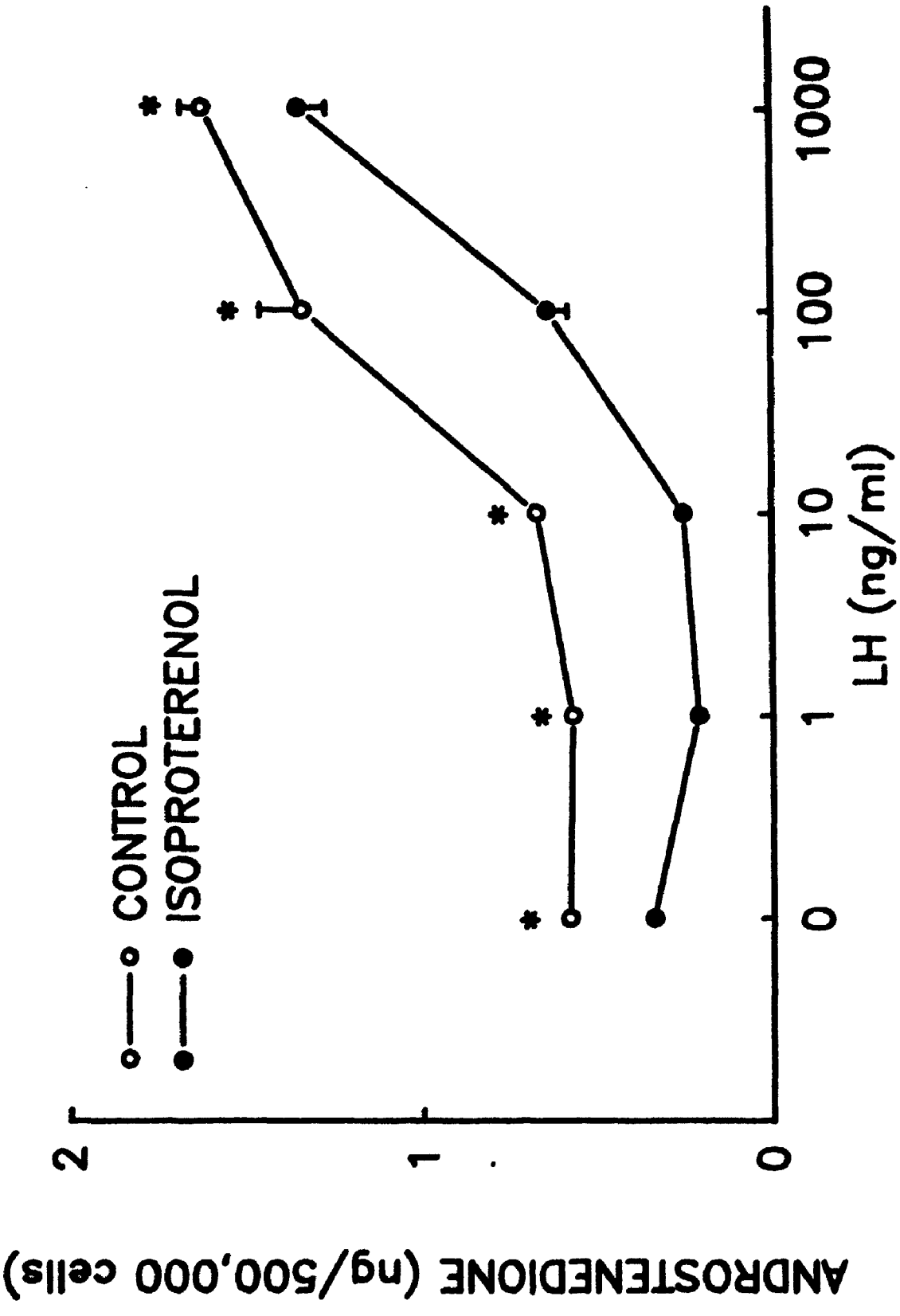
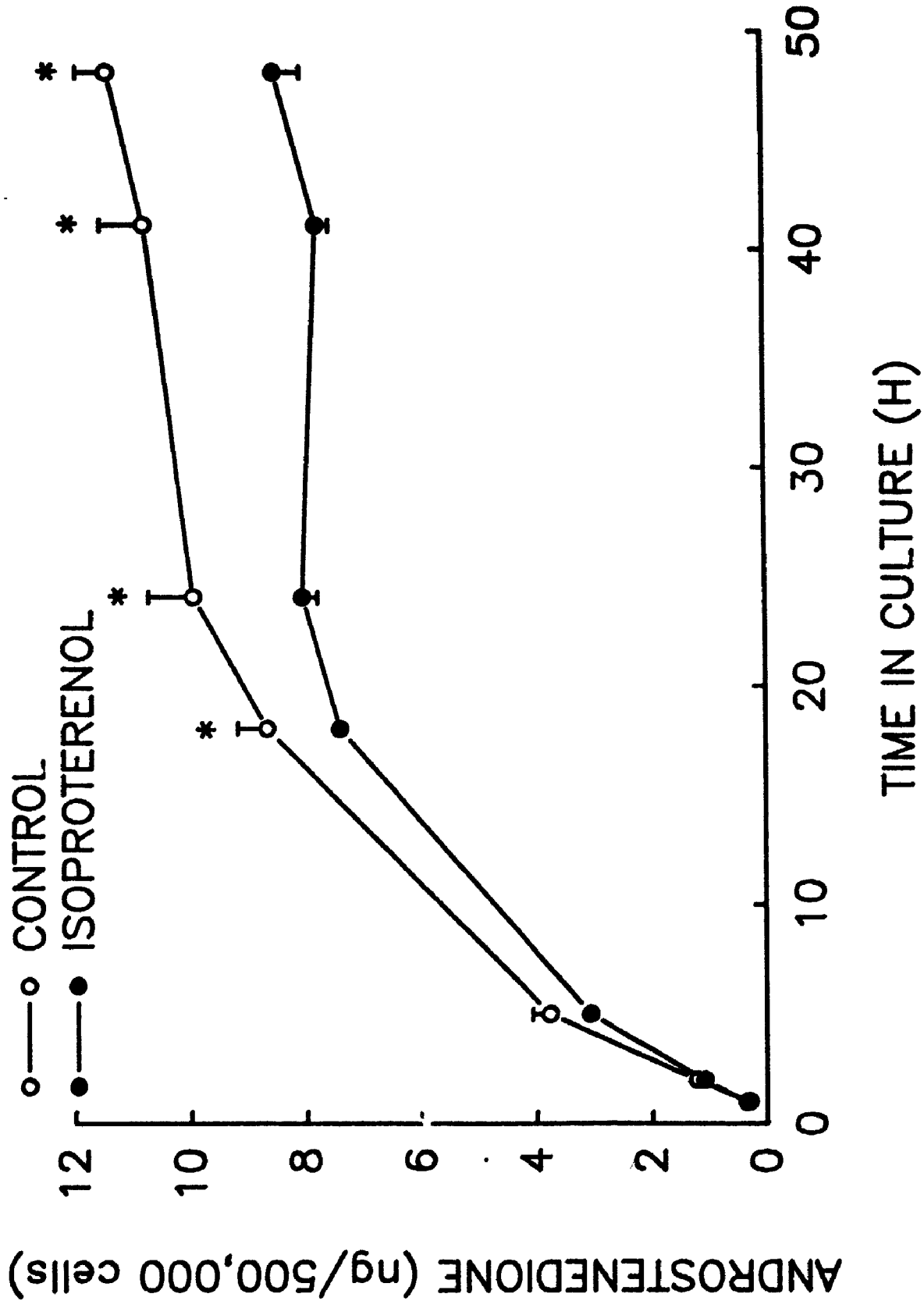

ANDROSTENEDIONE (ng/500,000 cells)
2
1
0
CONTROL
ISOPROTERENOL
0
1
10
100
1000
LH (ng/ml)

Figure 10. Isoproterenol inhibition of thecal androstenedione accumulation: Time course. LH-stimulated (250 ng/ml) theca cells were cultured with or without isoproterenol (10^{-4} M) and the medium was collected at varying times (1-48 h) after plating. Values are the mean ± S.E.M. of quadruplicate cultures from a single experiment. Analysis of variance revealed a significant ($p < 0.05$) interaction between the effects of isoproterenol and time. Values with asterisks are significantly ($p < 0.05$) different between cultures with and without isoproterenol. Isoproterenol inhibited androstenedione accumulation between 5 and 17 h after the start of treatment.


o—o CONTROL
●—● ISOPROTERENOL
ANDROSTENEDIONE (ng/500,000 cells)
TIME IN CULTURE (H)
0
2
4
6
8
10
12
0
10
20
30
40
50
*
*
*

6.2.3 Site of Isoproterenol Inhibition of Androstenedione Accumulation

To elucidate the site at which isoproterenol acts on thecal cells, we compared the accumulation of extracellular cyclic AMP in theca cells cultured in the absence and presence of LH (250 ng/ml), with or without isoproterenol (10^{-4} M). Extracellular cyclic AMP and androstenedione accumulation were measured at the end of 48 h of culture. As shown in Table 8, in theca cell cultures in which isoproterenol inhibited androstenedione accumulation by 30%, the addition of isoproterenol did not significantly ($p > 0.05$) affect LH-stimulated extracellular cyclic AMP accumulation.

To study the ability of isoproterenol to inhibit the action of other physiological and pharmacological agonists which activate adenylate cyclase and dibutyryl cyclic AMP, thecal cells were cultured in the presence of LH (250 ng/ml), dibutyryl cyclic AMP (0.5 mg/ml; 1mM), PGE_2 (1 μg/ml) or cholera toxin (0.1 μg/ml) in the absence or presence of isoproterenol (10^{-4} M). Dibutyryl cyclic AMP-stimulated cultures also contained the phosphodiesterase inhibitor IBMX (89 μg/ml; 400 μM). Isoproterenol significantly ($p < 0.05$) inhibited LH-, dibutyryl cyclic AMP-, PGE_2- and cholera toxin-stimulated androstenedione accumulation by 34, 29, 45 and 28% respectively (Figure 11).

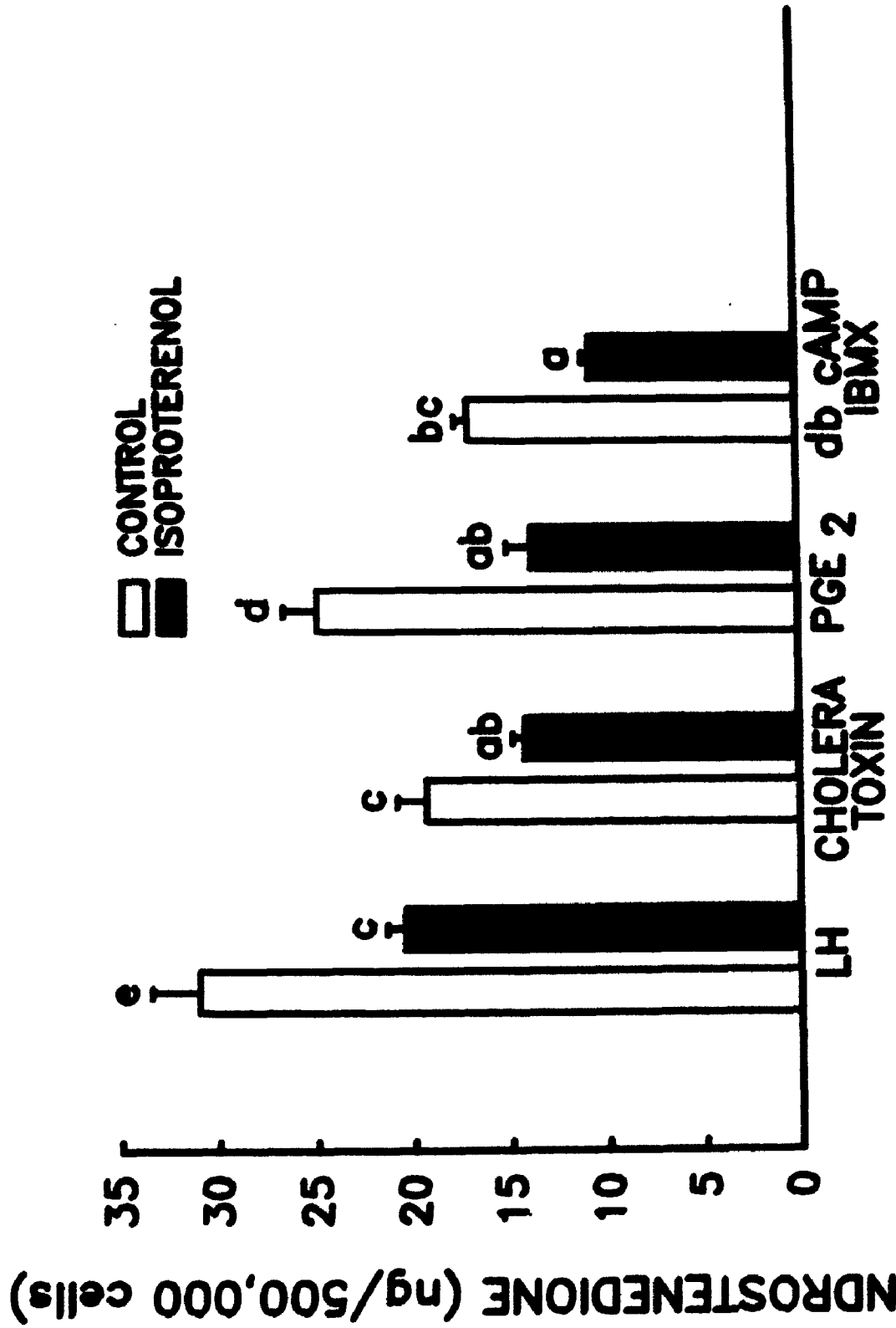
To investigate the site(s) in the steroidogenic pathway at which isoproterenol exerts its inhibitory effects, theca cells were cultured in the absence or presence of LH (250 ng/ml), with or without an inhibitory concentration of isoproterenol (10^{-4} M). The steroids pregnenolone, progesterone, 17α-hydroxyprogesterone, androstenedione, testosterone, estradiol and estrone were measured at the end of 48 h of culture. As

Table 8. Effect of isoproterenol on extracellular cyclic AMP accumulation.

Treatment[1]	Cyclic AMP (pmol/500,000 cells)	Androstenedione (ng/500,000 cells)
—	36.32 ± 0.87[a]	11.40 ± 0.54[b]
ISO	33.46 ± 1.06[a]	8.51 ± 0.46[a]

[1]LH-stimulated (250 ng/ml) theca cells were cultured with or without isoproterenol (ISO; 10^{-4} M) and extracellular cyclic AMP and androstenedione accumulation in culture media were measured after 48 h of culture. Data are the mean ± S.E.M. of quadruplicate cultures from a single experiment which was replicated one further time. Values with different superscripts are significantly ($p < 0.05$) different for each product.

Figure 11. Effect of isoproterenol on thecal androstenedione accumulation stimulated by LH, PGE_2, cholera toxin and dibutyryl cyclic AMP. Theca cells were cultured for 48 h in the presence of LH (250 ng/ml), PGE_2 (1 μg/ml), cholera toxin (0.1 μg/ml) or dibutyryl cyclic AMP (1 mM) and IBMX (400 μM) with or without isoproterenol (10^{-4} M) for 48 h. Data are the mean $\pm$ S.E.M. of quadruplicate cultures from a typical experiment which was replicated one further time. Values with different superscripts are significantly ($p < 0.05$) different. Isoproterenol inhibited the stimulation of androgen accumulation by all agents: LH, PGE_2, cholera toxin and dibutyryl cyclic AMP.


ANDROSTENEDIONE (ng/500,000 cells)
35
30
25
20
15
10
5
0
CONTROL
ISOPROTERENOL
e
c
c
ab
c
ab
d
ab
bc
a
LH
CHOLERA TOXIN
PGE 2
db cAMP IBMX

shown previously, LH stimulated significant ($p < 0.05$) increases in the accumulation of all steroids (Table 9). Treatment with isoproterenol decreased the accumulation of all steroids by basal and LH-stimulated cultures.

The inhibitory effect of isoproterenol on thecal androstenedione accumulation was not due to a toxic effect of catecholamines on the theca cells, since the DNA content of the cell layer measured after 48 h of culture in LH-stimulated cultures did not differ significantly in the absence (2.49 ± 0.07 μg/well) or presence (2.47 ± 0.08 μg/well) of isoproterenol (10^{-4} M).

6.2.4 Effect of β-Adrenergic Receptor Antagonists on the Isoproterenol Inhibition of Androstenedione Accumulation

The inhibition of thecal androstenedione accumulation by the β-adrenergic agonists noradrenaline and isoproterenol suggest the effect is mediated by a β-adrenergic receptor. To validate the specificity of the potential β-blockade of androstenedione accumulation the receptor at which catecholamines exert their inhibitory effects was studied further using β-adrenergic receptor antagonists.

Two β-adrenergic receptor antagonists, the non-specific antagonist DL-propranolol (10^{-4} M; Sigma) and the β_1 selective antagonist metoprolol (10^{-4} M; CIBA Geigy Canada Ltd., Mississauga, Ont) were used to determine if the inhibitory effects were mediated via β-adrenergic receptors. These concentrations of antagonists alone did not significantly ($p > 0.05$) affect thecal androstenedione accumulation. The antagonists were added to the cultures 30 min prior to the addition of the isoproterenol to allow time for the antagonists to bind to the β-receptors. Theca cells were

Table 9. Isoproterenol inhibition of thecal androstenedione accumulation: Steroidogenic profile

	Steroid Accumulation (ng/500,000 cells)			
Steroid[1]	CON	ISO	LH	LH+ISO
P_5	0.40±0.19^a	0±0^a	1.49±0.30^b	0.45±0.08^a
P_4	3.75±0.28^c	1.56±0.09^a	8.56±0.30^d	2.82±0.29^b
17α-OHP_4	7.15±0.43^{ab}	3.94±0.70^a	15.83±1.14^c	9.50±1.46^b
Adione	10.34±0.32^b	6.10±0.20^a	30.99±2.43^d	20.42±0.93^c
Test	0.88±0.06^a	0.49±0.03^a	3.75±0.20^c	1.85±0.13^b
E_2	0.99±0.07^b	0.44±0.07^a	2.60±0.20^c	1.08±0.07^b
E_1	0.60±0.03^b	0.41±0.05^a	1.19±0.04^d	0.91±0.02^c

[1]Thecal cells were cultured with (LH) or without (CON) LH (250 ng/ml) in the absence or presence of isoproterenol (ISO; 10^{-4} M). At the end of 48 h of culture the media was removed for RIA of pregnenolone (P_5), progesterone (P_4), 17α-hydroxyprogesterone (17α-OHP_4), androstenedione (Adione), testosterone (Test), estradiol (E_2) and estrone (E_1). Values represent the mean ± S.E.M. of quadruplicate cultures from a single experiment which was replicated one further time. Values with different superscripts are significantly ($p < 0.05$) different within each steroid group. Treatment of control and LH-stimulated theca cells with isoproterenol caused a decrease in the accumulation of all steroids measured.

cultured in the absence and presence of LH (250 ng/ml) with or without isoproterenol (10^{-5} M). Some cultures contained propranolol (10^{-4} M) or metoprolol (10^{-4} M). Addition of propranolol to control or LH-stimulated theca cell cultures caused a small but not statistically significant ($p > 0.05$) reversal of the isoproterenol inhibition of androstenedione accumulation (Table 10). Addition of metoprolol caused a complete reversal of the isoproterenol inhibition of LH-stimulated androstenedione accumulation and caused a small, but not statistically significant, reversal of the isoproterenol inhibition under control conditions (Table 11).

6.2.5 Effect of Other Putative Ovarian Neurotransmitters on Thecal Androstenedione Accumulation

The effects of other putative neurotransmitters previously shown to be present in the ovary and ovarian nerves or their agonists were added to theca cell cultures to determine if they affect androstenedione accumulation. Table 12 shows that the addition of gamma-amino-butyric acid (GABA; Sigma; 10^{-7}-10^{-4} M) to theca cells cultured in the absence and presence of LH (250 ng/ml) did not significantly ($p > 0.05$) affect androstenedione accumulation. Similarly, incubation of theca cells with dopamine (Sigma; 10^{-9}-10^{-4} M), vasoactive intestinal peptide (VIP; Sigma; 10^{-12}-10^{-9} M), neuropeptide Y (NPY; Sigma; 10^{-12}-10^{-9} M), oxytocin (Sigma; 10-1000 ng/ml), or the acetylcholine agonist carbamylcholine chloride (carbachol; Sigma; 10^{-9}-10^{-3} M) had no significant ($p > 0.05$) effect on control or LH-stimulated thecal androstenedione accumulation (data not shown).

Table 10. Effect of propranolol on the inhibition of thecal androstenedione accumulation by isoproterenol.

	Androstenedione (ng/500,000 cells)	
Treatment[1]	—	Propranolol
CON	11.15 ± 0.40[b]	11.99 ± 0.66[b]
ISO	6.34 ± 0.22[a]	6.92 ± 0.18[a]
LH	24.11 ± 1.40[d]	27.21 ± 2.02[d]
LH + ISO	14.66 ± 0.73[bc]	17.55 ± 0.48[c]

[1]Theca cells were cultured in the absence (CON) and presence of LH (250 ng/ml) with or without isoproterenol (ISO; 10^{-5} M) for 48 h. Some cultures contained the non-specific β-adrenergic antagonist propranolol (10^{-4} M) which was added 30 min prior to the addition of the isoproterenol to allow binding to the β receptors. Data are the mean ± S.E.M. of quadruplicate cultures from a single experiment which was replicated two further times. Values with different superscripts are significantly ($p < 0.05$) different. Propranolol caused a small but not statistically significant reversal of the isoproterenol inhibition of control and LH-stimulated androstenedione accumulation.

Table 11. Effect of metoprolol on the inhibition of thecal androstenedione accumulation by isoproterenol.

	Androstenedione (ng/500,000 cells)	
Treatment[1]	—	Metoprolol
CON	3.80 ± 0.20[a]	4.31 ± 0.37[ab]
ISO	2.89 ± 0.28[a]	4.07 ± 0.64[ab]
LH	7.16 ± 0.62[c]	7.11 ± 0.58[c]
LH + ISO	5.58 ± 0.26[b]	7.23 ± 0.45[c]

[1]Theca cells were cultured in the absence (CON) and presence of LH (250 ng/ml) with or without isoproterenol (ISO; 10^{-6} M) for 48 h. Some cultures contained the β-adrenergic antagonist metoprolol (10^{-4} M) which was added 30 min prior to the addition of isoproterenol to allow binding to the β receptors. Data are the mean ± S.E.M. of quadruplicate cultures from a single experiment which was replicated one further time. Analysis of variance revealed a significant ($p < 0.05$) interaction between the effects of metoprolol and isoproterenol. Values with different superscripts are significantly ($p < 0.05$) different. Metoprolol completely reversed the isoproterenol inhibition of LH-stimulated androstenedione accumulation and caused a small, but not statistically significant, reversal under control conditions.

Table 12. Effect of gamma amino butyric acid (GABA) on thecal androstenedione accumulation.

GABA (M)	Androstenedione (ng/500,000 cells)[1]	
	Control	LH
—	2.98 ± 0.17[a]	5.73 ± 0.22[b]
10^{-7}	3.10 ± 0.10[a]	6.08 ± 0.22[b]
10^{-6}	3.17 ± 0.09[a]	5.86 ± 0.16[b]
10^{-5}	3.15 ± 0.10[a]	5.76 ± 0.34[b]
10^{-4}	3.29 ± 0.08[a]	5.80 ± 0.14[b]

[1]Theca cells were cultured in the absence (Control) or presence of LH (250 ng/ml) with or without increasing concentrations of GABA (10^{-7}-10^{-4} M) for 48 h. Values are the mean ± S.E.M. of quadruplicate cultures from a single experiment which was replicated two further times. Values with different superscripts are significantly ($p < 0.05$) different. GABA did not affect control or LH-stimulated thecal androstenedione accumulation.

6.3 Discussion

These studies have shown that β-, but not α-, adrenergic stimulation inhibits basal and LH-stimulated porcine theca cell androgen accumulation. The effect is dose- and time-dependent, but independent of LH dose. Responsiveness to isoproterenol and noradrenaline suggest the response is mediated via a β-adrenergic receptor. The 10-fold lower effective dose for isoproterenol than noradrenaline reflects the greater potency of isoproterenol than noradrenaline at β-adrenergic receptors. The inhibitory effect of catecholamines on androgen accumulation was partially reversed by the β_1-receptor antagonist metoprolol. It is not clear why metoprolol, and not propranolol, was able to reverse the inhibitory effect of isoproterenol. The L-isomer of propranolol shows stereospecificity for β-adrenergic blockade while the D-isomer exhibits local anaesthetic activity (Barrett and Cullum, 1968). DL-propranolol (1 mM) has been shown to exhibit local anaesthetic activity which inhibits thyroid stimulating hormone stimulated adenylyl cyclase in bovine thyroid plasma membrane fractions (Marshall et al., 1975). A similar anaesthetic action of the D-isomer of propranolol may be occurring in our system, which prevents the reversal of the catecholamine effects.

In granulosa and luteal cell cultures catecholamines stimulate progesterone accumulation via a β-adrenergic receptor. Both immature and adult ovaries contain a well defined population of β-adrenergic receptors (Coleman et al., 1979; Jordan, 1981; Aguado et al., 1982). These have been characterized by biochemical and functional means to be of the β_2 subtype (Ratner et al., 1980; Veldhuis et al., 1980). Stimulation of these receptors by either catecholamines or specific agonists increases progesterone production from luteal cells (Condon and Black, 1976; Godkin

et al., 1977; Jordan et al., 1978; Harwood et al., 1980; Veldhuis et al., 1980; Norjavaara et al., 1982; Sheela Rani et al., 1983), granulosa cells (Adashi and Hsueh, 1981; Kliachko and Zor, 1981) or whole ovaries (Zsolnai et al., 1982). The response is mediated via an increase in cyclic AMP formation and is inhibited by propranolol (Condon and Black, 1976; Godkin et al., 1977; Kliachko and Zor, 1981). Although there are specific membrane receptors for catecholamines in rat ovarian granulosa (Ratner et al., 1980; Jordan et al., 1981; Aguado et al., 1982), luteal (Coleman et al., 1979; Harwood et al., 1980) and theca-interstitial cells (Hernandez et al., 1988b), there have been no reports of the type or capacity of catecholamine receptors in porcine theca cells.

The concentrations of catecholamines required to elicit significant effects were very high, and therefore the responses may not reflect a physiological mechanism. Veldhuis has reported the concentration of noradrenaline in the follicular fluid from small porcine preovulatory follicles to be 34 nM (Veldhuis et al., 1980) which is too low to inhibit thecal androstenedione accumulation in our cultures. These values do not necessarily reflect the local concentration of noradrenaline at the synapse of the nerve terminal on the theca cell. The catecholamine concentrations used in the present studies were above the normal physiological range of circulating or tissue catecholamines, raising the possibility that theca cells may normally be relatively unresponsive to catecholamines and therefore the responses observed may have little physiological significance. Alternatively, high doses of catecholamines may be required since the catecholamines are rapidly metabolized in culture. The catecholamines were added at the time of plating, and presumably were rapidly metabolized during the culture period. Attempts

to slow catecholamine metabolism in culture with the antioxidant ascorbic acid, or the monoamine oxidase inhibitor nialamide, did not influence the inhibitory effects of catecholamines. Both ascorbic acid and nialamide are unstable compounds and therefore, would also be metabolized in culture. Since we do not know the rate at which they are metabolized in culture, these studies do not prove that catecholamine degradation did not occur by oxidation or MAO activity.

The addition of the catechol-o-methyl transferase inhibitor, U-0521, however, significantly increased the inhibitory effects of various doses of isoproterenol. These observations suggest that porcine theca cells have active COMT enzymes and may account for the high catecholamine concentrations required to observe inhibitory effects in culture. COMT has been shown to be present in many mammalian tissues including the uterus (Hersey et al., 1982) and porcine ovarian follicular wall (Fernandez-Pardal et al., 1986).

The mechanism of the inhibition of thecal androstenedione accumulation by catecholamines is presently unknown. The finding that catecholamines do not alter extracellular cyclic AMP accumulation, but inhibit the stimulatory effect of dibutyryl cyclic AMP and other physiological and pharmacological agents which activate adenylate cyclase, suggests that the site of isoproterenol antagonism is distal to the site of cyclic AMP production. However, since the effects of isoproterenol on the secretion of cyclic AMP by these cells is not known, it is possible that isoproterenol may work through the cyclic AMP system by differentially affecting intracellular and extracellular cyclic AMP. Recently, Oikawa and Hsueh (1989) have shown that treatment of cultured rat granulosa cells

with isoproterenol, results in similar increases in intracellular and extracellular cyclic AMP accumulation.

The finding that catecholamines do not alter extracellular cyclic AMP implies that the inhibition does not involve changes in theca cell LH receptor number and/or affinity. These observations may also suggest that catecholamines are acting through an alternate second messenger system. Catecholamines caused a generalized suppression of theca cell steroidogenesis. It is not known if this is due to a specific inhibition of each steroidogenic enzyme or the suppression of an early step in the steroidogenic pathway.

The inhibitory effect of catecholamines is similar to that seen for prolactin, which also causes a general suppression of LH-stimulated steroidogenesis in rat theca-interstitial cells (Magoffin and Erickson, 1982b). Prolactin action is not due to decreased LH-stimulated cyclic AMP formation since prolactin does not alter LH receptor binding or LH stimulation of cyclic AMP formation, but inhibits cyclic AMP-stimulated steroidogenesis. Therefore, like catecholamines, prolactin inhibits LH stimulation of steroid production at a step distal to cyclic AMP formation. However, the inhibitory effect of prolactin has a more rapid onset, with significant declines in androgen production occurring within 2 h of treatment (Magoffin and Erickson, 1982b). GnRH (Magoffin et al., 1981; Magoffin and Erickson, 1982a) and estrogen (Leung et al., 1978; Tsang et al., 1979a; Magoffin and Erickson, 1982d; Hunter and Armstrong, 1987) also antagonize thecal androgen production; however, in contrast to catecholamines or prolactin, estrogen and GnRH cause a rapid and selective inhibition of the 17α-hydroxylase:$C_{17,20}$ lyase enzyme activities.

In all other ovarian cell types studied, catecholamines increase steroid production by increasing the levels of cyclic AMP (Godkin et al., 1977; Kliachko and Zor, 1981; Dyer and Erickson, 1985). There are reports in the literature that thyroid follicular cells respond to catecholamines in a similar manner as theca cells. The thyroid gland is richly innervated by adrenergic and cholinergic nerve fibers which terminate on blood vessels and in close proximity to the follicular cells (Ahren, 1986a). There is also evidence for nerve fibers containing a variety of putative neurotransmitter peptides, including VIP, substance P, CCK, NPY and calcitonin gene-related peptide (Ahren, 1986a). The role of these nerves in the physiology of the thyroid gland is not known. Similar to observations in theca cell cultures, several studies have demonstrated that noradrenaline, at concentrations of 10^{-4} or 10^{-5} M, inhibits thyroid stimulating hormone (TSH) induced thyroid hormone secretion under both in vivo (Ahren, 1985) and in vitro conditions (Maayan et al., 1977a,b; Muraki et al., 1982), without influencing cyclic AMP production. This inhibitory effect of noradrenaline was prevented by the α-adrenergic blocker phentolamine, but not by the β-adrenergic blocker propranolol, suggesting catecholamines are acting via an α-adrenergic receptor to suppress TSH-stimulated release of thyroid hormone (Maayan et al., 1977a,b; Muraki et al., 1982). The inhibitory effect of noradrenaline on TSH-stimulated thyroid hormone secretion is potentiated by NPY, which is present in sympathetic nerve terminals in the thyroid (Ahren, 1986b).

The mechanism of inhibition in the thyroid remains to be determined. It has been shown that noradrenaline causes dose-dependent increases in cyclic GMP in thyroid cells (Aiyoshi et al., 1978; Brandi et al., 1983). Alteration of the cyclic GMP/cyclic AMP ratio in thyroid cells by

noradrenaline may be responsible for the inhibitory effects of noradrenaline. This effect is interesting since cyclic GMP induces degradation of cyclic AMP in thyroid cells (Erneux et al., 1977). It is possible that a similar mechanism is responsible for the effects of noradrenaline on theca cells.

The results of this in vitro study indicate that sympathomimetic agents are capable of activating β-adrenergic receptors which leads to an inhibition of porcine theca cell androstenedione accumulation. The physiological importance of a direct inhibitory action of catecholamines on porcine theca cells has not been established. If the catecholamine inhibition of thecal androstenedione accumulation is of physiological significance there must be a way to supply the theca cells with catecholamines. The most likely source of noradrenaline would be as a neurotransmitter from the postganglionic sympathetic neurons which innervate the theca cells. Alternatively, adrenal catecholamines secreted into the circulation would reach the theca cells by their rich vascularization. There may also be cells within the ovary which are capable of noradrenaline production; however, there is no direct evidence for their existence. The combined facts that catecholamines are present in the theca and that they can inhibit androgen production, suggest that catecholamines may be important intra-ovarian regulators of theca cell activity. It is conceivable that the catecholamine actions are important during stress when an increased sympathetic discharge could play a role in inhibiting androgen production.

These studies did not provide conclusive evidence that catecholamines from the sympathetic nervous system control thecal androgen production. In addition, no effects of the putative ovarian neurotransmitters were

observed on thecal androgen accumulation. Therefore, a more direct means of studying the effect of the ovarian innervation on thecal steroidogenesis was attempted by adding rat superior ovarian nerve extracts to cultured theca cells to look for steroidogenic effects.

CHAPTER 7-EFFECT OF EXTRACTS OF RAT SUPERIOR OVARIAN NERVES ON PORCINE THECA CELL STEROIDOGENESIS

7.1 Introduction

Peripheral nerves are essential for the maintenance of many properties of their target organs. Neural influences are exerted (1) by the release of neurotransmitters, such as the nerves to skeletal muscle releasing the transmitter acetylcholine which produces contractile activity and (2) by the secretion of trophic factors. Trophic effects are long-term interactions between nerve cells and the tissues they innervate, that are responsible for the structural, chemical and functional integrity of the target tissue. It is believed that neurotrophic influences are independent of nerve transmission and are mediated by chemical substances that move by axoplasmic transport (Warnick et al., 1977).

In vitro bioassay systems have proven useful for assaying these neural factors (Lentz, 1971; Younkin et al., 1978). The nature and actions of the neurotrophic factors have been studied by adding homogenates of neural tissues (spinal cord, brain, peripheral nerves or sensory ganglia) to cultures of skeletal muscle cells (Oh, 1975, 1976; Markelonis and Oh, 1978). The nerve extracts enhanced the morphological development of the muscle cells, DNA and protein synthesis, formation of acetylcholinesterase and maintained muscle fibers for several months in the absence of innervation (Oh, 1976; Oh and Markelonis, 1978). In the absence of the extracts or innervation, cultured muscle fibers degenerated rapidly. In adults, the most potent trophic factors are found in peripheral nerves (Popiela, 1978). Currently it is believed that a neurohumoral factor is

responsible for these neurotrophic effects, however, the 'factor' has not yet been isolated or purified.

Peripheral nerve homogenates have also been used to study amphibian limb regeneration. Denervation of early regenerates leads to cessation of growth and restoration of the nerve supply results in renewed capacity to grow (Singer, 1974). Peripheral nerve homogenates can replace the neural trophic factors to promote the regeneration of denervated, amputated newt limb (Singer, 1974).

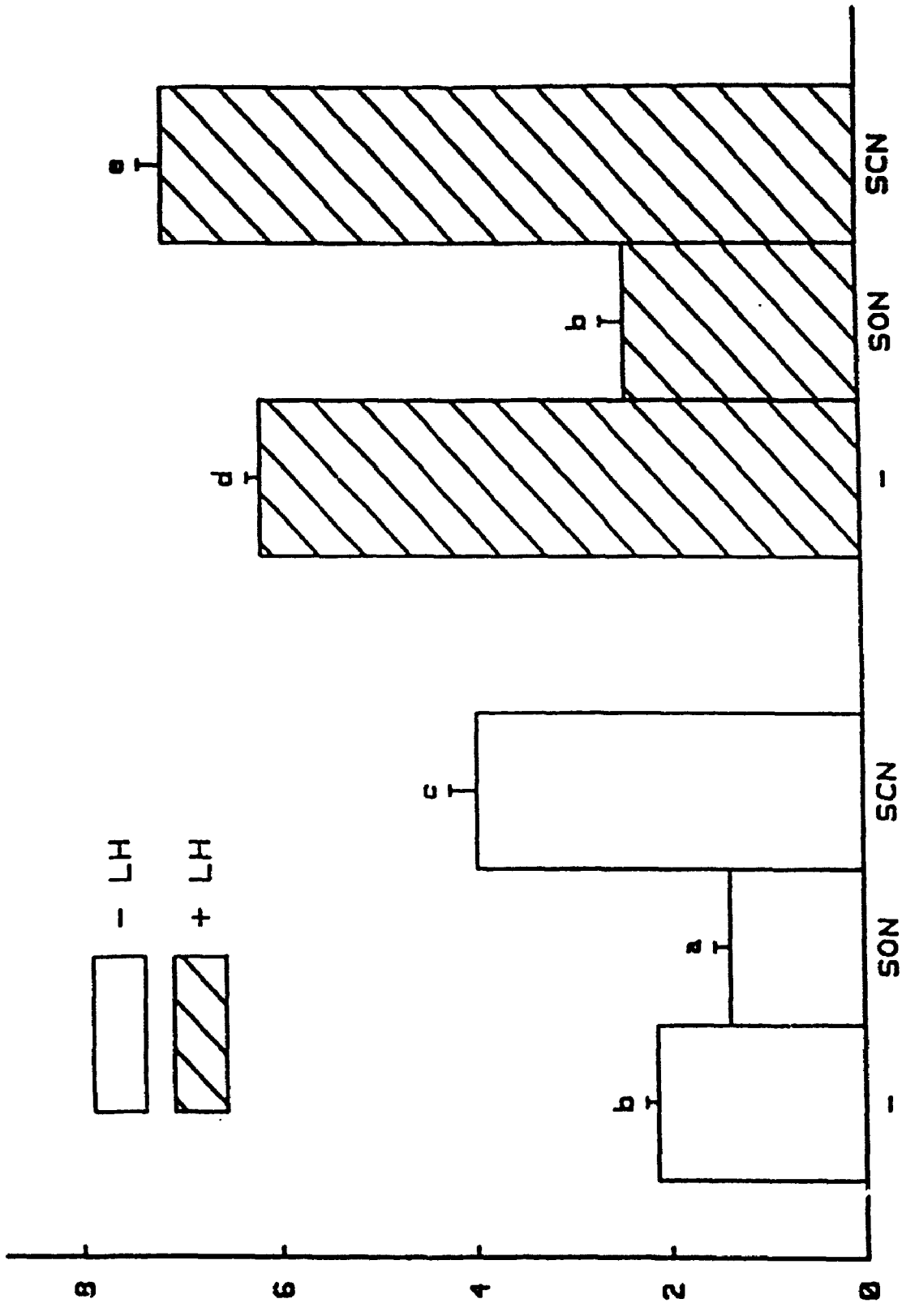
Based on observations from these two systems, a similar strategy was adopted to study the effects of peripheral nerves on ovarian theca cells. To my knowledge the use of nerve homogenates has not been used to study neural effects on endocrine organs. I have attempted to look at the direct effect of ovarian nerve factors on androgen production by porcine theca cells by adding homogenates of the superior ovarian nerve of rats to cultured theca cells.

7.2 Methods and Results

7.2.1 Effect of Rat Superior Ovarian Nerve Extracts on Theca Androgen Accumulation

Figure 12 shows the effect of extracts of rat superior ovarian nerves (SON) on basal and LH-stimulated androstenedione accumulation by cultured porcine theca cells. After 48 h of treatment with LH (250 ng/ml), the accumulation of androstenedione was increased by 2.9-fold over basal levels. Both basal and LH-stimulated androstenedione accumulation were significantly ($p < 0.05$) decreased by the addition of the extract of 20 mg SON/ml culture medium.

Figure 12. Effect of superior ovarian nerve (SON) and sciatic nerve (SCN) extracts on basal (-LH) and LH-stimulated (+LH; 250 ng/ml) androstenedione accumulation by cultured porcine theca cells. Values represent the mean ± S.E.M. of quadruplicate cultures in a single experiment which was replicated three further times. Values with different superscripts are significantly different ($p < 0.05$). The SON extract significantly decreased and the SCN extract significantly increased both basal and LH-stimulated androstenedione accumulation.


ANDROSTENEDIONE (ng/500,000 cells)
0
2
4
6
8
SCN
SON
SCN
SON
+ LH
- LH

The inhibitory effect of the SON extract on LH-stimulated androstenedione production was concentration-dependent over a 30-fold range from 11% inhibition in the presence of the extract of 2 mg SON/ml culture medium to 83% inhibition when the SON extract was increased to 60 mg SON/ml (Figure 13A).

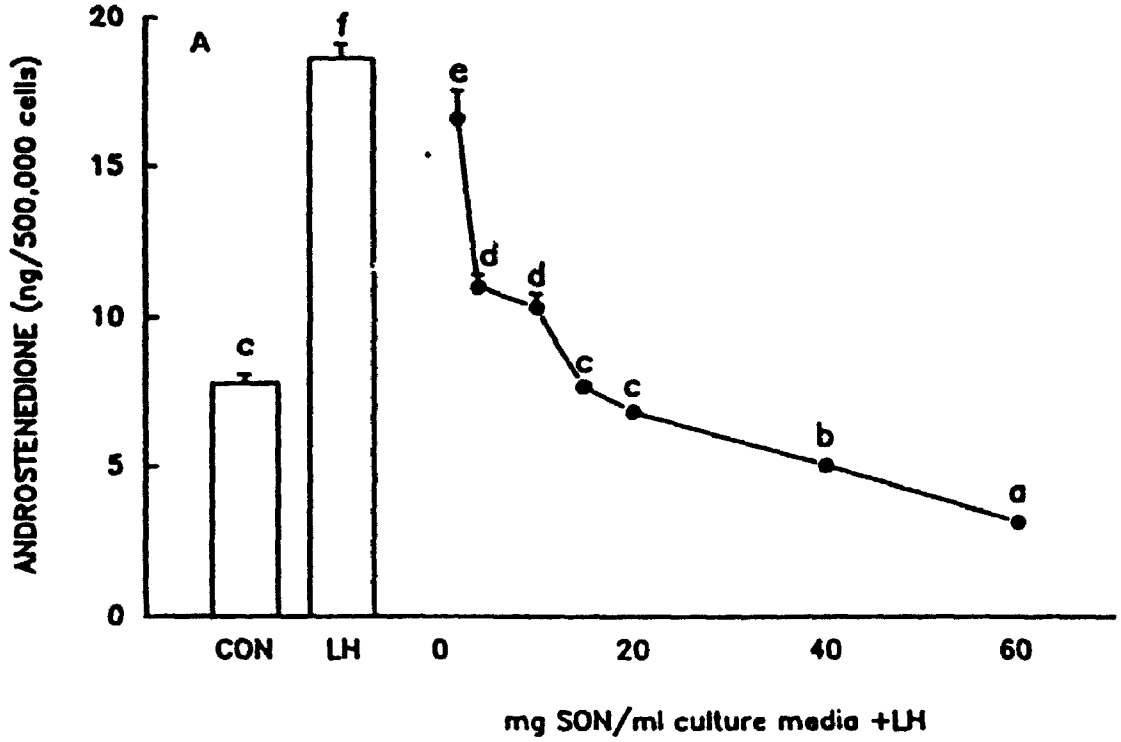
7.2.2 Control Nerves

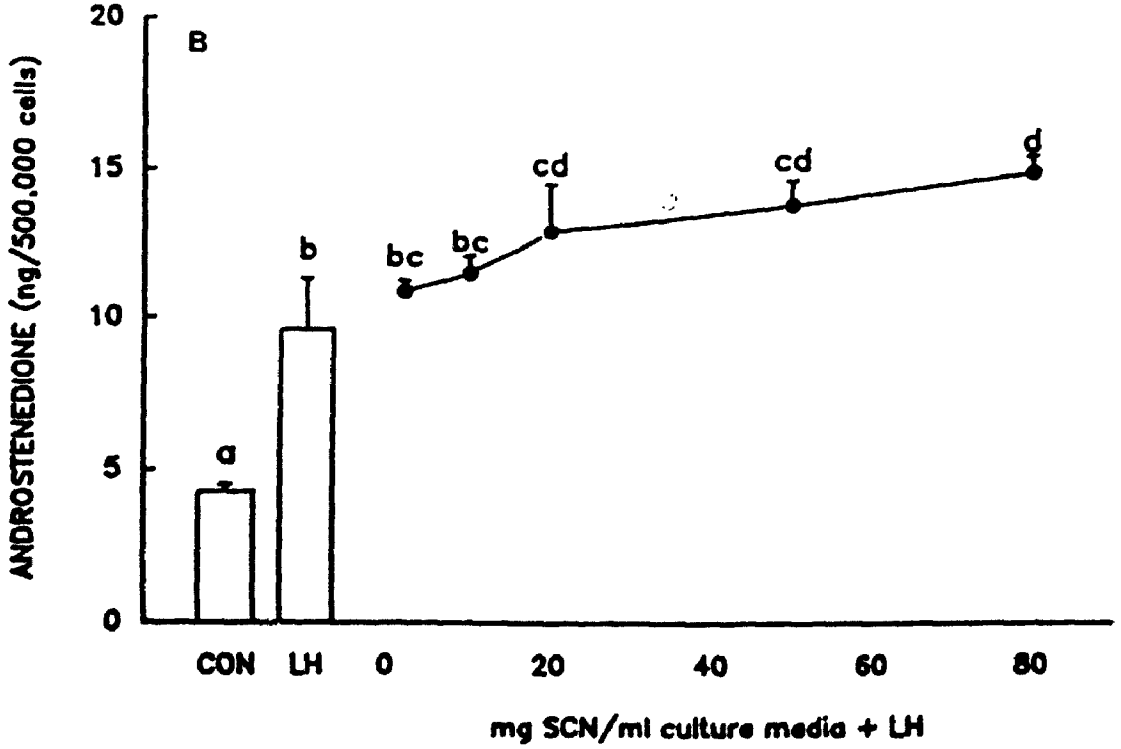
For control experiments, rat sciatic and cervical sympathetic trunk nerve extracts were used. In contrast to the inhibitory effect of the SON extract, both control and LH-stimulated androstenedione accumulation were significantly ($p < 0.05$) increased by the addition of the extract of 20 mg SCN/ml culture medium (Figure 12).

LH-stimulated theca cells cultured in the presence of the extract of 2 mg SCN/ml culture medium showed a 13% increase in androstenedione production over cells stimulated with LH alone, and in the presence of the extract of 80 mg SCN/ml culture medium, androstenedione accumulation was increased by 56% (Figure 13B). No cross reactivity of the SON or SCN extracts was seen in the radioimmunoassay for androstenedione.

In contrast to the stimulatory effect of the control SCN extract, the addition of similarly prepared rat cervical sympathetic trunk nerve extracts at the same concentration (20 mg tissue/μl DPBS) as the other neural tissue extracts inhibited androstenedione accumulation, in a similar manner as the SON extract. Cervical sympathetic trunk nerves were removed from adult female rats following sacrifice by ether overdose. The addition of cervical sympathetic trunk extracts to theca cells cultured in the absence (Control) or presence of LH (250 ng/ml) caused a

Figure 13. Concentration-dependence of the effects of nerve extracts on theca cell androgen accumulation. Theca cells were incubated without (CON) or with 250 ng/ml LH (LH) in the absence or presence of increasing amounts of nerve extracts for 48 h. Figure 13A shows the effects of the addition of the extract from 0-60 mg SON/ml culture medium and Figure 13B shows the effects of the addition of the extract from 0-80 mg SCN/ml culture medium, on LH-stimulated thecal androstenedione accumulation. Values represent the mean $\pm$ S.E.M. of quadruplicate cultures in a single experiment which was replicated one further time. Values with different superscripts are significantly different ($p < 0.05$). The effects of both the SON and SCN extracts on thecal androstenedione accumulation are dose-dependent.


A
ANDROSTENEDIONE (ng/500,000 cells)
20
15
10
5
0
c
f
e
d
d
c
c
b
a
CON
LH
0
20
40
60
mg SON/ml culture media +LH


B
ANDROSTENEDIONE (ng/500,000 cells)
20
15
10
5
0
a
b
bc
bc
cd
cd
d
CON
LH
0
20
40
60
80
mg SCN/ml culture media + LH

significant ($p < 0.05$) inhibition of androstenedione accumulation (Figure 14).

7.2.3 Suspensory Ligament Controls

Whereas SCN and cervical sympathetic trunk nerves could be isolated relatively free of other tissues, the SON was contained in the suspensory ligament. Microscopic examination of fixed and stained segments of rat suspensory ligaments showed the ligament to contain longitudinally oriented smooth muscle bundles among which blood vessels and a large bundle of nerve fibers could be seen. At the periphery of the sections a few adipose cells could be observed. This was in agreement with the previous observations of Musgrove et al. (1978) and Gabella (1976). Experiments were done to test whether extracts of adipose or muscle tissue in the suspensory ligament were responsible for the inhibitory effects of the SON extract on thecal androgen production. LH-stimulated (250 ng/ml) theca cells were cultured with the extracts of adipose tissue from the pad of fat surrounding the ovary or with muscle from the abdominal wall, prepared in an identical manner to the nerve extracts. Figure 15 shows no significant ($p > 0.05$) effect of the addition of DPBS alone or two different extracts of adipose tissue on thecal androstenedione accumulation. Although the levels of androstenedione in cultures incubated with the muscle(1) extract did not differ significantly from cultures with LH alone, cultures incubated with the muscle(2) extract showed significantly ($p < 0.05$) greater androstenedione accumulation than cultures stimulated with LH alone.

To determine if substances of ligament origin were responsible for the inhibitory effects of the SON extracts, inguinal ligaments were collected

Figure 14. Effects of cervical sympathetic trunk (CST) nerve extracts (20 mg tissue/μl DPBS) on basal (CONTROL) and LH-stimulated (LH; 250 ng/ml) androstenedione accumulation by cultured porcine theca cells. Values represent the mean $\pm$ S.E.M. of quadruplicate cultures in a single experiment. Values with different superscripts are significantly different ($p < 0.05$). The cervical sympathetic trunk nerve extract caused a small, but statistically significant inhibition of both basal and LH-stimulated androstenedione accumulation.

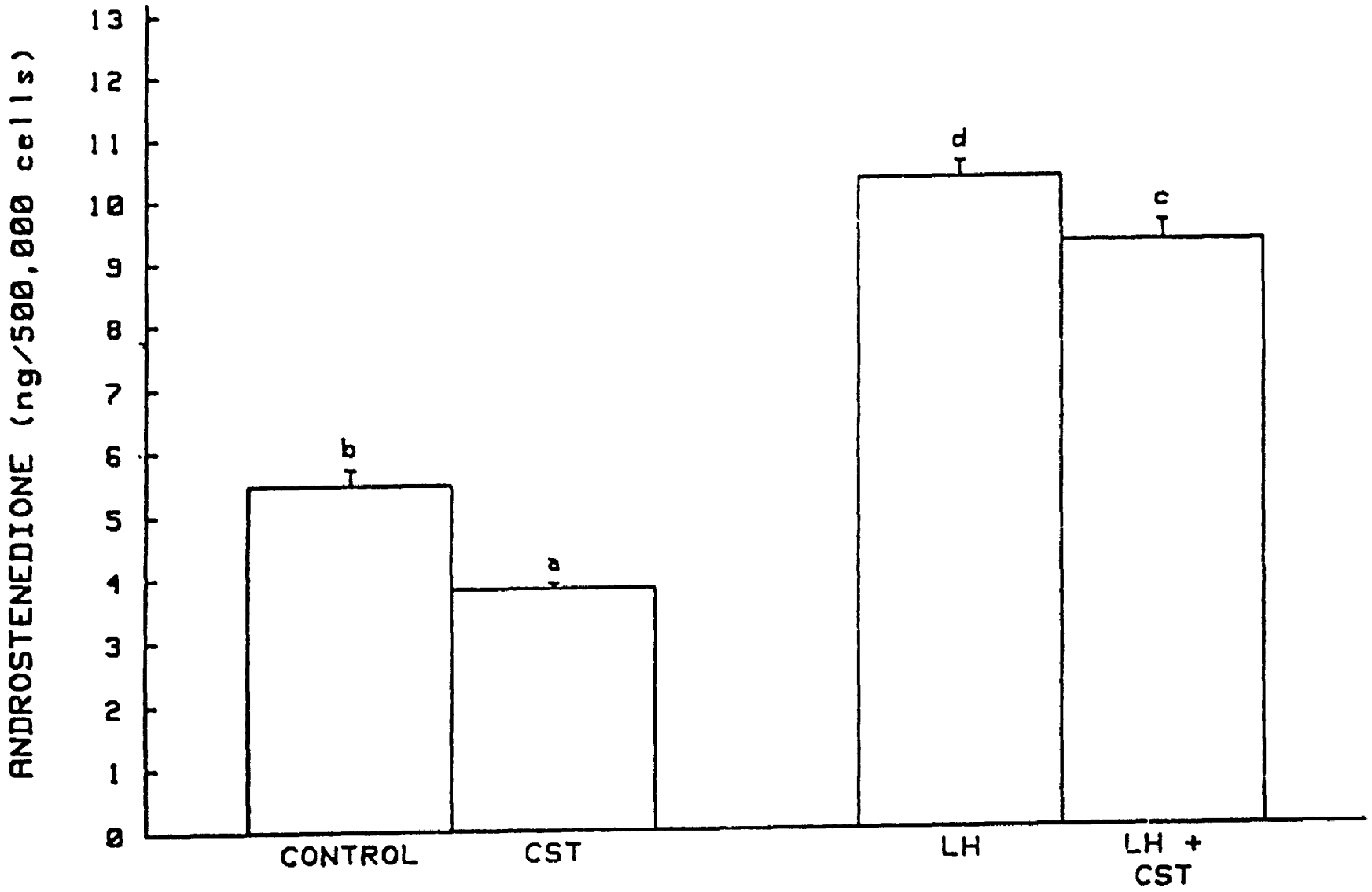
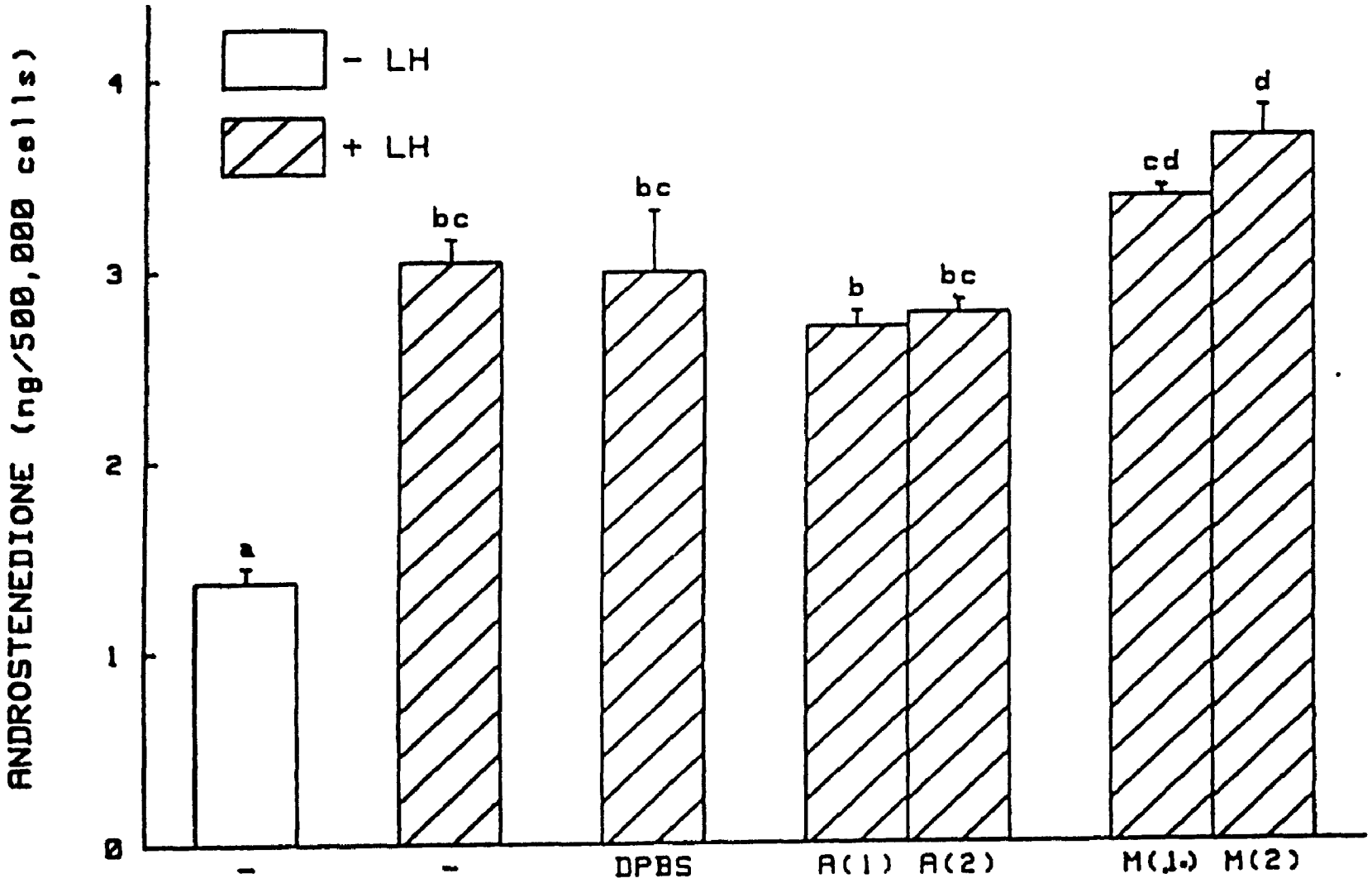

ANDROSTENEDIONE (ng/500,000 cells)
13
12
11
10
9
8
7
6
5
4
3
2
1
0
b
a
d
c
CONTROL
CST
LH
LH +
CST

Figure 15. Effects of adipose and muscle tissue extracts and DPBS on LH-stimulated theca cell androgen accumulation. LH-stimulated (250 ng/ml) theca cells were cultured in the presence of two separate extracts of adipose tissue [A(1) and A(2)] and muscle tissue [M(1) and M(2)] for 48 h. Values represent the mean ± S.E.M. of eight replicate cultures in a single experiment which was replicated two further times. Values with different superscripts are significantly different ($p < 0.05$). Androstenedione accumulation by LH-stimulated theca cells cultured in the presence of DPBS or extracts of adipose tissue did not differ significantly from that of cultures stimulated with LH alone. One of the two muscle extracts had no significant effect and the other caused a small but significant ($p < 0.05$) increase in LH-stimulated androstenedione accumulation.

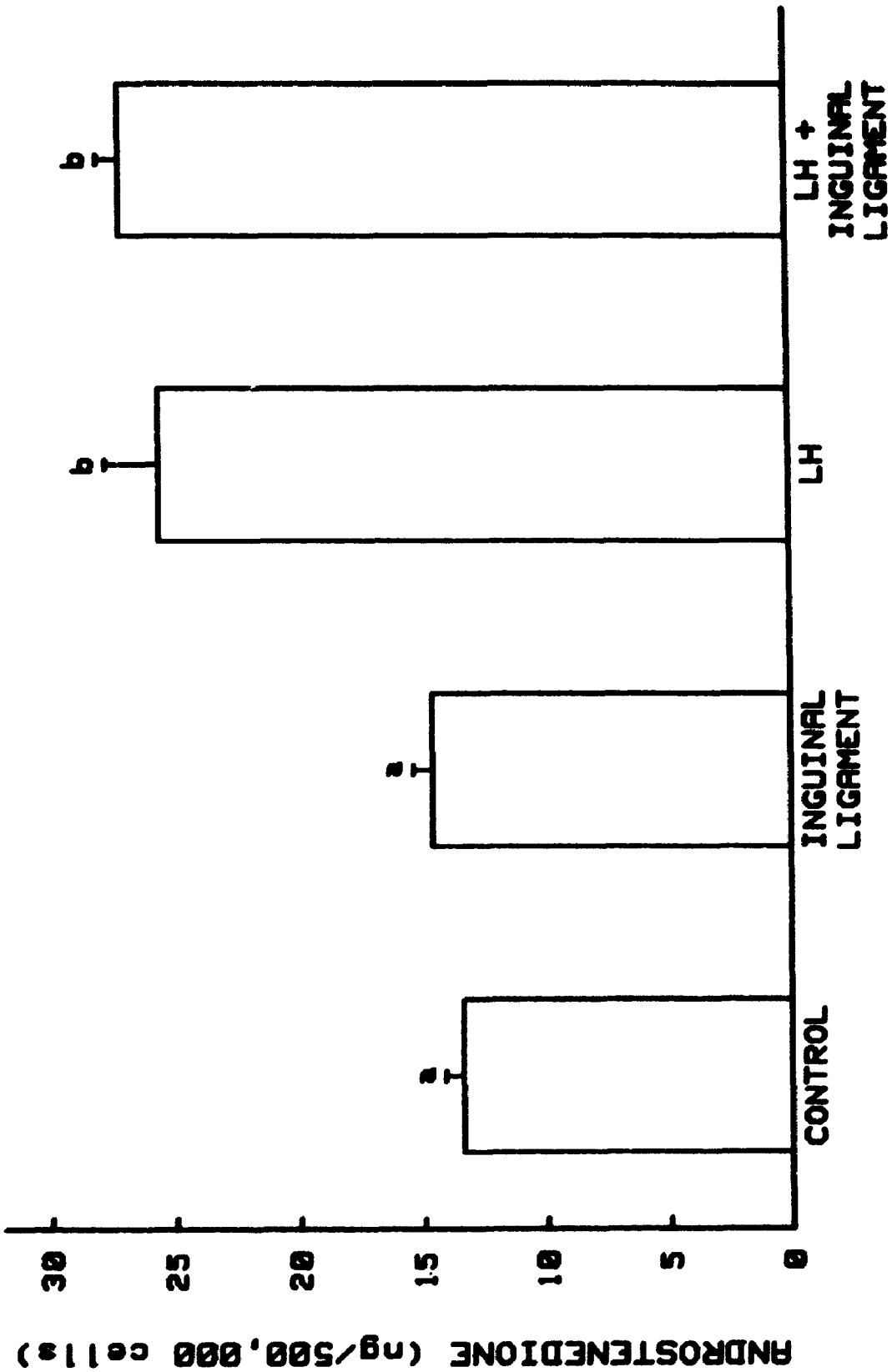

ANDROSTENEDIONE (ng/500,000 cells)
- LH
+ LH
0
1
2
3
4
a
bc
bc
b
bc
cd
d
-
-
DPBS
A(1)
A(2)
M(1)
M(2)

from the lower abdomen of adult female rats and experiments were done to test whether extracts of another ligament could cause the inhibitory effects of the SON extract on thecal androstenedione accumulation. Theca cells were cultured under control and LH-stimulated (250 ng/ml) conditions, with similar concentrations of the extract of inguinal ligaments prepared in an identical manner to the superior ovarian nerve extracts. Figure 16 shows no significant ($p > 0.05$) effect of the addition of inguinal ligament extracts on basal or LH-stimulated androstenedione accumulation.

The connective tissue and ligamentous tissues cannot be stripped away from the nerve fibers in the suspensory ligament. Therefore an experiment was done to determine if a procedure which caused degeneration of the SON fibers in the suspensory ligament, thus leaving only the ligamentous and connective tissues intact, would cause a loss of the SON extracts inhibitory activity on thecal androgen accumulation.

To cause the degeneration of the superior ovarian nerve fibers, silk sutures were tightly tied unilaterally around the left suspensory ligaments of adult female rats under Avertin anaesthesia. The right suspensory ligaments were left intact and used as controls. After 21 days, the ligated and control SON's were excised and stored at -20°C until extracted. The ligated SON's were excised distal to the ligature. Ligated and control superior ovarian nerve extracts were prepared in a similar manner and at the same concentration as previously described for SON extracts and added to LH-stimulated (250 ng/ml) porcine theca cell cultures. The control right intact SON extract caused a significant ($p < 0.05$) inhibition of thecal androstenedione accumulation while androstenedione accumulation in cultures containing the extract of the

Figure 16. Effect of inguinal ligament extracts on porcine theca cell androstenedione accumulation. Porcine theca cells were cultured in the absence (CONTROL) and presence of LH (LH; 250 ng/ml) with or without extracts of inguinal ligaments for 48 h. Values represent the mean $\pm$ S.E.M. of quadruplicate cultures from a single experiment which was replicated one further time. Values with different superscripts are significantly different ($p < 0.05$). Inguinal ligament extracts did not significantly ($p > 0.05$) affect basal or LH-stimulated theca cell androstenedione accumulation.

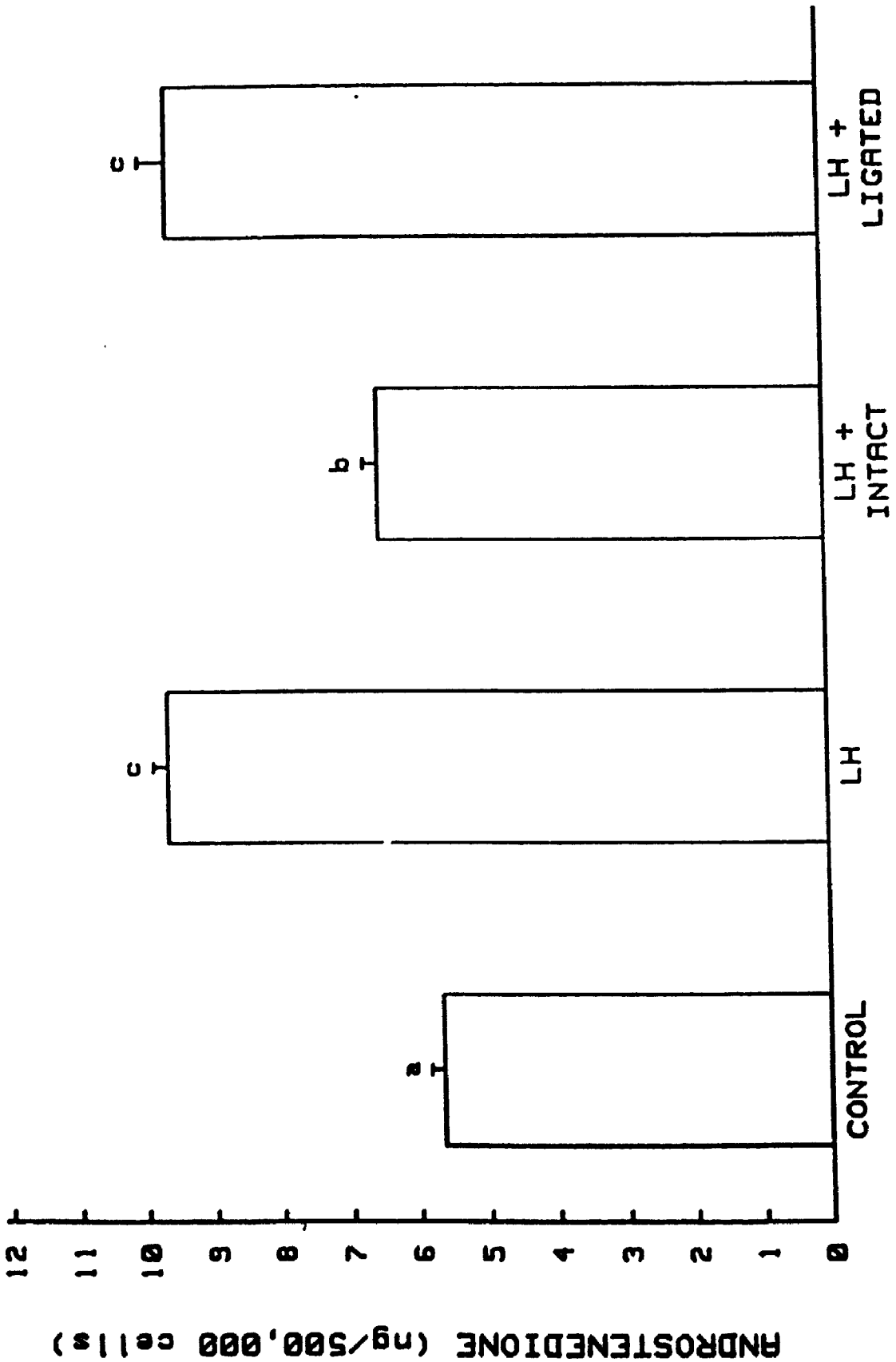

ANDROSTENEDIONE (ng/500,000 cells)
0
5
10
15
20
25
30
a
a
b
b
CONTROL
INGUINAL LIGAMENT
LH
LH + INGUINAL LIGAMENT

left ligated SON's was not significantly ($p > 0.05$) different from cultures stimulated with LH alone (Figure 17). Ovarian noradrenaline concentrations were measured by HPLC, as an indication of degeneration of the SON fibers. The noradrenaline concentration of the control right ovary (226 ± 24 ng/g tissue; n=6), with the intact SON was significantly ($p < 0.05$) greater than in the left ovary (156 ± 40 ng/g tissue) with the ligated SON. This observation suggests that ligation of the SON for 21 days causes a degeneration of the SON fibers, resulting in a decreased ovarian noradrenaline concentration.

7.2.4 Species Effects

In all experiments, the actions of rat SON extracts on androgen accumulation by porcine theca cell cultures have been studied. Concerns of possible species effects were addressed by culturing rat theca cells with rat SON extracts. Rat theca cells were obtained from immature (28 day old) rats treated for 48 h with 6IU PMSG (Equinex; Ayerst Laboratories Inc.) to induce follicular development, as described by Fortune and Armstrong (1977). The animals were killed and the largest ovarian follicles (0.9-1.1 mm in diameter) were isolated and trimmed of interstitial tissue. The follicles were cut in half and the inside of the follicles were scraped with a glass needle to clean the theca of granulosa cells. Scraped pieces of theca (two follicles per culture well) were cultured in 1 ml of DMEM (with the same antibiotic supplements as described previously in Section 4.1) in each well of 24-well Falcon tissue culture plates. Due to variability in follicle size it was necessary to normalize the androstenedione accumulation to the protein content of the rat theca cell cultures. Following culture, the medium was collected and

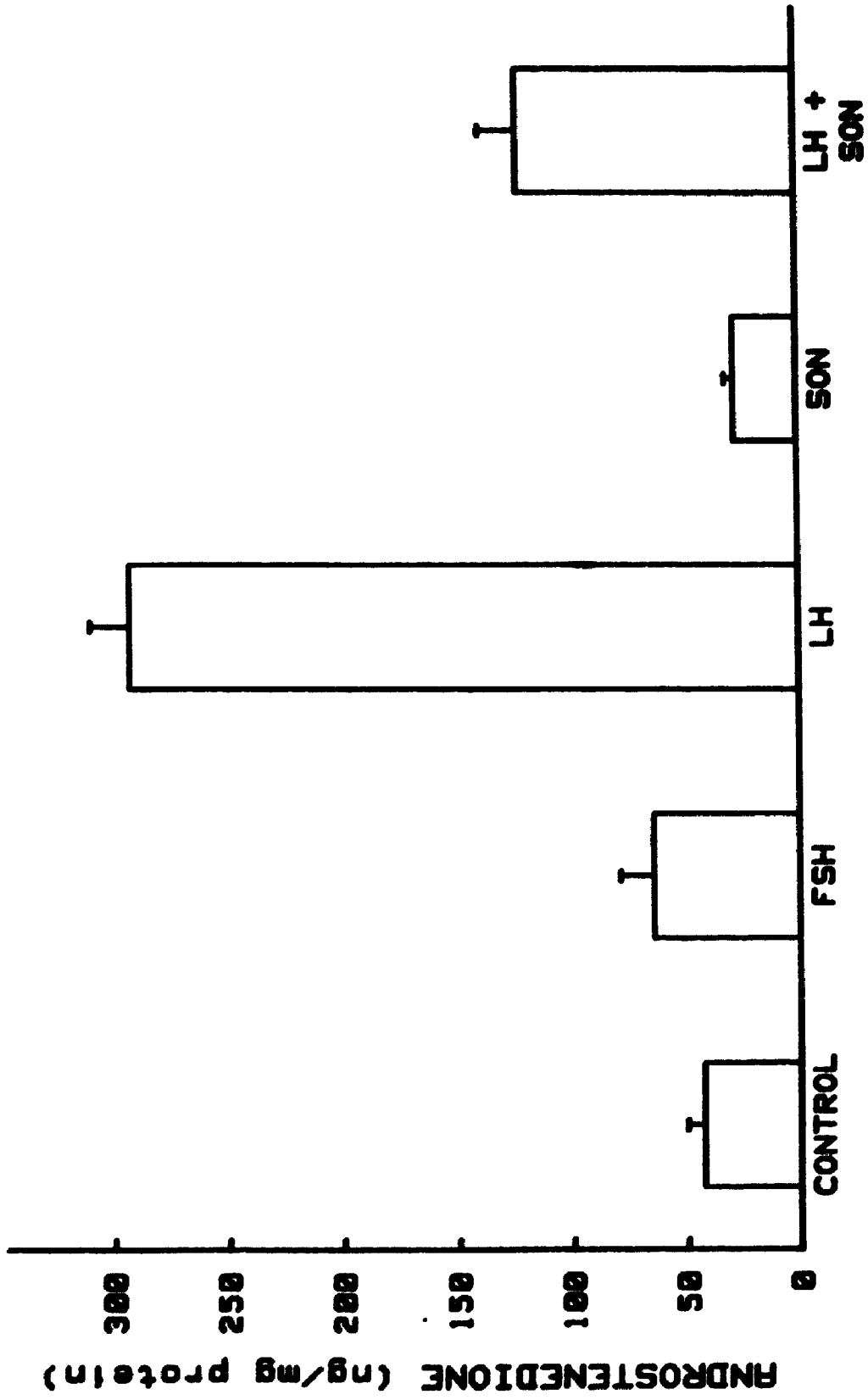
Figure 17. Effect of ligamentous and connective tissue extracts on porcine thecal androstenedione accumulation. LH-stimulated (250 ng/ml) theca cells were cultured in the presence of SON extracts prepared from intact suspensory ligaments or from ligated suspensory ligaments for 48 h. Values represent the mean ± S.E.M. of quadruplicate cultures from a single experiment which was replicated two further times. Values with different superscripts are significantly ($p < 0.05$) different. SON extracts prepared from intact suspensory ligaments inhibited androstenedione accumulation. SON extracts from suspensory ligaments in which the nerve fibers had degenerated following ligation, leaving only the connective tissue and ligamentous tissues of the suspensory ligament, had no significant inhibitory effect on LH-stimulated androstenedione accumulation.


ANDROSTENEDIONE (ng/500,000 cells)
0
1
2
3
4
5
6
7
8
9
10
11
12
a
c
b
c
CONTROL
LH
LH + INTACT
LH + LIGATED

the theca fragments were removed from the medium by centrifugation at 1000g for 10 min. The protein content was measured by the method of Lowry et al. (1951). To study the effect of rat SON extracts on rat theca cell androstenedione accumulation, rat theca cells were cultured in the absence (Control) and presence of LH (250 ng/ml) with or without rat SON extracts for 48 h. Rat theca cells responded to LH with significantly ($p < 0.05$) increased androstenedione accumulation, but were unresponsive to FSH (500 ng/ml) (Figure 18). Addition of rat SON extracts to rat thecal cell cultures significantly ($p < 0.05$) inhibited both basal and LH-stimulated androstenedione accumulation as it did in the porcine theca cell culture system.

These observations were independently confirmed in a collaborative study with Dr. Dennis Magoffin of the University of Southern California, San Diego at La Jolla, California, using the theca-interstitial cell culture model prepared from hypophysectomized immature rats (Magoffin and Erickson, 1982). Theca-interstitial cells were obtained from hypophysectomized immature (21 day old) female Sprague-Dawley rats. Four days after hypophysectomy the animals were sacrificed and the ovaries were removed, cut into small pieces and incubated at 37°C for 90 min with collagenase-DNase solution (4 mg/ml collagenase, 10 μg/ml DNase and 10 mg/ml bovine serum albumin in Medium 199). The dispersed cells were collected and washed. The cells (20,000 cells/well) were incubated in 96-well tissue culture plates for 48 h in the presence of LH (100 ng/ml) and various concentrations of SON extract (0-100 μl/well). The medium was frozen at -20°C until assayed for androsterone by a specific radioimmunoassay (Magoffin and Erickson, 1982). Androsterone is the major androgen produced by this culture system. The addition of rat SON extracts

Figure 18. Effect of rat SON extracts on rat theca cell androstenedione accumulation. Rat theca cells were cultured in the absence (Control) or presence of FSH (500 ng/ml) or LH (250 ng/ml), with or without rat SON extracts for 48 h. Values represent the mean ± S.E.M. of quadruplicate cultures from a single experiment which was replicated one further time. Rat SON extracts inhibit both basal and LH-stimulated rat theca cell androstenedione accumulation.


ANDROSTENEDIONE (ng/mg protein)
0
50
100
150
200
250
300
CONTROL
FSH
LH
SON
LH + SON

to LH-stimulated (100 ng/ml) rat theca-interstitial cells caused a dose-dependent inhibition of androsterone accumulation (Figure 19) over a similar dose range that was effective in the porcine theca cell culture system.

7.2.5 Cross Reactivity of Nerve Extracts in the Estradiol Radioimmunoassay

While investigating the possible cross reactivity of the nerve extracts in the androstenedione radioimmunoassay, their cross reactivity was also tested in the estradiol radioimmunoassay. Nerve extracts (10-200 μl) were used to prepare inhibition curves, and assayed in the same assay with a standard curve for estradiol (5-640 pg). SON extracts produced an inhibition curve that showed displacement of the binding of standard estradiol, over a range of 0-150 μl SON extract, whereas SCN extracts produced no inhibition (Figure 20).

Aliquots of 100 μl of nerve extracts were then assayed for immunoreactive estradiol. SON extracts contained 522 $\pm$ 174 pg/ml (range 42-1980 pg/ml; n=10) immunoreactive estradiol, whereas SCN extracts contained only 15 $\pm$ 8 pg/ml (range non-detectable-37 pg/ml; n=5) immunoreactive estradiol which is close to the sensitivity of the assay. Extracts of adipose and muscle tissue, inguinal ligaments and cervical sympathetic trunk nerves all had undetectable levels of immunoreactive estradiol.

7.3 Discussion

This study has demonstrated that both basal and LH-stimulated androstenedione production by dispersed porcine theca cells are

Figure 19. Inhibition of rat theca-interstitial cell androsterone accumulation by rat SON extracts. LH-stimulated (100 ng/ml) rat theca-interstitial cells were cultured with or without increasing amounts of rat SON extract. Values represent the mean ± S.E.M. of quadruplicate cultures from a single experiment Where no error bar is shown, the S.E.M. is within the limit of the symbol. The SON extract caused a dose-dependent inhibition of LH-stimulated rat theca-interstitial cell androsterone accumulation.

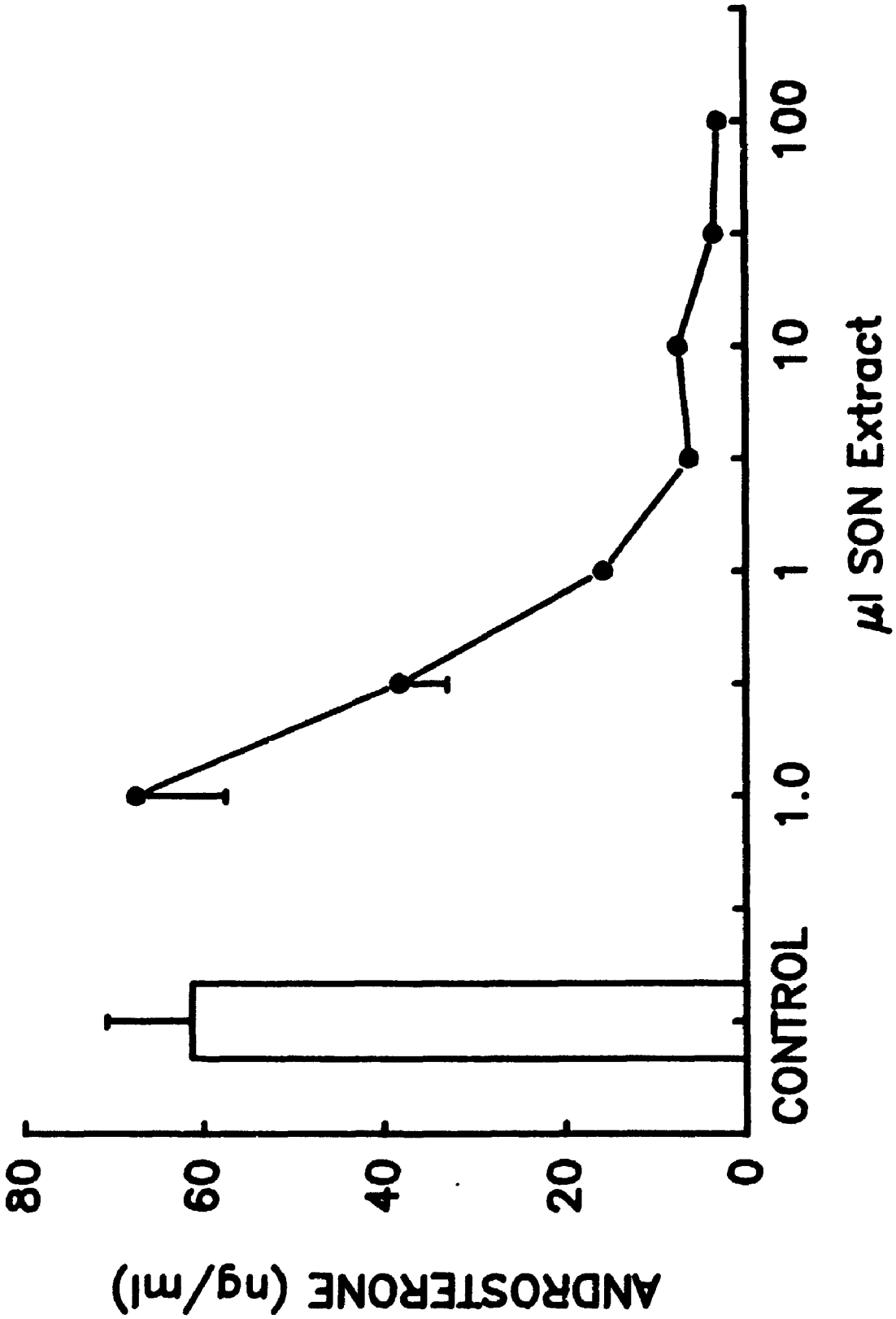
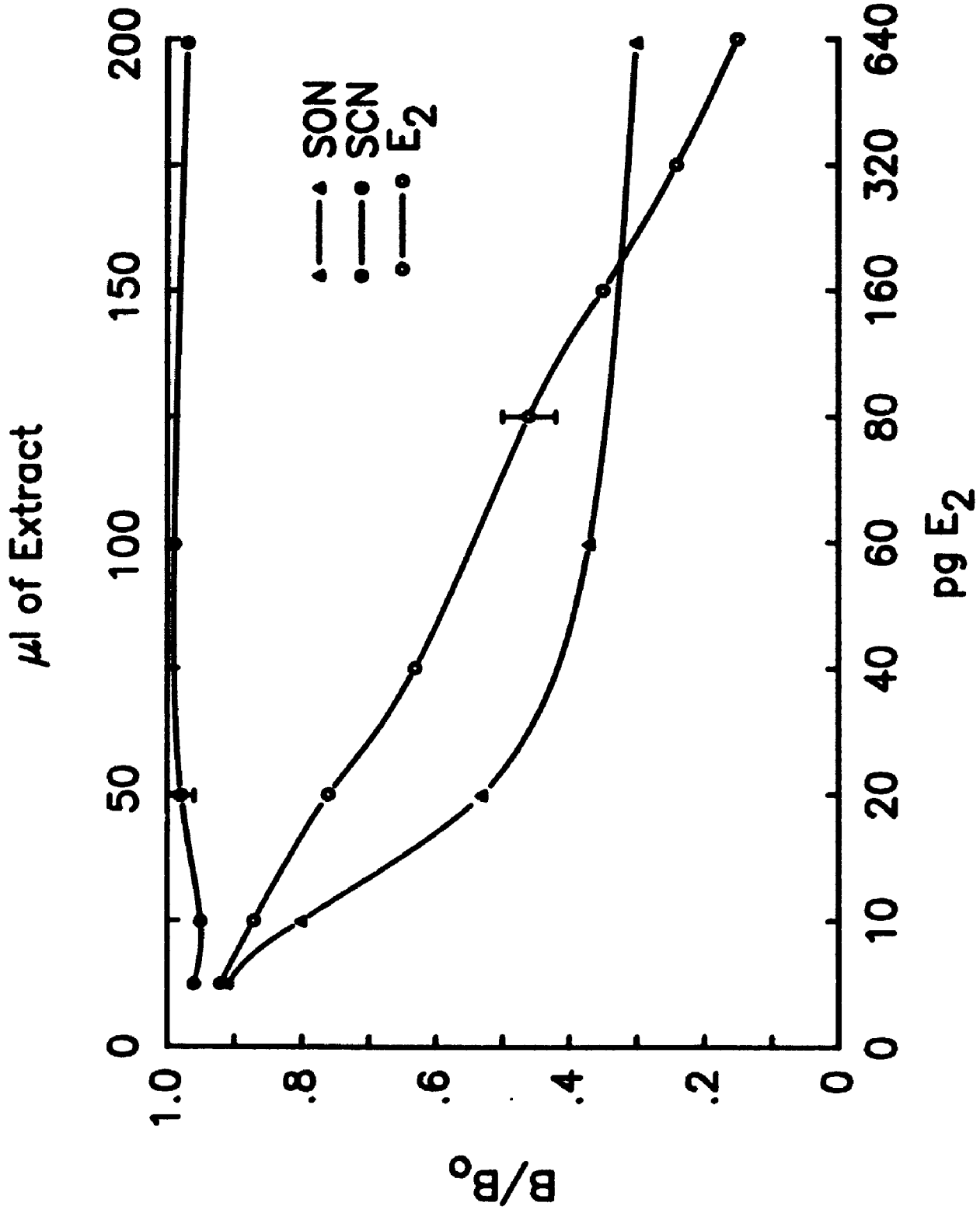

ANDROSTERONE (ng/ml)
80
60
40
20
0
CONTROL
1.0
1
10
100
µl SON Extract

Figure 20. Cross reactivity of superior ovarian and sciatic nerve extracts in the estradiol radioimmunoassay. Nerve extracts (10-200 μl) were used to prepare inhibition curves, and assayed in the same assay with the standard curve of estradiol for comparison. Data are the mean $\pm$ S.E.M. of triplicate determinations in a single experiment which was replicated one further time. Where no error bar is shown, the S.E.M. is within the limit of the symbol. The SCN extracts produced no inhibition, whereas the SON extracts produced a displacement of the binding of standard estradiol, over a range of 0-150 μl extract. Binding in the absence of added nerve extract (B_o) was 35%.


SON
SCN
E_2
µl of Extract
pg E_2
B/B_o
0
50
100
150
200
0
10
20
40
60
80
160
320
640
1.0
.8
.6
.4
.2
0

significantly decreased by extracts of rat SON and significantly increased by extracts of rat sciatic nerves. Preliminary experiments using cervical sympathetic trunk extracts indicate the presence of a factor, albeit less potent, which inhibits androgen accumulation, suggesting that the SON active factor(s) could be common to other neural and even non-neural tissues.

Sciatic nerves and cervical sympathetic trunk nerves were chosen as non-ovarian nerve controls because of their large size and ease of collection. It may be argued that other visceral nerves, such as splanchnic or renal nerves, would have provided a better control, but collecting sufficient quantities of these nerves for experiments would have been difficult. Sciatic and cervical sympathetic trunk nerves could be excised free of surrounding tissues so that these extracts were not contaminated by other tissues.

However, since the SON was isolated by excising the suspensory ligament which also contained smooth muscle, adipose cells and connective tissue, other control studies were necessary using extracts of adipose and muscle tissues. In the control studies, adipose extracts as well as DPBS alone did not affect thecal androgen accumulation. The only observed effects of the muscle extracts were stimulatory, so the presence of these factors in the SON extract would cause us to underestimate the inhibitory effect of the SON extract.

The possibility that the active factor in the SON extract originates in the ligament, for example a product of extravascular white blood cells or connective tissue degradation products, has also been eliminated. An extract from another ligament, the inguinal ligament from the groin, which was assumed to have a similar ligamentous and connective tissue

composition as the suspensory ligament, but without a nerve associated with it, had no effect on thecal androgen production. The effect of the ligamentous and connective tissues in the suspensory ligament were tested directly using a procedure to cause degeneration of the nerve fibers in the suspensory ligament, while leaving the other tissues intact. Transection or ligation of a peripheral nerve causes a series of destructive changes in the distal stump called Wallerian degeneration (Ochs, 1982). Normally, the perikaryon produces molecules that are carried distally by axoplasmic flow and that support the axon (Forman, 1987). Once the axon is separated from the perikaryon by transection, crushing or ligation, the distal stump of the axon becomes swollen and irregular during the first day after transection and breaks up into fragments by the third to fifth day (Ochs, 1982). Impulse conduction ceases completely after 72 hours. In myelinated nerves the myelin sheath is broken into short segments during the first week of degeneration; during the second week chemical degradation of the myelin begins until finally the myelin undergoes complete degradation. Therefore, a 21 day ligation of suspensory ligaments would be expected to leave only ligamentous and connective tissues intact. Evidence for degeneration of the nerve fibers in the SON was supported by the significantly ($p < 0.05$) decreased ovarian noradrenaline concentration in the ovary associated with the ligated suspensory ligament. Previous reports have shown that denervation of the rat ovary by sectioning the suspensory ligament or ovarian plexus nerve greatly reduced or virtually abolished both the catecholamine-staining nerve fibers and reduced the amount of chemically determined noradrenaline in the ovary (Lawrence and Burden, 1980). In previous experiments we have shown that ovarian catecholamine concentrations are reduced by sectioning

the plexus nerve alone (47% decrease), SON alone (67% decrease), or both the plexus nerve and SON (82% decrease). The presence of noradrenaline in the ovary following sectioning of both nerves suggests the ovary may have intrinsic cells capable of noradrenaline synthesis, or alternatively, the noradrenaline could be contained in the vascular system.

It is unlikely the effects of the nerve extracts are due to noradrenaline, since noradrenaline is rapidly decomposed and would not have survived the conditions to which the extracts were subjected. The observation that similarly prepared sciatic nerves did not inhibit sterodiogenesis, and in fact significantly stimulated androgen accumulation, suggests that the inhibitory effect of the SON extract was due to factor(s) present in either the nerve fibers of the SON or their surrounding glial cells, and not to non-specific actions of the nerve extracts.

The action of the nerve extract was not species-specific. Concerns regarding the testing of the effects of rat SON extracts on porcine thecal androgen production were addressed by demonstrating that the SON extract inhibited androgen production by two different preparations of rat theca cells.

The segments of nerves excised for the preparation of nerve extracts contain both afferent and efferent axons, but not nerve terminals where synaptic transmitters, such as noradrenaline, acetylcholine and peptides are stored. It is therefore possible that the nerve extracts contain molecules such as putative neurotransmitter peptides that are produced within neuronal cell bodies, and are moved by axoplasmic transport down axons to their terminals (Iversen, 1967), in this case in the ovary. Although the noradrenergic innervation of the ovary has been well

described, it is only recently that peptidergic nerves have been shown to be widely distributed throughout the autonomic nervous system. Immunohistochemcial studies have demonstrated nerve fibers containing substance P, VIP, neuropeptide Y, CCK-8, somatostatin and calcitonin gene-related peptide in the ovary of a variety of mammalian species (Dees et al., 1985; Papka et al., 1985; McNeill and Burden, 1986; Calka et al., 1988). Recently studies have shown that these peptides can act as neurotransmitters themselves or they can interact with the classical neurotransmitters to modify the target tissues responsiveness to nerve stimulation (for reviews see Hokfelt et al., 1980; Hokfelt et al., 1986). The coexistence of several synaptic messengers has been observed in many neuronal systems, and therefore the peptides may coexist with the classical transmitters in ovarian nerve terminals. Therefore tne effects of nerve homogenates have to be interpreted carefully, since there may be more than one active factor, and the actions of these factors may depend on their interactions with other factors.

To investigate the effect of SON extracts in vivo, osmotic minipumps could be used to deliver SON extracts under the ovarian bursa to both intact and denervated rat ovaries at a constant rate for a period of up to 7 days. Ovarian androgen content and ovarian venous androgen concentration could be determined to see if androgen production was effected in vivo by SON extracts. This study would have to assume that the inhibitory factor in the SON extract was stable in the minipumps for 7 days at body temperature, and also that the active factor was able to penetrate into the ovary.

The demonstration of a factor immunologically similar to estradiol in the SON extract is exciting, as estradiol is a well described inhibitor

of thecal androgen production. The estradiol immunoreactivity measured in the SON extract was lower than the doses reported to be effective for inhibiting thecal androgen production in vitro (Tsang et al., 1979a; Magoffin and Erickson, 1981; Magoffin and Erickson, 1982d; Hunter and Armstrong, 1987). These observations suggest three possibilities: 1) that estradiol is present in the SON extract but not in high enough concentration to inhibit thecal androgen production in vitro and therefore some other factor is responsible for the inhibitory effect of the SON extract on thecal androgen accumulation; 2) a molecule immunologically similar to estradiol, that cross reacts in the estradiol RIA, is present in the SON extract, and depending on the degree of cross reaction, this molecule could be present in much higher concentrations than estradiol; or 3) the factor may be immunologically similar to estradiol but be a much more potent inhibitor of thecal androstenedione production than estradiol.

These observations suggest the presence of sex steroids in the SON. There have been no reports of steroids in peripheral nerves. Estrogen is, however, present in specific regions of the human brain, the anterior and posterior pituitary, the retina and cranial nerves (Pfaff and Keiner, 1973; Hammond et al., 1983b; Lanthier and Patwardhan, 1986). In autoradiographic studies, neurons, but not glial cells, have been shown to contain putative estrogen receptors (Pfaff and Keiner, 1973; McEwen, 1978). These neurons are concentrated in the medial preoptic area, anterior and medial-basal hypothalamus, amygdala and to a lesser extent in the midbrain (Pfaff and Keiner, 1973). The cranial nerves from human cadavers have been shown by radioimmunoassay to contain progestins, androgens and estrogens (Lanthier and Patwardhan, 1986). The function of gonadal steroids in the nervous system is relatively unknown; however, it

has been suggested that estrogen plays a role in the integration of reproductive behaviours, gonadotropin release, the triggering of puberty and in the sexual differentiation of the developing brain (McEwen, 1980).

The isolated nerve factors are soluble; however the extraction procedure employed was not entirely effective. If the pellet remaining following homogenization and centrifugation was reextracted with DPBS, a significant amount of activity could be removed from both the SON and SCN pellets (data not shown). It will be necessary to develop a more efficient extraction procedure to increase the yield of active factor(s) prior to purification and quantitation studies.

Initial homogenization of the SON and SCN nerves were done in the presence and absence of ascorbic acid (Sigma; 1 μg/ml) and soybean trypsin inhibitor (Sigma; 0.1 mg/ml) to prevent oxidation and proteolytic degradation, respectively, of any active factor that may be present in the nerve homogenates. However, there were no differences in the activities of the extracts prepared with or without the inhibitors, therefore their use was discontinued (data not shown).

There is no way of proving that the contents of the nerve in the ligament accurately reflect the contents at the nerve terminals in the theca cells since the nerve terminals are embedded in the ovarian theca and stroma and are inaccesible. Therefore the assumption will be made that the substances extracted from the axons are the same as the substances in the nerve terminals in the theca cell layer.

The results of these studies indicate that the nerves to the ovary contain factor(s) capable of influencing theca cell androstenedione production. These neural factors may play an important regulatory role in controlling steroidogenesis <u>in vivo</u>, in addition to the well described

hormonal control mechanisms. The presence of immunoreactive estradiol in the SON extract is particularly interesting since estrogen is a well described inhibitor of ovarian androgen production; however, the concentrations of immunoreactive estradiol in the SON extracts were lower than the doses of estradiol previously reported (Tsang et al., 1979a; Magoffin and Erickson, 1981; Magoffin and Erickson, 1982d; Hunter and Armstrong, 1987) to be required to inhibit porcine thecal androstenedione accumulation in vitro. To extend the present observations the inhibitory effect of estrogens on thecal androgen accumulation will be examined in the following chapter.

CHAPTER 8-EFFECT OF ESTRADIOL-17β AND CATECHOLESTROGENS ON PORCINE THECA CELL ANDROSTENEDIONE ACCUMULATION

8.1 Introduction

Estradiol-17β (estradiol) has been shown to have an inhibitory action on ovarian androgen production both *in vivo* (Leung et al., 1978; Leung and Armstrong, 1979a) and *in vitro* (Tsang et al., 1979a; Magoffin and Erickson, 1982d; Hunter and Armstrong, 1987). The action of estradiol is mediated by a selective inhibition of the 17α-hydroxylase:C_{17-20} lyase enzyme complex, with no change in LH receptor binding capacity or LH-stimulated adenylate cyclase activity (Magoffin and Erickson, 1981).

Recent studies have shown the presence of the estrogen-2- and 4-hydroxylase enzymes, which convert estradiol to the catecholestrogens 2- and 4-hydroxyestradiol, in both porcine granulosa and theca cells (Hammond et al., 1986). Catecholestrogens and their methoxylated metabolites are also present in micromolar quantities in human (Dehennin et al., 1984) and equine (Silberzahn et al., 1985) follicular fluid. Therefore, the inhibitory actions of estradiol on thecal androstenedione accumulation could be due, at least in part, to catecholestrogen activity on theca cells. It has been suggested that some of the effects of estradiol in the hypothalamus, anterior pituitary (Ball and Knuppen, 1980; Fishman, 1983; MacLusky et al., 1983), blastocyst (Mondschien et al., 1985; Dey et al., 1986) and granulosa cells (Spicer and Hammond, 1987b) are mediated via conversion to catecholestrogens.

The direct effects of catecholestrogens on steroidogenesis by porcine theca cells have not been reported. The present studies were designed to investigate the effects and mechanism of action of catecholestrogens on

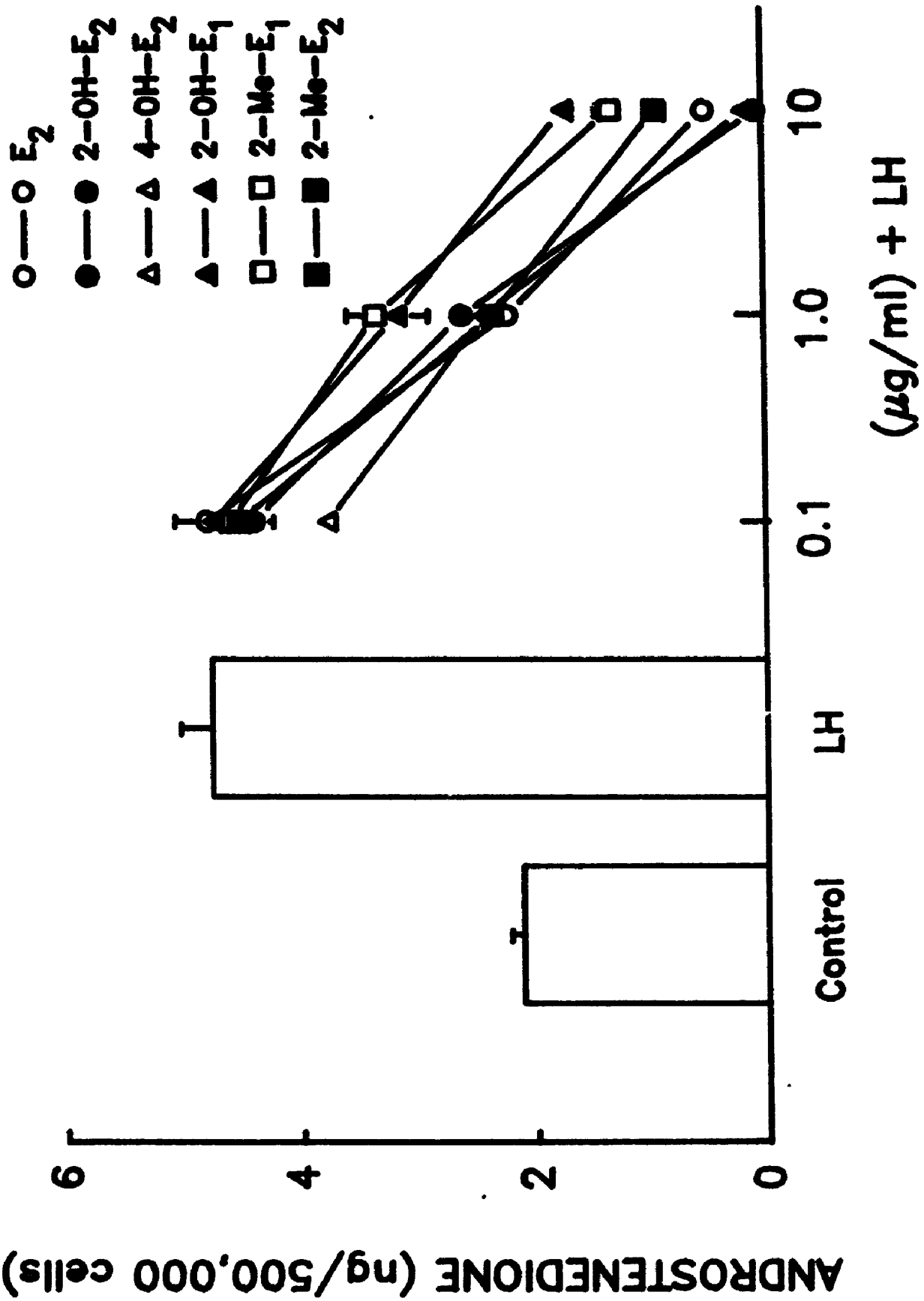
androgen production by porcine theca cells in vitro and to compare their actions to those of estradiol. The inhibitory effects of estradiol and the catecholestrogens will be compared to those of the SON extract, to see if they could be the inhibitory factor in the SON extract.

8.2 Methods and Results

8.2.1 Effect of Estradiol-17β and Catecholestrogens on Thecal Androstenedione Accumulation

To evaluate the effects of estradiol and catecholestrogens on basal and LH-stimulated theca cell androstenedione accumulation, theca cells were cultured in the absence or presence of LH (250 ng/ml), with or without increasing concentrations (0.1, 1.0 and 10 μg/ml) of estradiol (E_2; Steraloids, Wilton NH) or the catecholestrogens 2-hydroxyestradiol (2-OH-E_2; Steraloids), 4-hydroxyestradiol (4-OH-E_2; Steraloids), 2-hydroxyestrone (2-OH-E_1; Steraloids), or their methoxylated metabolites, 2-methoxyestrone (2-Me-E_1; Organon Inc., W. Orange, NJ) or 2-methoxyestradiol (2-Me-E_2; Sigma). Prior to use, the purity of all catecholestrogens was checked by high performance liquid chromatography on a C_{18} μBondapak column (300x3.9 mm; Waters Associates, Canada) and detected at 280 nm. The column was eluted isocratically with 70% methanol/water, flow rate 1 ml/min. After 48 h of treatment of theca cells with LH, the accumulation of androstenedione in the medium was increased by 2.6-fold over control levels (Figure 21). Treatment with increasing concentrations of either estradiol or any of the catecholestrogens tested, resulted in a significant ($p < 0.05$) dose-dependent inhibition of LH-stimulated thecal androstenedione accumulation. Following 48 h of

Figure 21. Concentration-dependence of the effects of estradiol and catecholestrogens on theca cell androstenedione accumulation. Theca cells were incubated without (Control) or with 250 ng/ml LH (LH) in the absence or presence of increasing concentrations (0.1-10 μg/ml) of E_2, 2-OH-E_2, 4-OH-E_2, 2-OH-E_1, 2-Me-E_1 or 2-Me-$E_{2\text{ for }48\text{ h}}$. Values represent the mean $\pm$ S.E.M. of quadruplicate cultures from a single experiment which was replicated one further time. Where no error bar is shown the S.E.M. is within the limit of the symbol. Estradiol and all of the catecholestrogens tested caused a significant ($p < 0.05$) inhibition of androstenedione accumulation.

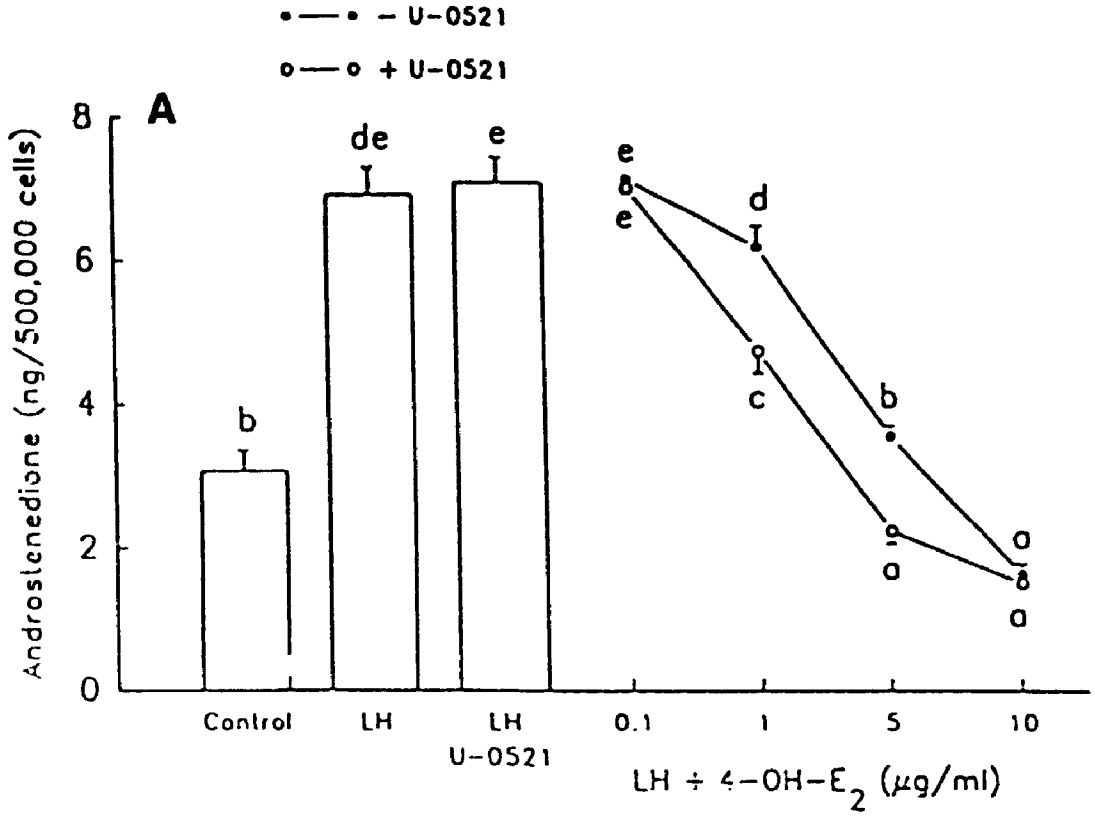

ANDROSTENEDIONE (ng/500,000 cells)
0
2
4
6
Control
LH
0.1
1.0
10
(μg/ml) + LH
O—O E2
●—● 2-OH-E2
Δ—Δ 4-OH-E2
▲—▲ 2-OH-E1
□—□ 2-Me-E1
■—■ 2-Me-E2

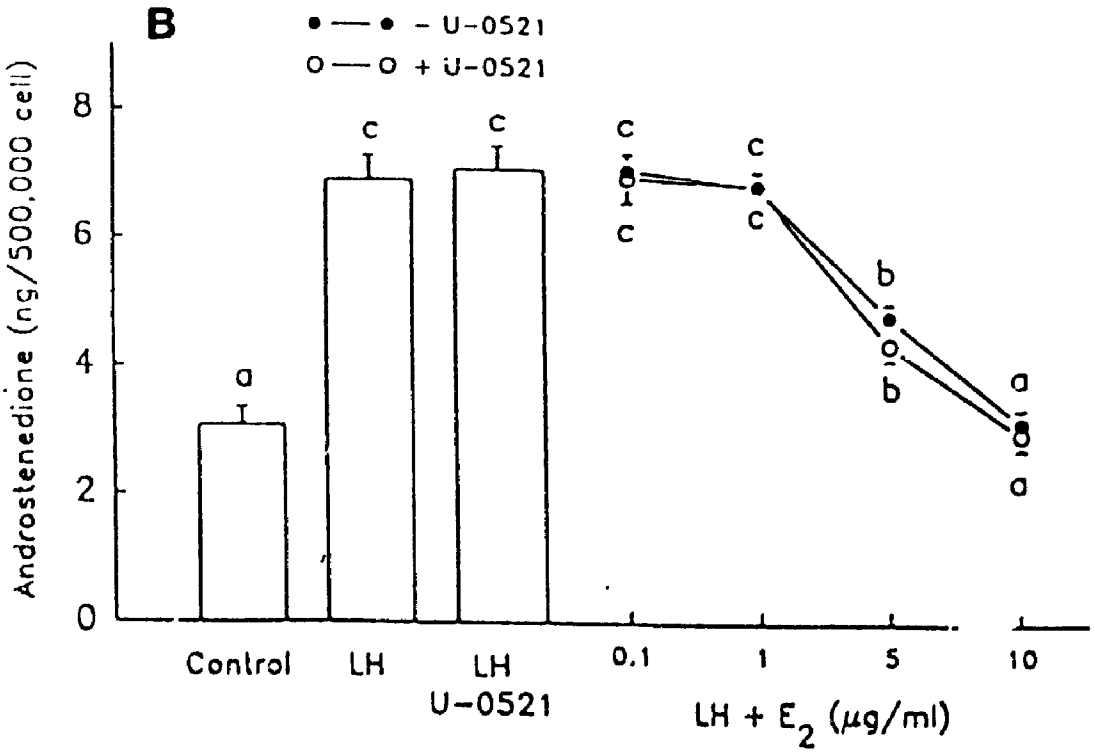
culture in the presence of LH and the maximal concentration of each agent tested (ie. 10 μg/ml) androstenedione accumulation was decreased by 89, 99, 97 64, 72 and 80% for E_2, 2-OH-E_2, 4-OH-E_2, 2-OH-E_1, 2-Me-E_1 and 2-Me-E_2, respectively.

The catechol-o-methyl transferase inhibitor U-0521 has been shown to protect catecholestrogens from enzymatic breakdown (Spicer et al., 1987). To determine if the addition of U-0521 enhanced the inhibitory effect of 4-OH-E_2 on theca cell androstenedione accumulation, theca cells were cultured in the absence or presence of LH (250 ng/ml) and increasing concentrations of 4-OH-E_2 (0-10 μg/ml) with or without U-0521 (3.6 μg/ml; 10 μM). The addition of U-0521 alone did not significantly affect thecal androstenedione accumulation (Figure 22A). Theca cell androstenedione accumulation was significantly ($p < 0.05$) inhibited by 4-OH-E_2 at concentrations greater than 1 μg/ml. In the presence of U-0521 the inhibitory effect of 1 and 5 μg/ml 4-OH-E_2 was significantly ($p < 0.05$) enhanced. At the maximal concentration of 4-OH-E_2 tested (10 μg/ml) there was no significant effect of the addition of U-0521. In addition, a dose of 4-OH-E_2 (1 μg/ml) which alone was not capable of inhibiting androstenedione accumulation was rendered effective by the addition of U-0521. The inhibition of androstenedione accumulation by E_2 was not significantly affected by concurrent treatment with U-0521 (Figure 22B). The addition of the antioxidant ascorbic acid (1 μg/ml) to the culture medium had no significant effect on the inhibition of thecal androgen accumulation by catecholestrogens or estradiol (data not shown).

To compare the time course of estradiol and catecholestrogen inhibition of thecal androstenedione accumulation, theca cells were cultured in the absence or presence of LH (250 ng/ml), with or without an inhibitory dose

Figure 22. Effect of the catechol-o-methyl transferase inhibitor U-0521 on the inhibition of thecal androstenedione accumulation by 4-OH-E_2 (A) and E_2 (B). Theca cells were incubated without (Control) or with 250 ng/ml LH (LH) with increasing concentrations of 4-OH-E_2 or E_2 (0-10 μg/ml), with or without U-0521 (10 μM) for 48 h. Values represent the mean $\pm$ S.E.M. of quadruplicate cultures in single experiment which was replicated one further time. Where no error bar is shown the S.E.M. is within the limit of the symbol. Values with different superscripts are significantly different ($p < 0.05$). U-0521 enhanced the inhibitory effect of 4-OH-E_2, but did not alter the effect of E_2, on thecal androstenedione accumulation


• — • – U-0521
o — o + U-0521
A
Androstenedione (ng/500,000 cells)
8
6
4
2
0
b
de
e
e
d
c
b
a
a
a
Control
LH
LH
U-0521
0.1
1
5
10
LH + 4-OH-E2 (μg/ml)


B
• — • – U-0521
o — o + U-0521
Androstenedione (ng/500,000 cell)
8
6
4
2
0
a
c
c
c
c
c
b
b
a
a
Control
LH
LH
U-0521
0.1
1
5
10
LH + E2 (μg/ml)

of E_2 (1[illegible] μg/ml) or 4-OH-E_2 (10 μg/ml) for different incubation times (2-48 h). Time dependent increments in androstenedione accumulation were observed in both basal and LH-stimulated cultures in the absence of E_2 or 4-OH-E_2 (Figure 23). Treatment of basal or LH-stimulated cultures with either E_2 or 4-OH-E_2 caused a significant ($p < 0.05$) inhibition of androstenedione accumulation by 2 h and at all subsequent times studied.

8.2.2 Site of the Inhibition of Thecal Androstenedione Accumulation by Estradiol and Catecholestrogens

The following studies were designed to investigate the site of the inhibitory action of catecholestrogens on theca cell androstenedione accumulation, and to compare their site with that of estradiol.

To determine if the estradiol or catecholestrogen inhibition of thecal androstenedione accumulation involved an inhibition of the generation of cyclic AMP, theca cells were incubated under control and LH-stimulated (250 ng/ml) conditions, in the presence or absence of E_2 (10 μg/ml) or 4-OH-E_2 (10 μg/ml) and extracellular cyclic AMP accumulation was measured at the end of 48 h of culture. LH significantly ($p < 0.05$) stimulated cyclic AMP production over control levels (Table 13). Addition of E_2 (10 μg/ml) or 4-OH-E_2 (10 μg/ml) at concentrations which are inhibitory to androstenedione accumulation did not significantly affect basal or LH-stimulated extracellular cyclic AMP accumulation. Measurement of androstenedione accumulation by the same cultures showed that treatment with either E_2 or 4-OH-E_2 resulted in a significant ($p < 0.05$) inhibition of both basal and LH-stimulated androstenedione accumulation.

The ability of estradiol and catecholestrogens to inhibit androgen accumulation stimulated by other physiological and pharmacological

Figure 23. Time course of 4-OH-E_2 and E_2 inhibition of thecal androstenedione accumulation. Theca cells were cultured in the absence (Control) or presence of LH (250 ng/ml), with or without an inhibitory dose of E_2 (10 μg/ml) or 4-OH-E_2 (10 μg/ml). The media were collected at varying times (2-48 h) after plating. Values represent the mean $\pm$ S.E.M. of quadruplicate cultures from a single experiment. Where no error bar is shown the S.E.M. is within the limit of the symbol. Both E_2 and 4-OH-E_2 significantly inhibited androstenedione production under basal and LH-stimulated conditions by 2 h and at all subsequent time points studied.

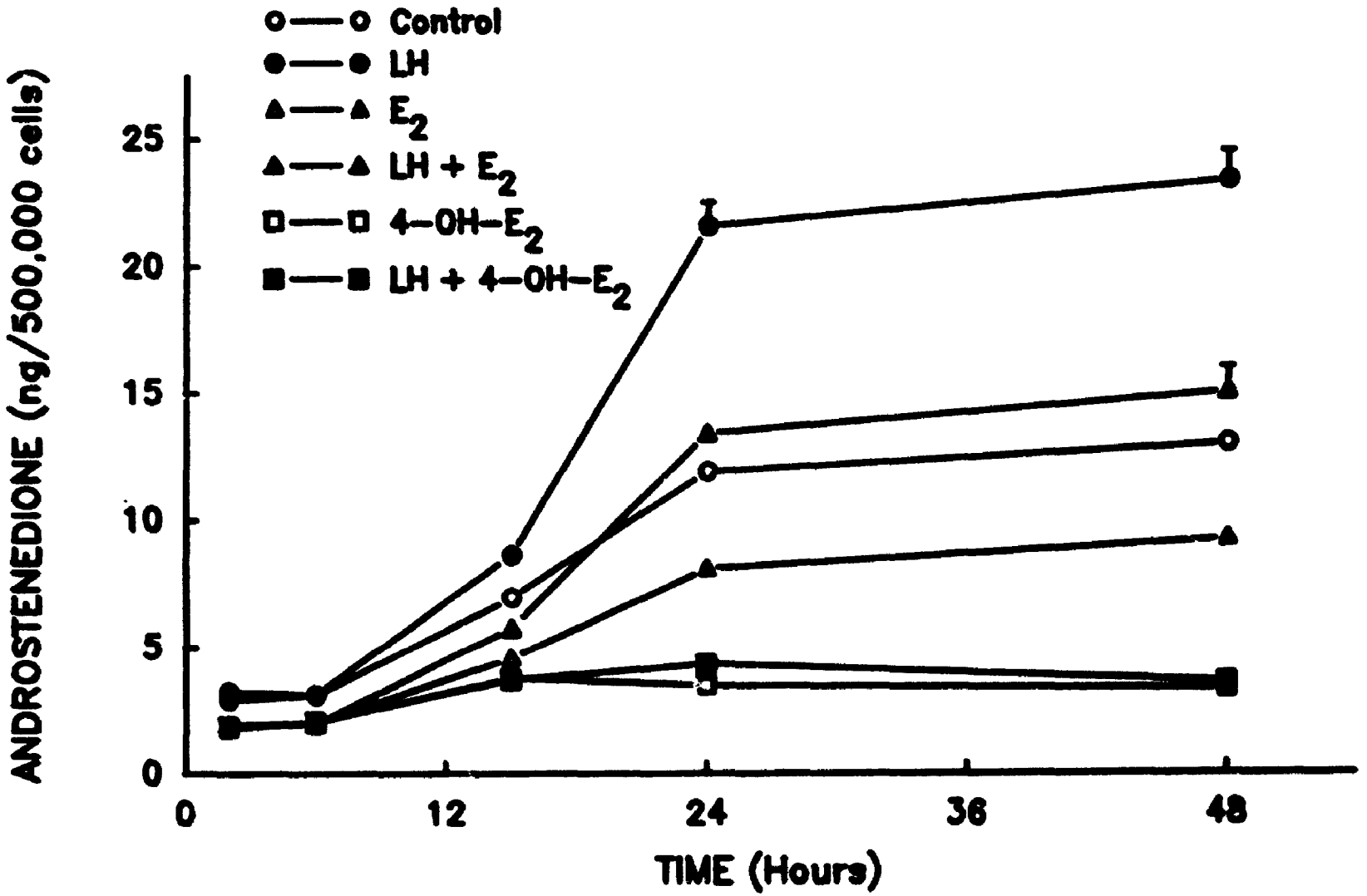

Control
LH
E2
LH + E2
4-OH-E2
LH + 4-OH-E2
ANDROSTENEDIONE (ng/500,000 cells)
0
5
10
15
20
25
0
12
24
36
48
TIME (Hours)

Table 13. Effect of E_2 and 4-OH-E_2 on basal and LH-stimulated extracellular cyclic AMP and androstenedione accumulation by porcine theca cells.

Treatment[1]	Cyclic AMP (pmol/500,000 cells)	Androstenedione (ng/500,000 cells)
Control	2.58 ± 0.17[a]	11.12 ± 0.32[b]
E_2	2.65 ± 0.24[a]	7.27 ± 0.11[a]
4-OH-E_2	2.70 ± 0.07[a]	7.11 ± 0.26[a]
LH	8.71 ± 0.12[b]	20.65 ± 0.81[d]
LH + E_2	8.90 ± 0.28[b]	13.01 ± 0.45[c]
LH + 4-OH-E_2	8.76 ± 0.40[b]	8.45 ± 0.35[a]

[1]Theca cells were incubated in the absence (Control) or presence (LH) of LH (250 ng/ml) with or without E_2 (10 μg/ml) or 4-OH-E_2 (10 μg/ml) for 48 h. Values represent the mean ± S.E.M. of quadruplicate cultures from a single experiment which was replicated one further time. Values with different superscripts are significantly different ($p < 0.05$). Both E_2 and 4-OH-E_2 inhibited thecal androstenedione accumulation without affecting the accumulation of extracellular cyclic AMP.

activators of adenylate cyclase, as well as dibutyryl cyclic AMP, was also examined. Theca cells were cultured in the presence of LH (250 ng/ml), dibutyryl cyclic AMP (0.5 mg/ml; 1 mM) or PGE_2 (10 μg/ml) in the presence and absence of an inhibitory dose of E_2 (10 μg/ml) or 4-OH-E_2 (10 μg/ml). Dibutyryl cyclic AMP-stimulated cultures also contained the phosphodiesterase inhibitor IBMX (89 μg/ml; 400 μM). All three agents caused marked increases in androstenedione accumulation over control levels (Figure 24). 4-OH-E_2 significantly ($p < 0.05$) inhibited LH-, dibutyryl cyclic AMP- and PGE_2-stimulated androstenedione accumulation by 65, 64 and 59%, respectively. E_2 significantly ($p < 0.05$) inhibited the stimulatory effect of LH, dibutyryl cyclic AMP and PGE_2 on thecal androstenedione accumulation by 32, 35 and 46%, respectively. In all experiments in which both estradiol and 4-OH-E_2 were tested at 10 μg/ml, the percent inhibition of 4-OH-E_2 (Control 55.0 ± 7.9%; n=6; LH 72.7 ± 3.7%; n=13) was significantly ($p < 0.05$) greater than that by estradiol (Control 45.5 ± 6.1%; n=6; LH 58.2 ± 5.9%; n=13).

To investigate the site(s) in the steroidogenic pathway at which catecholestrogens and estradiol exert their inhibitory effects, theca cells were cultured in the absence or presence of LH (250 ng/ml), with or without an inhibitory concentration of E_2 (10 μg/ml) or 4-OH-E_2 (10 μg/ml), and the steroids pregnenolone, progesterone, 17α-hydroxyprogesterone and androstenedione were measured at the end of 48 h of culture. As shown in Table 14, treatment of cultured porcine theca cells with E_2 or 4-OH-E_2 significantly ($p < 0.05$) decreased both basal and LH-stimulated 17α-hydroxyprogesterone and androstenedione accumulation, while significantly ($p < 0.05$) increasing pregnenolone production. Pregnenolone accumulation in cultures treated with E_2 were significantly ($p < 0.05$)

Figure 24. Effect of E_2 and 4-OH-E_2 on LH-, dibutyryl cyclic AMP- and PGE_2-stimulated androstenedione accumulation by porcine theca cells. Theca cells were cultured in the absence (CON) or presence of 250 ng/ml LH, dibutyryl cyclic AMP (1 mM) and MIX (400 μM) or PGE_2 (10 μg/ml), with or without E_2 (10 μg/ml) or 4-OH-E_2 (10 μg/ml) for 48 h. Values represent the mean $\pm$ S.E.M. of quadruplicate cultures from a single experiment which was replicated one further time. Values with different superscripts are significantly ($p < 0.05$) different. Both E_2 and 4-OH-E_2 caused a significant inhibition of androstenedione accumulation stimulated by all three agents.

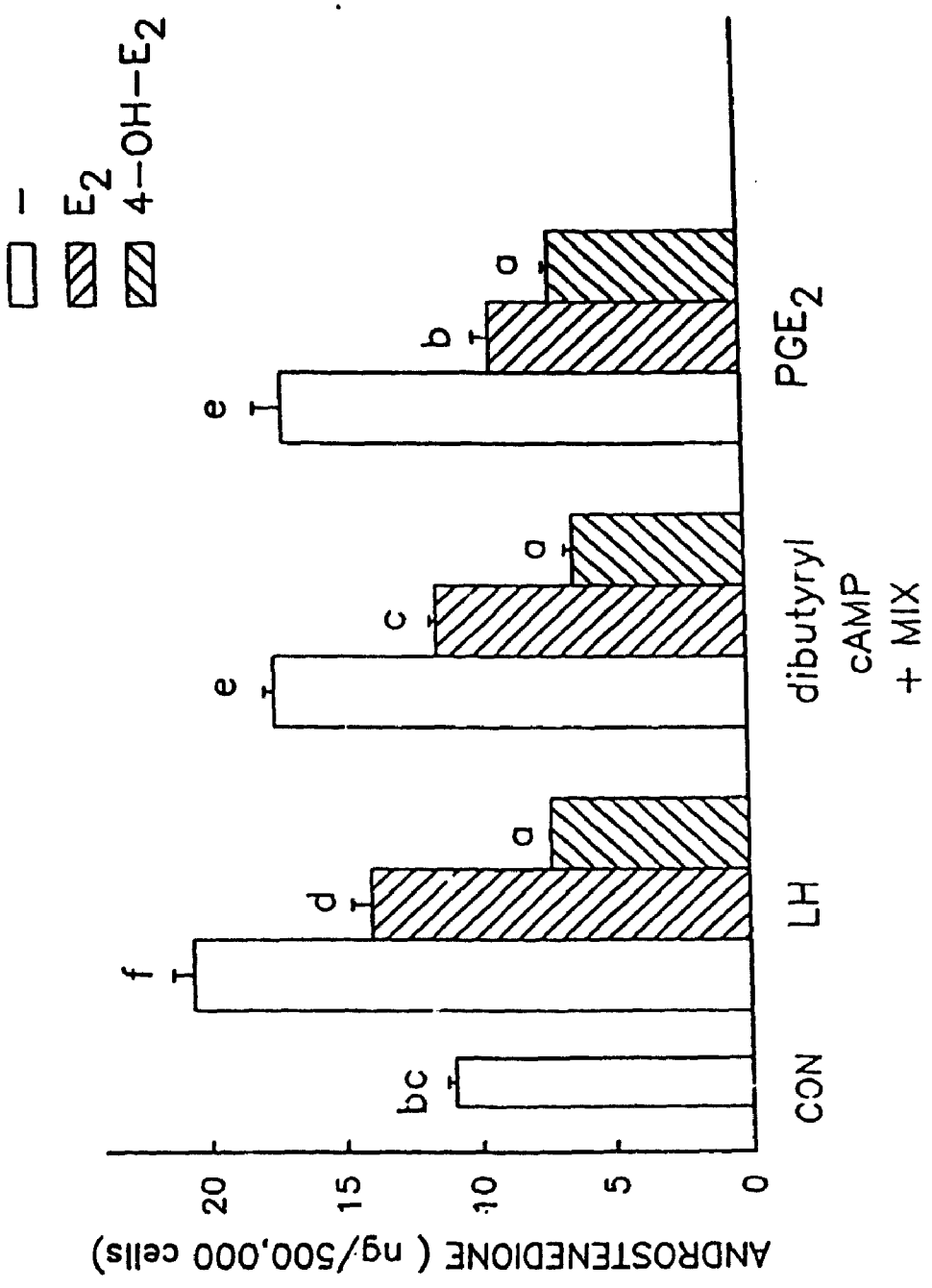

ANDROSTENEDIONE (ng/500,000 cells)
0
5
10
15
20
CON
LH
dibutyryl cAMP + MIX
PGE2
bc
f
d
a
e
c
a
e
b
a
–
E2
4–OH–E2

Table 14. Effect of E_2 and 4-OH-E_2 on the accumulation of pregnenolone, progesterone, 17α-hydroxyprogesterone and androstenedione by porcine theca cells.

	Steroid Accumulation (ng/500,000 cells)			
Treatment[1]	Preg	Prog	17α-OH-P_4	Adione
Control	0±0[a]	4.94±0.40[a]	3.21±0.08[c]	0.35±0.02[b]
E_2	8.92±2.70[c]	5.81±1.95[ab]	0.65±0.22[ab]	0.25±0.01[a]
4-OH-E_2	1.04±0.06[b]	5.99±1.00[ab]	0.39±0.03[a]	0.25±0.01[a]
LH	0.08±0.08[a]	6.63±0.95[ab]	6.00±0.39[d]	1.42±0.04[e]
LH + E_2	12.55±1.08[d]	7.10±1.14[ab]	0.96±0.14[b]	0.41±0.02[c]
LH + 4-OH-E_2	1.54±0.10[b]	8.01±1.16[b]	0.34±0.03[a]	0.47±0.02[d]

[1]Theca cells were cultured with (LH) or without (Control) LH (250 ng/ml) in the absence or presence of E_2 (10 μg/ml) or 4-OH-E_2 (10 μg/ml). At the end of 48 h of culture the media was removed for radioimmunoassay of pregnenolone (Preg), progesterone (Prog), 17α-hydroxyprogesterone (17α-OH-P_4) and androstenedione (Adione). Values represent the mean ± S.E.M. of quadruplicate cultures from a single experiment which was replicated three further times. Values with different superscripts are significantly different ($p < 0.05$). Treatment of theca cells with E_2 or 4-OH-E_2 significantly decreased 17α-hydroxyprogesterone and androstenedione accumulation, while significantly increasing pregnenolone accumulation.

greater than in 4-OH-E_2 treated cultures. Progesterone accumulation in the presence of estradiol or 4-OH-E_2 showed small but statistically insignificant increases over control cultures. The sum of the four steroids measured was significantly ($p < 0.05$) greater in the presence of estradiol than in the presence of 4-OH-E_2 (Control 15.63 vs. 7.67 ng/ml; LH 21.02 vs. 10.36 ng/ml).

The inhibitory effects of estradiol and catecholestrogens on thecal androstenedione accumulation were not due to decreases in theca cell number, since the DNA content measured after 48 h of culture in control and LH-stimulated cultures did not differ significantly in the absence or presence of estradiol (10 μg/ml) or 4-OH-E_2 (10 μg/ml) (Table 15).

8.2.3 Cross Reactivity of Catecholestrogens in the Estradiol Radioimmunoassay

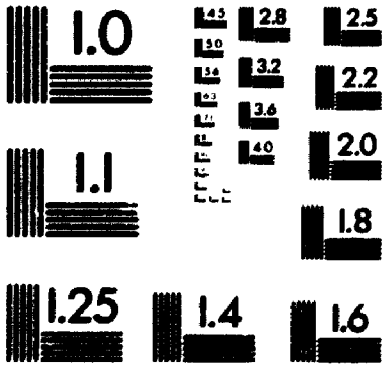
Studies were carried out to determine the degree of cross reactivity of the various catecholestrogens in the estradiol radioimmunoassay. Various concentrations (0-1 μg) of catecholestrogens were used to prepare inhibition curves, and run in the same assay with the standard curve of estradiol for comparison. Cross reactivity was expressed as the mass of standard estradiol which inhibited binding by 50% divided by the mass of the potential cross reacting steroid that inhibited binding by 50% expressed as a percentage. As shown in Figure 25, all of the catecholestrogens cross reacted in the estradiol assay to various degrees; 4-OH-E_2 12,857%, 2-OH-E_2 15%, 2-Me-E_2 4.5%, 2-OH-E_1 0.16% and 2-Me-E_1 0.02%.

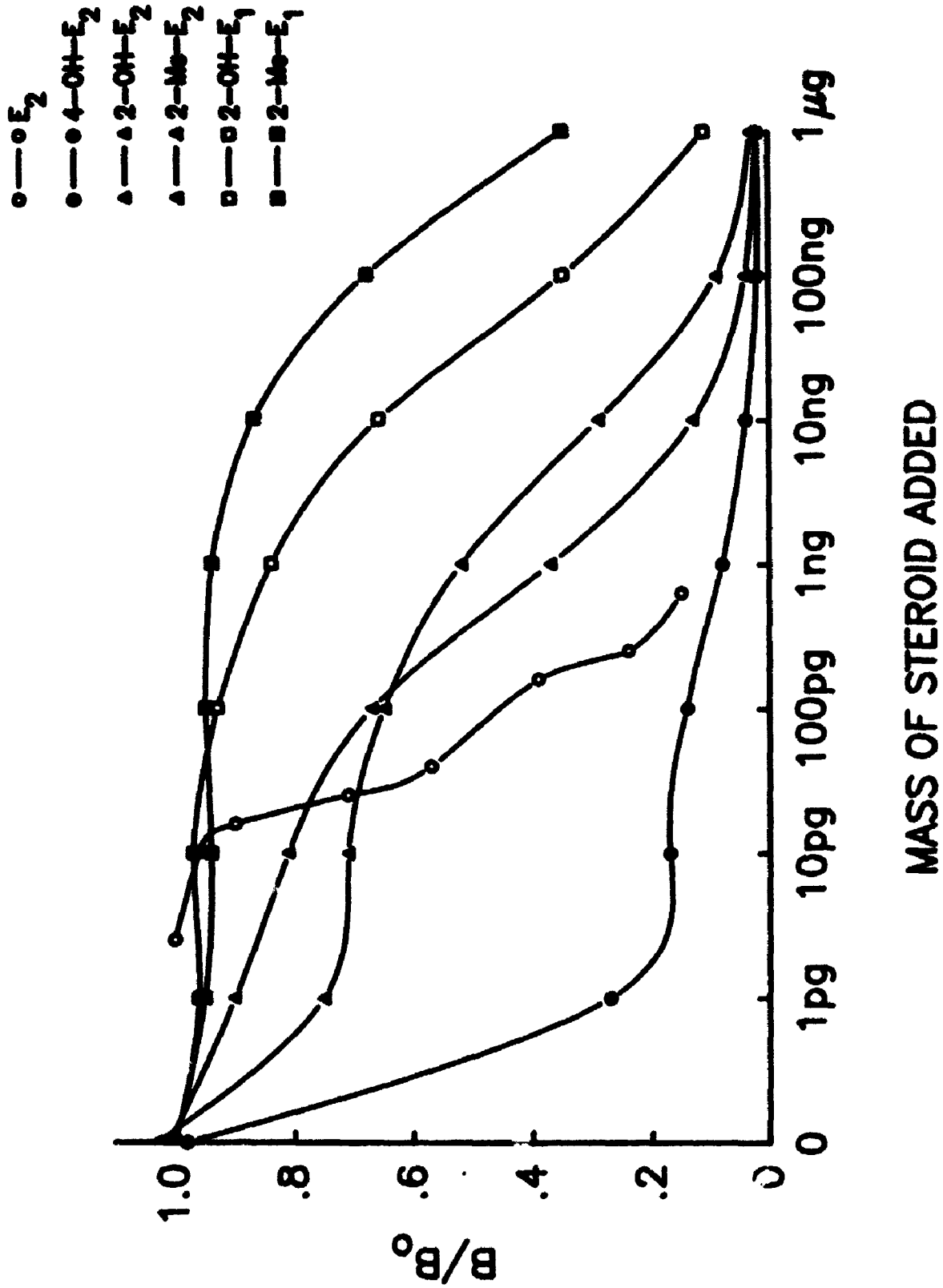
Table 15. Effect of E_2 and 4-OH-E_2 on the DNA content of theca cell cultures.

	DNA content (μg/well)	
Treatment[1]	Control	LH
___	1.30 ± 0.15[a]	1.14 ± 0.05[a]
E_2	1.18 ± 0.19[a]	1.25 ± 0.12[a]
4-OH-E_2	0.98 ± 0.09[a]	1.27 ± 0.16[a]

[1]Theca cells were cultured with (LH) or without (Control) 250 ng/ml LH in the absence and presence of E_2 (10 μg/ml) or 4-OH-E_2 (10 μg/ml). At the end of 48 h of culture the media was removed and the DNA content of the cell layer was determined. Values represent the mean ± S.E.M. of triplicate cultures from a single experiment. Values with different superscripts are significantly ($p < 0.05$) different. Treatment of theca cells with E_2 or 4-OH-E_2 does not significantly effect the DNA content of the cultures.

Figure 25. Cross reactivity of the catecholestrogens in the estradiol radioimmunoassay. Various concentrations (0-1 μg) of catecholestrogens were used to prepare inhibition curves, and assayed in the same assay with the standard curve of estradiol for comparison. Data are the mean $\pm$ S.E.M. of triplicate determinations from a single experiment which was replicated two further times. Where no error bar is shown, the S.E.M. is within the limit of the symbol. All of the catecholestrogens tested cross reacted to various degrees in the estradiol assay.


1.0
1.1
1.25
1.4
1.6
1.8
2.0
2.2
2.5
2.8
3.2
3.6
4.0
4.5
5.0
5.6
6.3
7.1


o—o E2
●—● 4-OH-E2
▲—▲ 2-OH-E2
▲—▲ 2-Me-E2
□—□ 2-OH-E1
■—■ 2-Me-E1
B/Bo
1.0
.8
.6
.4
.2
0
0
1pg
10pg
100pg
1ng
10ng
100ng
1 µg
MASS OF STEROID ADDED

8.3 Discussion

The role of estradiol and catecholestrogens as factors that can inhibit both basal and LH-stimulated theca cell steroidogenesis was investigated using porcine theca cells. The inhibitory effects of estradiol and catecholestrogens were dose- and time-dependent. These findings indicate for the first time that catecholestrogens, like estradiol, can inhibit both basal and LH-stimulated porcine theca cell androstenedione accumulation in vitro. Doses of estradiol and catecholestrogens which caused significant inhibitory effects on thecal androstenedione accumulation were in excess of 1 μg/ml and maximal inhibition occurred at 10 μg/ml. The inhibitory effects observed were variable among experiments, which was possibly due to variability in the stage of sexual development of the ovaries of the pigs provided by the abattoir on any given day, and the stage of development may affect the theca cells' responsiveness to estradiol and catecholestrogens. These concentrations of estradiol and catecholestrogens were much higher than the levels of immunoreactive estradiol-like material measured in the SON extract, assuming similar cross reactivity to estradiol. Similar to the SON extract, the catecholestrogens cross reacted to varying degrees in the estradiol radioimmunoassay.

Catechol-o-methyl transferase (COMT) has been shown to be present in many mammalian tissues including the uterus (Hersey et al., 1982) and porcine ovarian follicular wall (Fernandez-Pardal et al., 1986). The observation that the COMT inhibitor U-0521 enhanced the inhibitory action of catecholestrogens, but not E_2, suggests the presence of COMT in porcine theca cells. Spicer et al. (1987) have reported that U-0521 slowed the rate of degradation of [^{3}H]-2-OH-E_2 by half in cultured porcine granulosa

cells. These authors have also reported that the main metabolite of 2-OH-E_2 comigrates with 2-Me-E_2. Since 2-Me-E_2 was shown in our studies to be an effective inhibitor of thecal androstenedione production, methylation of 2-hydroxyestradiol by theca cells would not remove the inhibitory effects of the catecholestrogens. 2-Me-E_2 has also been shown to be an effective stimulator of progesterone production by cultured porcine granulosa cells (Spicer et al., 1987).

The predominant catecholestrogen found in follicular fluid of mares and women is 2-Me-E_2, with concentrations in preovulatory follicles ranging from 10-310 ng/ml (Dehennin et al., 1984; Silberzahn et al., 1985) which are well below the levels found to be effective in our theca cell cultures. However, since it has been demonstrated that theca cells contain the hydroxylase enzymes for catecholestrogen production, the intracellular levels of catecholestrogens in theca cells may be considerably higher. In addition, 2-OH-E_2 is metabolized by porcine granulosa cells at a rate several fold higher than that for E_2 (Spicer et al., 1987). This rapid breakdown in culture may also be a major determinant of their potency.

The site at which estradiol acts to inhibit thecal androgen production has been described previously (Leung and Armstrong, 1979a; Tsang et al., 1979a, Magoffin and Erickson, 1981). The failure of estradiol or catecholestrogens to prevent the LH-stimulated increase in cyclic AMP production indicates that the inhibitory influence of these compounds occurs distal to the generation of cyclic AMP. This suggests that the catecholestrogens do not affect thecal LH receptor number and/or affinity or the LH sensitive adenylate cyclase system. These conclusions are supported by the findings that in cultures containing estradiol or 4-OH-E_2 in which androstenedione production was inhibited, there was no

significant inhibition of theca cell cyclic AMP production, and, in addition, androstenedione production in response to dibutyryl cyclic AMP was also inhibited.

To determine the site in the steroid biosynthetic pathway where estradiol and catecholestrogens exert their inhibitory effects, we examined the steroid metabolic profile in the presence of these two agents. The results suggest the antagonistic effects of both agents on ovarian androgen biosynthesis are expressed through a selective inhibition of the 17α-hydroxylase:C_{17-20} lyase enzyme complex. Inhibition of this enzyme complex results in an accumulation of pregnenolone and progesterone and an inhibition of 17α-hydroxyprogesterone and androstenedione production. However, the data also suggest that there are additional site(s) of action where the two agents differ, since the accumulation of pregnenolone and total steroids were significantly greater in cultures treated with estradiol than with 4-OH-E_2. The site in the steroid biosynthetic pathway at which catecholestrogens act to inhibit theca cell androgen production is similar to that described for estradiol (Leung and Armstrong, 1979a; Magoffin and Erickson, 1982d) and gonadotropin releasing hormone (Magoffin and Erickson, 1982a). Since all of the metabolites of androstenedione have not been measured in these studies, the possibility that estradiol and catecholestrogens are decreasing androstenedione accumulation by increasing androstenedione metabolism cannot be ruled out.

The rapidity of the inhibition of androgen accumulation by both estradiol and catecholestrogens raises the possibility that these agents are not acting through the classical estrogen receptor, and instead, may involve an allosteric interaction with the 17α-hydroxylase and C_{17-20} lyase enzymes, resulting in the inhibition of their activities. Preincubation

of theca cells with a variety of antiestrogens (tamoxifen, nafoxidine or CI-628) did not prevent the inhibitory effect of either estradiol or the catecholestrogens on thecal androstenedione accumulation (data not shown).

The receptor at which the catecholestrogens exert their effects on theca cell steroidogenesis is unclear. Many studies have been done to suggest that catecholestrogens possibly act through catecholamine receptors (Paden et al., 1982; Hiemke et al., 1983), estradiol receptors (Fishman, 1981; Hersey et al., 1981), androgen receptors (Hudson and Hillier, 1985) or alternatively, distinct catecholestrogen receptors may exist (Parvizi et al., 1985; Etchegoyen et al., 1986; Vandewalle et al., 1985).

It has been suggested that catecholestrogens act in a local autocrine or paracrine manner since the rapid clearance of catecholestrogens from the circulation makes it unlikely that they act as circulating hormones (Merriam et al., 1980). Since both porcine granulosa and theca cells possess the 2- and 4-hydroxylase enzymes for catecholestrogen production (Hammond et al., 1986) the inhibitory action of catecholestrogens may be an autocrine action within the theca cell itself or a paracrine action from adjacent theca cells or even granulosa cells. If so, catecholestrogens may act with estradiol as an intraovarian feedback mechanism for the regulation of ovarian androgen production.

Catecholestrogen formation has been shown to occur in the brain and pituitary tissue of rat fetuses (Fishman and Norton, 1975; Ball and Knuppen, 1980; Paul et al., 1983). The enzyme activity for the formation of catecholestrogens is evenly distributed throughout the brain with the exception of the hypothalamus, which has significantly greater activity (Ball and Knuppen, 1980; Paul et al., 1983). The neural tissues can

convert estrone and estradiol to 2- and 4-hydroxylated derivatives (Fishman and Norton, 1975). Catecholestrogens are involved in hypothalamic gonadotropin release (Naftolin et al., 1975a); however, their place in physiology remains to be determined.

The mechanism of catecholestrogen action in the brain is unknown; however, several possibilities have been suggested. First, the interaction of catecholestrogens with estradiol receptors in the brain suggests that they may act as estrogens or antiestrogens (Paul and Skolnick, 1977; Martucci and Fishman, 1979). Second, catecholestrogens may interfere with catecholamine synthesis by inhibiting tyrosine hydroxylase activity (Lloyd and Weiss, 1979), or they may block catecholamine degradation through competitive inhibition of the COMT enzyme (Breuer and Koster, 1974; Ghraf and Hiemke, 1983) suggesting a close relationship between catecholestrogens and catecholamines. In addition, catecholestrogens double the number of β-adrenergic receptors in cultured porcine granulosa cells (Spicer and Hammond, 1987a). Third, catecholestrogens bind to neuronal cell membranes (Schaeffer et al., 1980), suggesting that catecholestrogens may function as neurotransmitters. Catecholestrogens may also play important roles in steroid-neurotransmitter interactions. It is difficult to study the endogenous role of catecholestrogens since they are very unstable and specific antagonists and hydroxylase inhibitors are not available.

These studies have shown that estradiol and catecholestrogens cause a similar inhibition of thecal androstenedione accumulation, however they differ in their effects on pregnenolone accumulation. These observations will be further investigated by examining the biochemical properties of

the inhibitory factor in the SON extract and by comparing these properties to those of estradiol and catecholestrogens.

CHAPTER 9-BIOCHEMICAL PROPERTIES OF THE INHIBITORY ACTIVITY OF THE SUPERIOR OVARIAN NERVE EXTRACT

9.1 Introduction

This chapter describes studies aimed at initial biochemical characterization of the inhibitory factor. Simple tests can be done to determine if the active factor is a protein or peptide, its relative molecular size and its stability to temperature. The demonstration of immunoreactive estradiol activity in the SON extract have suggested the presence of an estrogen or estrogen-like molecule in the extract; however, the concentration of estradiol immunoreactivity is too low to inhibit thecal androstenedione accumulation *in vitro*. The results of these studies will be compared to the responses of estradiol and catecholestrogens in similar tests. We now have two methods for assessing the activity of the SON extract, 1) its immunoreactive estradiol concentration as measured by radioimmunoassay and 2) its ability to inhibit androstenedione accumulation.

9.2 Methods and Results

9.2.1 Charcoal Treatment

Charcoal treatment is used as a non-specific means of removing small molecules from suspensions. To determine the effect of charcoal treatment on the ability of nerve extracts to influence androstenedione accumulation, a portion of the SON and SCN extracts were treated: 50 mg/ml Norit A charcoal (Sigma), mixed, allowed to sit 15 min at 4°C, mixed again and then centrifuged at 1,600 g at 4°C; following centrifugation, the

supernatant was decanted and added to theca cell cultures with untreated extracts.

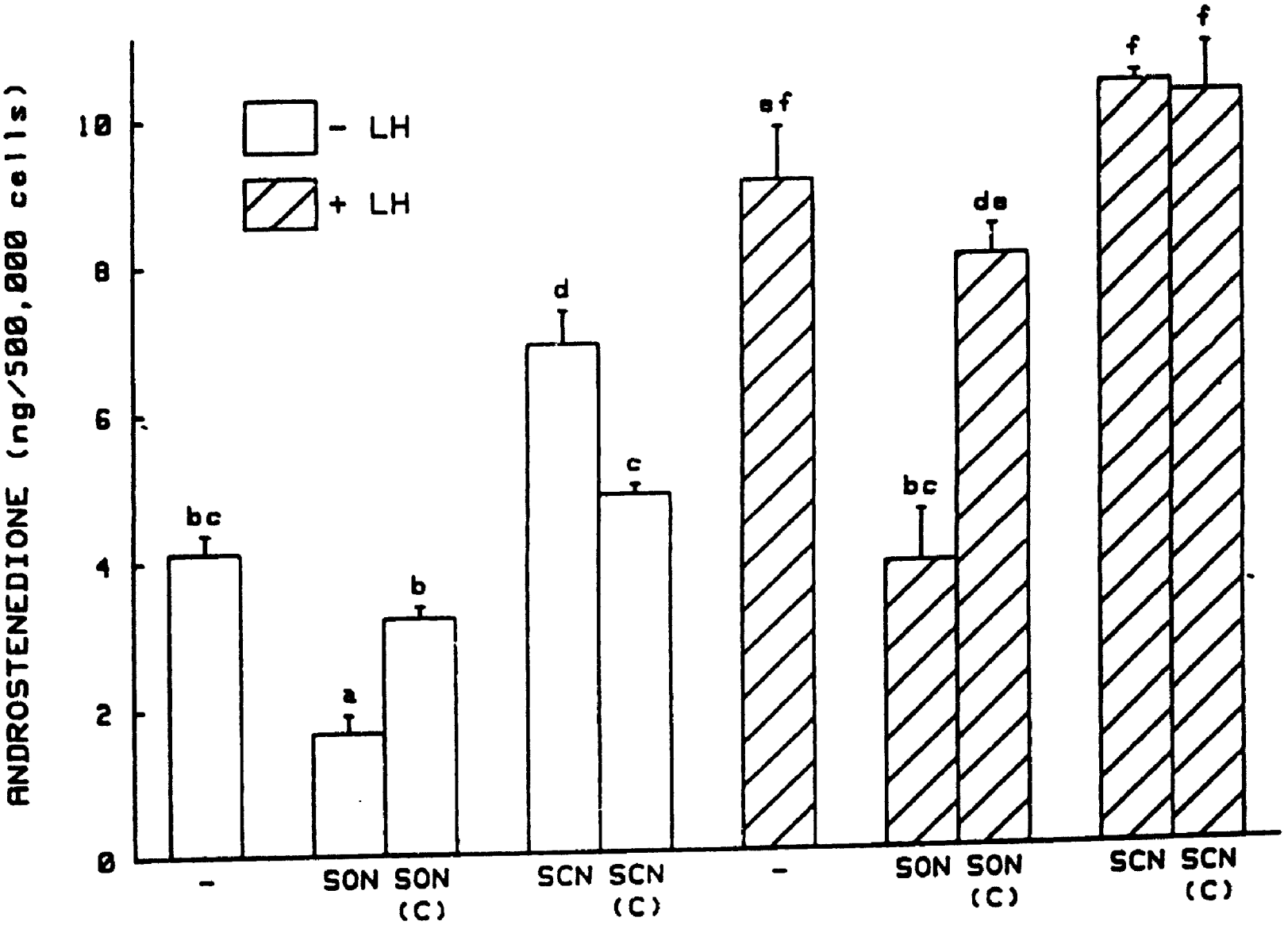
The effects of charcoal treatment of the nerve extracts are shown in Figure 26. Charcoal treatment of the SON extract caused a significant ($p < 0.05$) reduction of the inhibitory activity of the SON extract on thecal androstenedione accumulation to levels that were not significantly different from those in cultures without extracts. Charcoal treatment also reduced the immunoreactive estradiol concentration in the SON extract from 273 pg/ml in untreated extracts to 13 pg/ml in charcoal-treated SON extracts.

Charcoal treatment of the SCN extract caused a significant ($p < 0.05$) decrease of the stimulatory activity of the SCN extract on thecal androstenedione accumulation in the absence, but not in the presence of LH, to levels that were not significantly different from cultures without extracts. Testosterone levels in the LH-stimulated theca cell cultures containing the charcoal treated SCN were not significantly different from cultures without extracts.

9.2.2 Chymotrypsin Treatment

To determine if the inhibitory factor in the SON extract was a large peptide or protein, the effect of treatment with the relatively non-specific protease chymotrypsin on the inhibitory activity of the SON extract was determined by incubating a portion of the SON extract for 1 h at room temperature in 1% NH_4HCO_3 with α-chymotrypsin (Sigma) at a concentration of 25 μg/mg protein. For controls, theca cells were incubated in the presence of the chymotrypsin vehicle or chymotrypsin alone and the test fractions were incubated with the chymotrypsin vehicle

Figure 26. Effect of charcoal treatment on the activity of the SON and SCN extracts influencing theca cell androgen accumulation. Theca cells were cultured without (-LH) or with 250 ng/ml LH (+LH) in the presence of either untreated SON or SCN extracts or charcoal-treated extracts, SON(C) and SCN(C) for 48 h. Values represent the mean ± S.E.M. of quadruplicate cultures in a single experiment which was replicated two further times. Analysis of variance revealed significant ($p < 0.05$) interactions between the effects of LH and charcoal treatment and charcoal treatment and the nerve extracts. Values with different superscripts are significantly different ($p < 0.05$). Charcoal treatment of the SON extract caused a significant ($p < 0.05$) removal of its inhibitory activity on theca cell androstenedione accumulation to levels that were not significantly different than in cultures without extracts. Charcoal treatment of the SCN extract significantly ($p < 0.05$) removed the stimulatory activity of the SCN extract on thecal androstenedione accumulation in the absence, but not in the presence of LH.

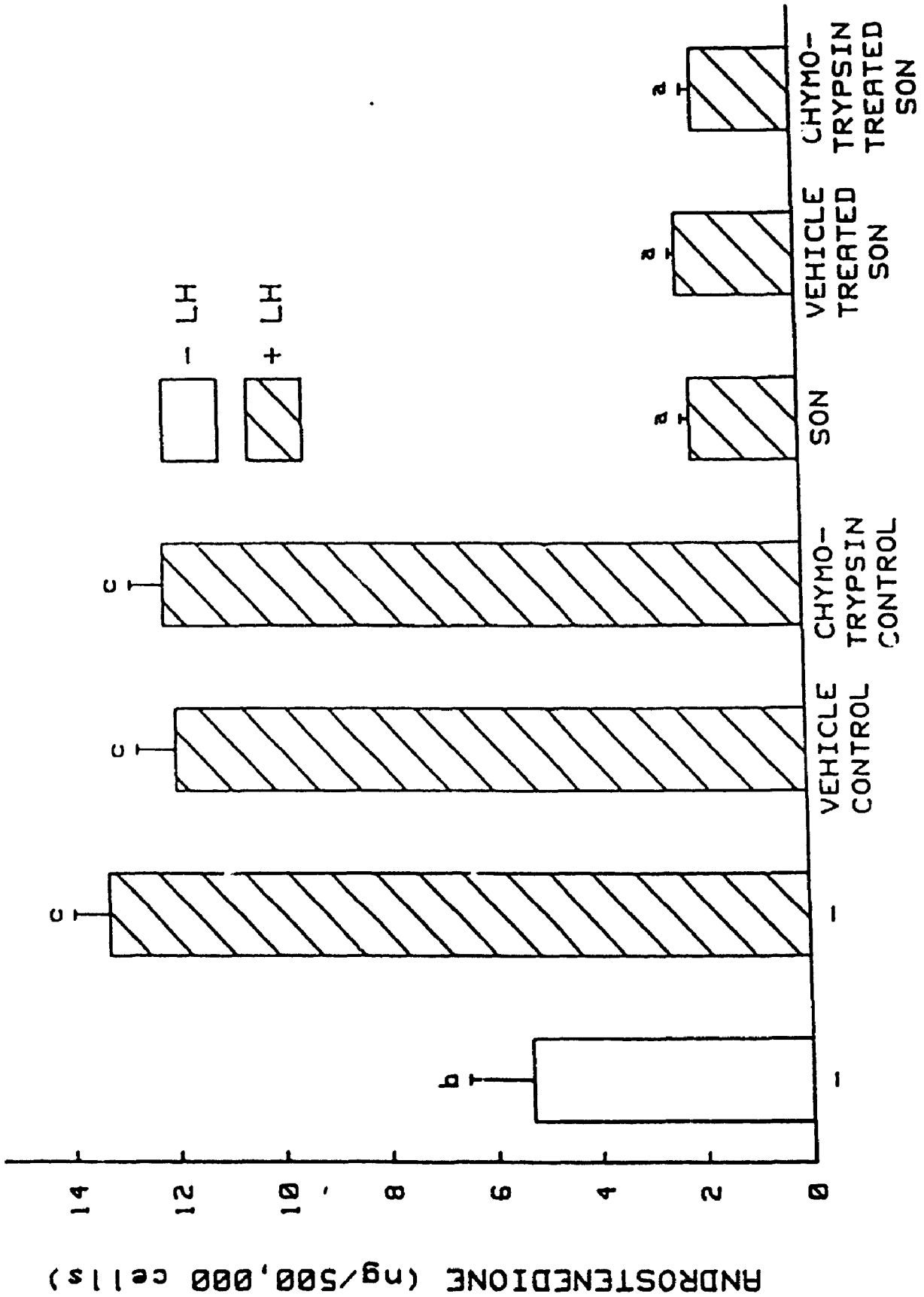

ANDROSTENEDIONE (ng/500,000 cells)
10
8
6
4
2
0
- LH
+ LH
bc
a
b
d
c
ef
bc
de
f
f
-
SON
SON (C)
SCN
SCN (C)
-
SON
SON (C)
SCN
SCN (C)

under the same conditions. Control and chymotrypsin treated samples were lyophilized, redissolved in culture medium and added to LH-stimulated (250 ng/ml) theca cell cultures.

Figure 27 shows that chymotrypsin treatment of the SON extract did not significantly affect the inhibitory activity of the SON extract on LH-stimulated theca cell androstenedione accumulation. LH-stimulated androstenedione accumulation was not significantly affected by pretreatment with chymotrypsin or chymotrypsin vehicle. As a positive control, LH was digested with chymotrypsin in a similar manner as described above. Chymotrypsin-treated LH lost its ability to stimulate thecal androstenedione accumulation above the levels seen in unstimulated cultures (data not shown). The lack of effect of chymotrypsin on the inhibitory activity of the SON extract suggests that the inhibitory factor in the SON extract is not a large peptide or protein.

To further investigate the possibility that the active factor in the SON extract is not a large peptide or protein, the effect of deproteination on the inhibitory activity of the SON extract was examined. An SON extract was prepared in the usual manner and a portion was incubated in 5 ml acetone:ethanol (1:1)/g tissue for 12 hours at room temperature, after which the suspension was centrifuged, the supernatant decanted, dried under nitrogen and reconstituted in culture media. The untreated (raw SON) and deproteinated SON extracts were added to LH-stimulated theca cell cultures. Both the raw and deproteinated SON extracts significantly ($p < 0.05$) inhibited androstenedione accumulation and both had comparable concentrations of immunoreactive estradiol (raw SON 575 pg/ml vs deproteinated SON 810 pg/ml) (Figure 28). Deproteination

Figure 27. Effect of chymotrypsin treatment on the inhibitory activity of the SON extract. Untreated, vehicle- and chymotrypsin-treated SON extracts were added to LH-stimulated (250 ng/ml) theca cells cultured for 48 h. Values represent the mean ± S.E.M. of quadruplicate cultures from a single experiment which was replicated one further time. Values with different superscripts are significantly different ($p < 0.05$). Chymotrypsin treatment did not affect the inhibitory activity of the SON extract.


ANDROSTENEDIONE (ng/500,000 cells)
0
2
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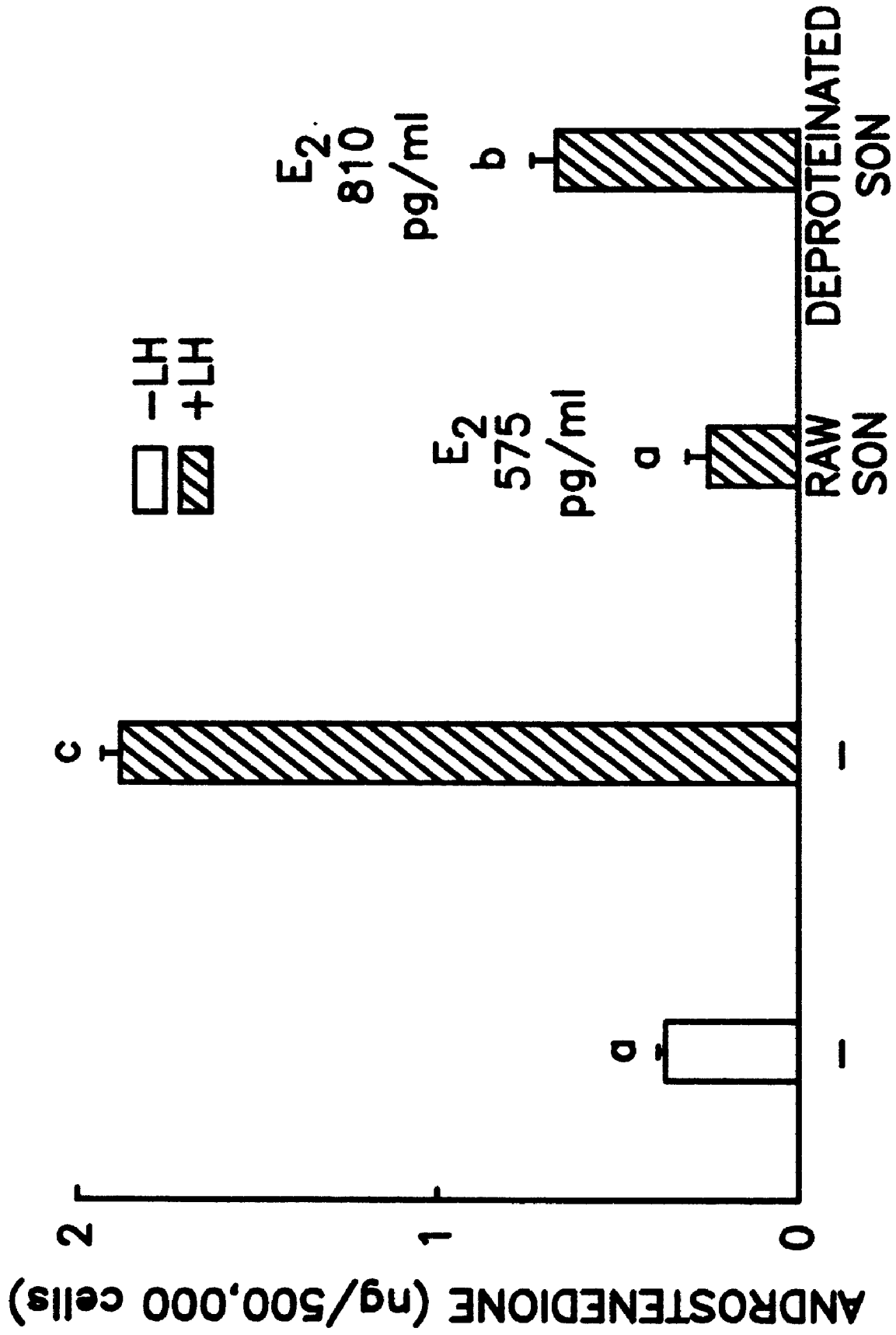
6
8
10
12
14
- LH
+ LH
VEHICLE CONTROL
CHYMO-TRYPSIN CONTROL
SON
VEHICLE TREATED SON
CHYMO-TRYPSIN TREATED SON
a
b
c

Figure 28. Effect of deproteination on the inhibitory activity of the SON extract. Untreated (raw SON) and deproteinated SON extracts were added to LH-stimulated (250 ng/ml) theca cells cultured for 48 h. Values represent the mean ± S.E.M. of quadruplicate cultures from a single experiment which was replicated two further times. Values with different superscripts are significantly different ($p < 0.05$). Immunoreactive estradiol concentration in each sample is shown above the corresponding histogram bar. Both raw and deproteinated SON extracts inhibit thecal androstenedione accumulation, and both contain significant amounts of immunoreactive estradiol.


ANDROSTENEDIONE (ng/500,000 cells)
2
1
0
a
c
−LH
+LH
E2 575 pg/ml
a
RAW SON
E2 810 pg/ml
b
DEPROTEINATED SON

of the SCN extract caused the complete loss of its activity stimulatory to thecal androstenedione accumulation (data not shown).

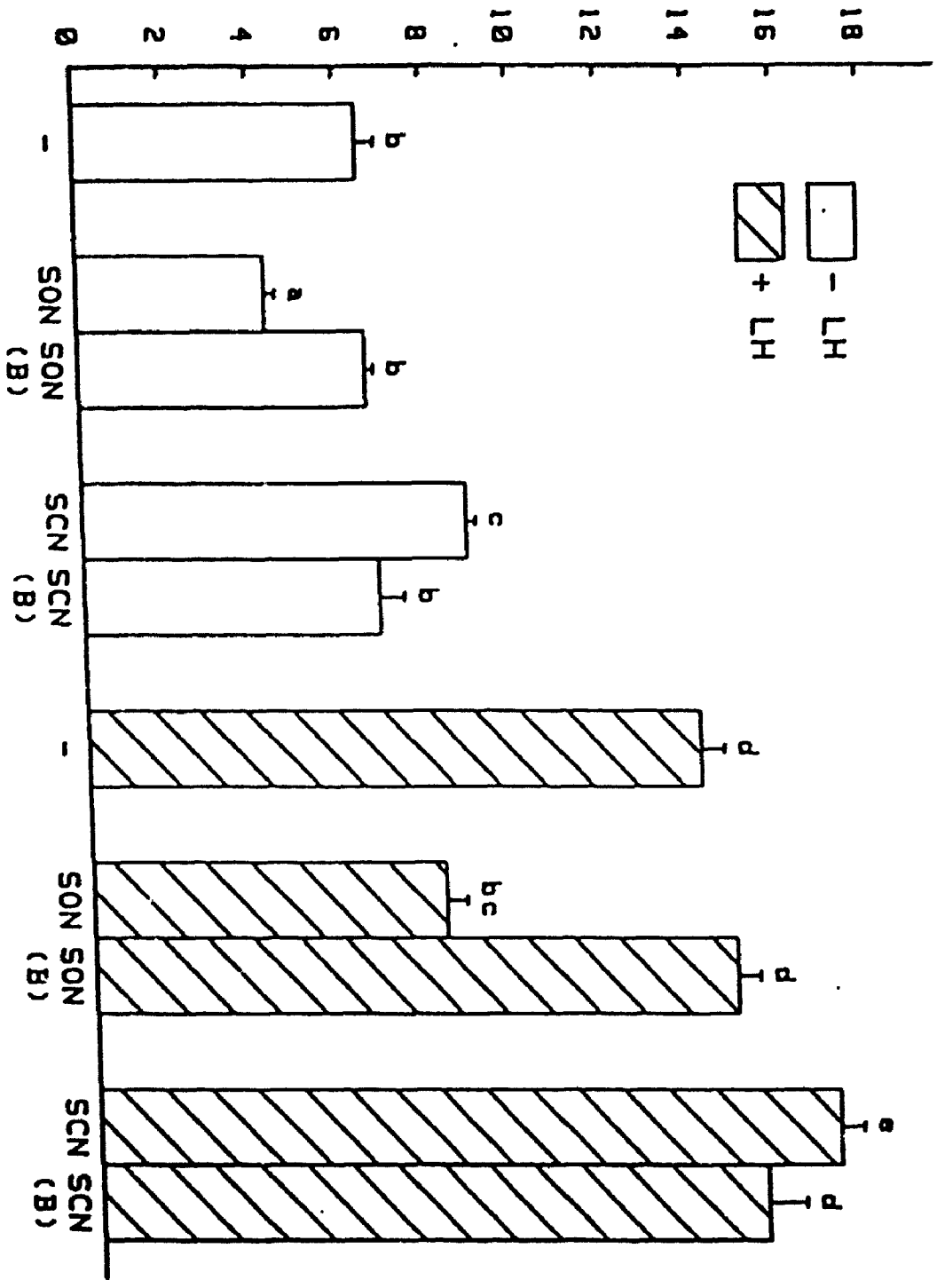
9.2.3 Heat Denaturation

To test if the active factor(s) in the nerve extracts were heat labile, SON and SCN extracts were prepared in the normal manner and divided in two portions. One portion remained untreated and the other was placed in a boiling water bath for 30 min. After boiling the extracts were cooled to room temperature and added to control and LH-stimulated theca cell cultures with untreated extracts.

Figure 29 shows that the unboiled SON extract significantly ($p < 0.05$) inhibited and the unboiled SCN extract significantly ($p < 0.05$) increased both basal and LH-stimulated androstenedione accumulation. Boiling of the SON and SCN extracts for 30 min completely removed ($p < 0.05$) their activities influencing thecal androstenedione accumulation. The levels of androstenedione in both basal and LH-stimulated cultures containing the boiled extracts were not significantly different from the levels in cultures without extracts. The concentrations of Na^+ and K^+ ions in the boiled nerve extracts did not differ significantly from those in untreated extracts, indicating that little, if any, evaporation of the extract had occurred during boiling (data not shown). Boiling also decreased the immunoreactive estradiol concentration in the SON extract from 235 pg/ml in untreated extracts to 8 pg/ml in boiled SON extracts.

Boiling the nerve extracts caused a precipitation of proteins in the extracts. The nerve extracts contain approximately 1 mg protein/ml. If the active factor is a steroid or some other small molecule then it could bind to the proteins and be removed from the extract when they are precipitated

Figure 29. Effect of boiling on the activity of the SON and SCN extracts influencing theca cell androstenedione accumulation. Theca cells were cultured without (-LH) or with 250 ng/ml LH (+LH) in the presence of either untreated SON or SCN extracts or boiled extracts, SON(B) and SCN(B) for 48 h. Values represent the mean ± S.E.M. of quadruplicate cultures in a single experiment which was replicated one further time. Analysis of variance revealed significant ($p < 0.05$) interactions between the effects of LH and the nerve extracts, LH and boiling, and boiling and the nerve extracts. Values with different superscripts are significantly different ($p < 0.05$). Boiling caused a complete loss of the inhibitory activity of the SON extract and of the stimulatory activity of the SCN extract to levels that were not significantly different than in cultures without extracts.


ANDROSTENEDIONE (ng/500,000 cells)
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18
-
(B) NOS SON
(B) SCN NOS
-
(B) SON NOS
(B) NOS SCN
+ LH
- LH
a
b
c
d
bc

during boiling. To investigate this possibility, SON and SCN extracts were prepared in the normal manner and each divided into two portions. One portion was added to the cultures directly and the remainder was boiled for 30 min to precipitate the proteins. The proteins in the boiled extracts were pelleted by centrifugation and the supernatants were added to LH-stimulated (250 ng/ml) theca cell cultures with unboiled extracts. As before, boiling caused a complete loss of the inhibitory activity of the SON extract and the stimulatory activity of the SCN extract on thecal androstenedione accumulation (Figure 30). The protein pellets precipitated from boiled extracts were then extracted with 3 x 3 vol diethyl ether, dried under nitrogen, resuspended in culture medium and added to LH-stimulated theca cell cultures. The extract of the SON precipitated protein pellet caused a significant ($p < 0.05$) inhibition of androstenedione accumulation, in a similar manner to the untreated SON extract. These data suggest that the inhibitory factor in the SON extract was precipitated with the proteins when the extract was boiled. The diethyl ether extract of the SCN precipitated protein pellet had no significant stimulatory effect on thecal androstenedione accumulation.

To re-examine if the SON extract's inhibitory factor is stable to heat, samples of the deproteinated SON extract were prepared by acetone:ethanol precipitation as described previously, and divided into two portions. One portion remained untreated and the other was boiled for 30 min. Following deproteination, there was no precipitation of proteins from the SON extract during the boiling procedure. After boiling, the extracts were cooled to room temperature and added to control and LH-stimulated (250 ng/ml) theca cell cultures with untreated SON extracts. Figure 31 shows that boiling the deproteinated SON extract has no significant effect on

Figure 30. Extraction of nerve extracts active factors from precipitated proteins following boiling. Theca cells were cultured in the absence (-LH) or presence of 250 ng/ml LH (+LH) for 48 h. SON and SCN extracts were prepared and added to the cultures directly (SON and SCN), or boiled for 30 min prior to being added to the cultures [SON(B) and SCN(B)]. The proteins precipitated from the extracts during boiling were extracted with diethyl ether, dried under nitrogen and resuspended in culture media before being added to LH-stimulated theca cell cultures [Extract SON(B) and Extract SCN(B)]. Values represent the mean $\pm$ S.E.M. of quadruplicate cultures from a single experiment which was replicated one further time. Values with different superscripts are significantly different ($p < 0.05$). As described previously, the SON extract significantly inhibited and the SCN extract significantly stimulated thecal androstenedione accumulation. The effects of both extracts were completely removed by boiling for 30 min. An ether extract of the precipitated proteins following boiling contained the inhibitory activity of the SON extract but not the stimulatory activity of the SCN extract.

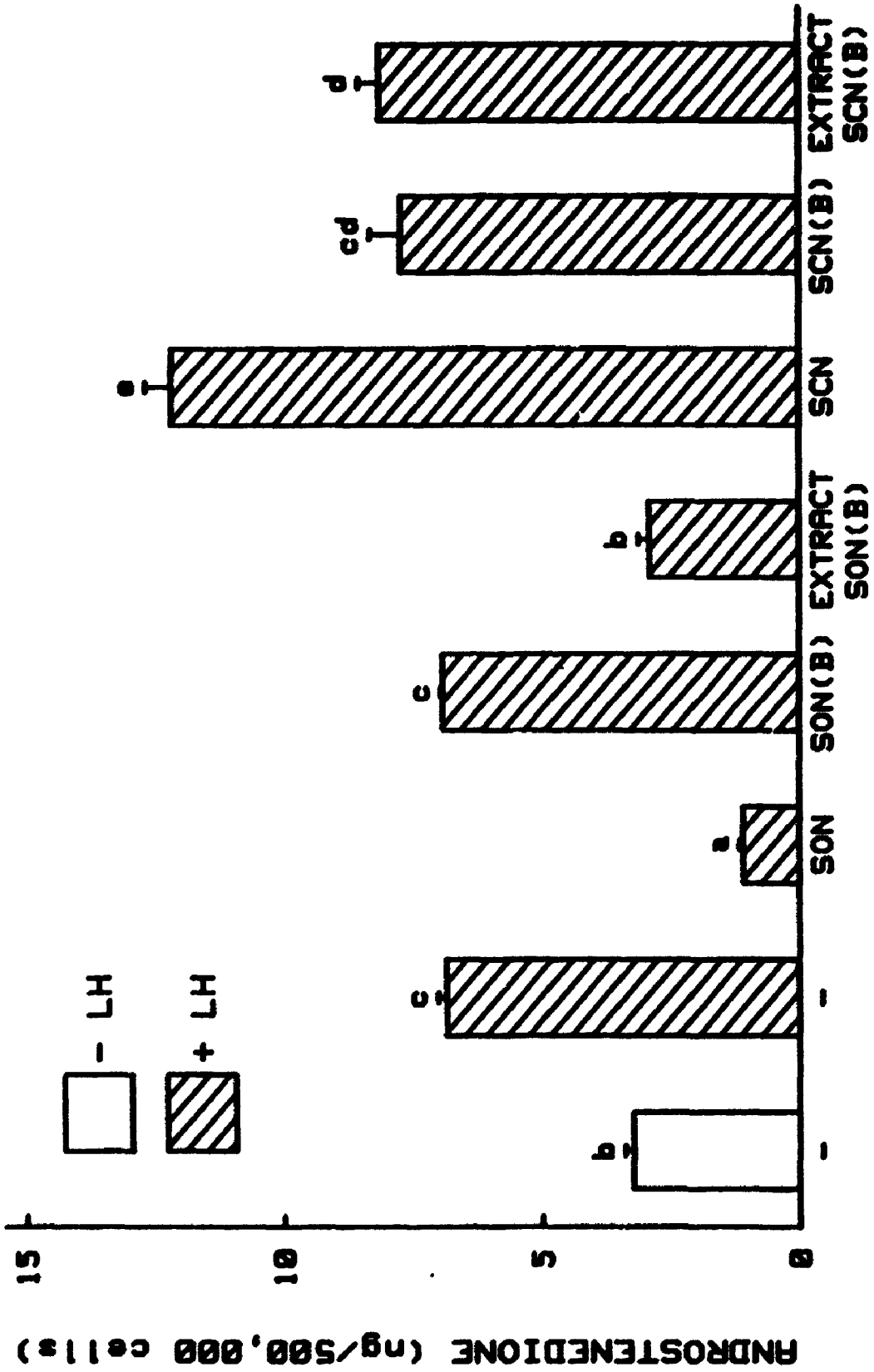
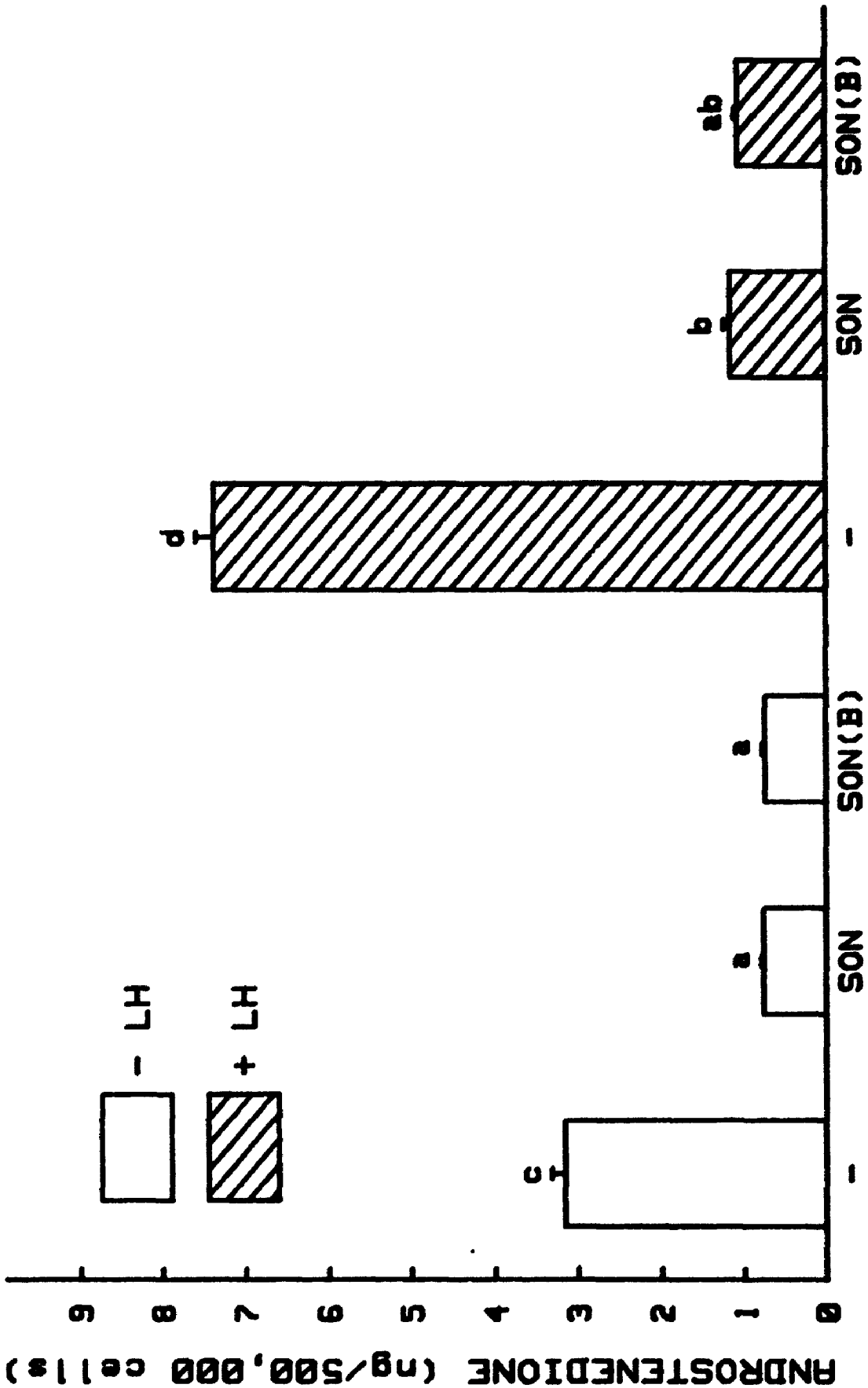

ANDROSTENEDIONE (ng/500,000 cells)
0
5
10
15
- LH
+ LH
a
b
c
d
e
cd
SON
SON(B)
SON(B) EXTRACT
SCN
SCN(B)
SCN(B) EXTRACT

Figure 31. Effect of boiling on the activity of deproteinated SON extracts inhibitory to thecal androstenedione accumulation. Theca cells were cultured without (-LH) or with 250 ng/ml LH (+LH) in the presence of either untreated (SON) or boiled [SON(B)] deproteinated SON extracts for 48 h. Values represent the mean ± S.E.M. of quadruplicate cultures from a single experiment which was replicated one further time. Values with different superscripts are significantly different ($p < 0.05$). Boiling had no effect on the inhibition of thecal androstenedione accumulation by deproteinated SON extracts.

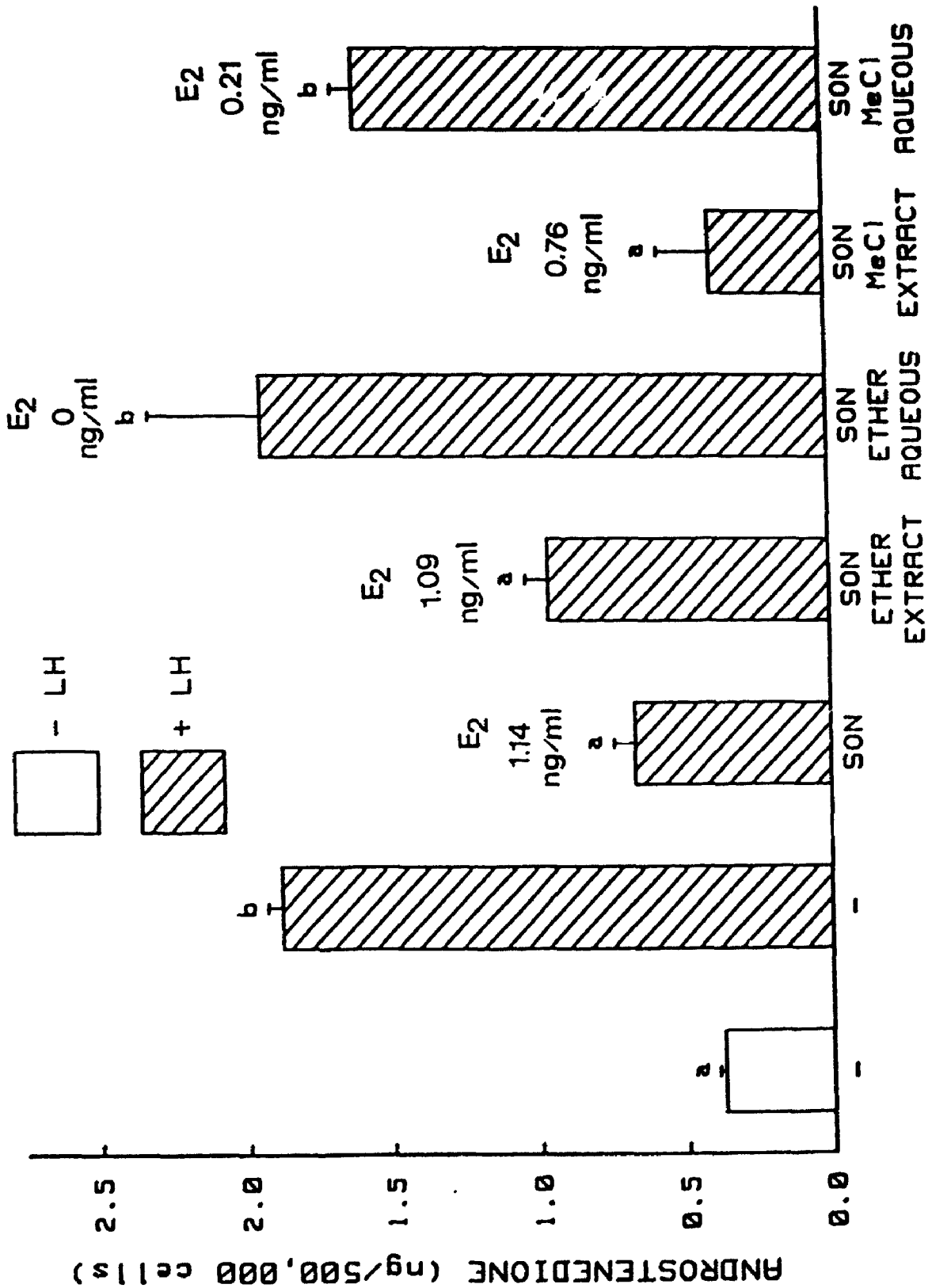

ANDROSTENEDIONE (ng/500,000 cells)
0
1
2
3
4
5
6
7
8
9
LH +
LH -
c
1
a
SON
a
SON(B)
d
1
b
SON
ab
SON(B)

the inhibitory activity of the SON extract on control or LH-stimulated thecal androstenedione accumulation. The level of androstenedione accumulation in cultures containing the boiled SON extract did not differ from those containing the untreated SON extract.

9.2.4 Organic Solvent Extraction of SON Extracts

SON extracts were extracted with diethyl ether and methylene chloride. to determine if the immunoreactive estradiol activity and the activity inhibitory to thecal androstenedione accumulation were extractable with organic solvents. The deproteinated SON extract was extracted with 3x3 vol of diethyl ether or methylene chloride. The solvent phases were dried under nitrogen and resuspended in culture media. The aqueous and solvent phases were tested for inhibitory effects on LH-stimulated (250 ng/ml) thecal androstenedione accumulation and for estradiol immunoreactivity. The results, presented in Figure 32, show that the diethyl ether and methylene chloride extracts both caused a significant ($p < 0.05$) inhibition of LH-stimulated androstenedione accumulation, while the aqueous phases after either extraction had no significant effect on androstenedione accumulation. The levels of androstenedione accumulation in the presence of the diethyl ether and methylene chloride solvent phases were not significantly different from the levels in cultures containing unextracted SON extracts. The immunoreactive estradiol concentration of each sample is shown above the corresponding histogram bar. The solvent phase of the diethyl ether (1.09 ng/ml) and methylene chloride (0.76 ng/ml) extractions were found to contain nearly all of the immunoreactive estradiol originally present in the unextracted SON extract (1.14 ng/ml).

Figure 32. Organic solvent extraction of the SON extract. Deproteinated SON extracts were extracted with 3x3 vol fresh diethyl ether or methylene chloride. The solvent phases were dried and resuspended in culture media and added along with the aqueous phases to LH-stimulated (250 ng/ml; LH) theca cells cultured for 48 h. The concentration of immunoreactive estradiol in each sample is shown above the corresponding histogram bar. Values represent the mean $\pm$ S.E.M. of quadruplicate cultures from a single experiment which was replicated one further time. Values with different superscripts are significantly ($p < 0.05$) different. Most of the extracts' activity inhibitory to thecal androstenedione accumulation and their immunoreactive estradiol were found in the solvent phase of each extraction.


ANDROSTENEDIONE (ng/500,000 cells)
2.5
2.0
1.5
1.0
0.5
0.0
- LH
+ LH
a
b
-
-
SON
E2 1.14 ng/ml
a
SON ETHER EXTRACT
E2 1.09 ng/ml
a
SON ETHER AQUEOUS
E2 0 ng/ml
b
SON MeCl EXTRACT
E2 0.76 ng/ml
a
SON MeCl AQUEOUS
E2 0.21 ng/ml
b

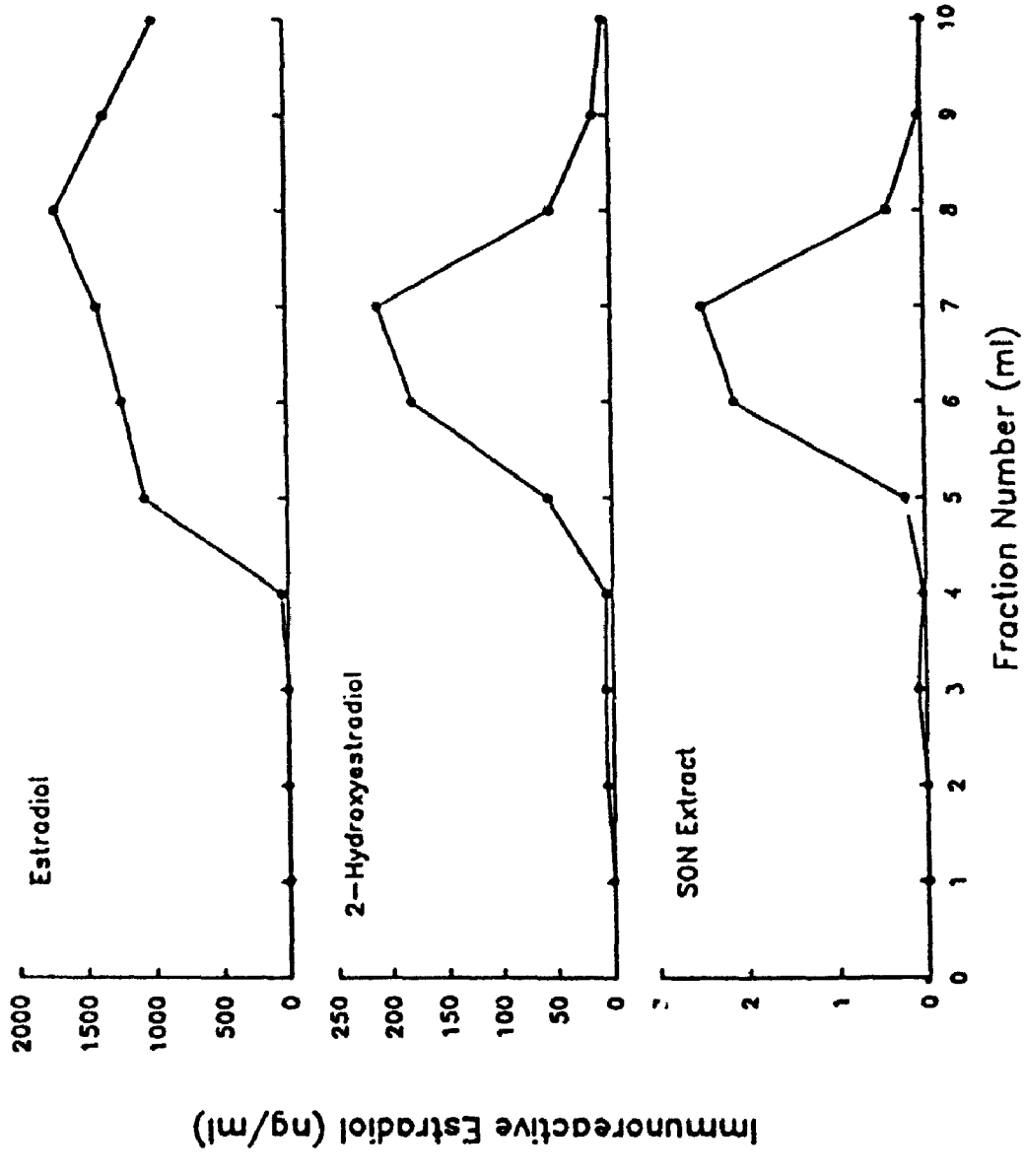
9.2.5 Column Chromatography of SON Extracts

Sephadex LH-20 column chromatography is a procedure routinely used to separate estrogenic steroids in biological fluids. To determine if the SON extract contained an estrogenic compound, SON extracts were run on columns with standard estradiol and the catecholestrogen, 2-OH-E_2, for comparison.

LH-20 column chromatography was carried out as described previously (Karsch et al., 1973). Sephadex LH-20 (10 g; Pharmacia Fine Chemicals. Piscataway, NJ)) was soaked in 100 ml methanol overnight and 5 ml of the slurry was added to 5 ml pipettes containing glass beads. The column was washed with 20 ml column solvent (heptane: chloroform: methanol: water, 500: 500: 75: 3; v/v/v/v). Estradiol elutes from the column in the 4-8 ml fractions and estrone appears between 2-3 ml. Portions of the deproteinated SON extract (320 mg starting material), were extracted with 3x3 vol of diethyl ether and the extracts dried under nitrogen and redissolved in column solvent. The extracts were applied to the columns, and 10 ml of column solvent was added. One ml fractions were collected from the columns, dried under nitrogen and resuspended in culture media. Aliquots of each 1 ml fraction were added to LH-stimulated theca cell cultures to bioassay the activity inhibitory to thecal androstenedione accumulation and also to assay for immunoreactive estradiol. Samples of estradiol (50 μg) and 2-OH-E_2 (50 μg) were run through similar columns for comparison to the SON extract.

Figure 33 shows the immunoreactive estradiol elution profiles for estradiol, 2-OH-E_2 and the SON extract in the 1 ml fractions from the Sephadex LH-20 columns. The immunoreactive estradiol appears in the 5-8 ml fractions for all three of the samples tested. When aliquots of the 1 ml fractions from the LH-20 columns were added to LH-stimulated theca cell

Figure 33. Immunoreactive estradiol elution profile for estradiol, 2-hydroxyestradiol and the SON extract in fractions from Sephadex LH-20 column chromatography. Samples of estradiol (50 μg), 2-hydroxyestradiol (50 μg) and SON extract (320 mg starting tissue) were run on Sephadex LH-20 columns and 1 ml fractions were collected, dried under nitrogen and resuspended in culture media as described in Section 9.2.5. Aliquots of each fraction were assayed for immunoreactive estradiol in the estradiol radioimmunoassay. Values represent a single determination of immunoreactive estradiol in each fraction from a single experiment which was repeated one further time. For estradiol and 2-hydroxyestradiol the immunoreactive estradiol was found in the 5-8 ml fractions and in the SON extract the immunoreactive estradiol was found in the 5-8 ml fractions.


Estradiol
2000
1500
1000
500
0
2-Hydroxyestradiol
250
200
150
100
50
0
SON Extract
2
1
0
0 1 2 3 4 5 6 7 8 9 10
Fraction Number (ml)
Immunoreactive Estradiol (ng/ml)

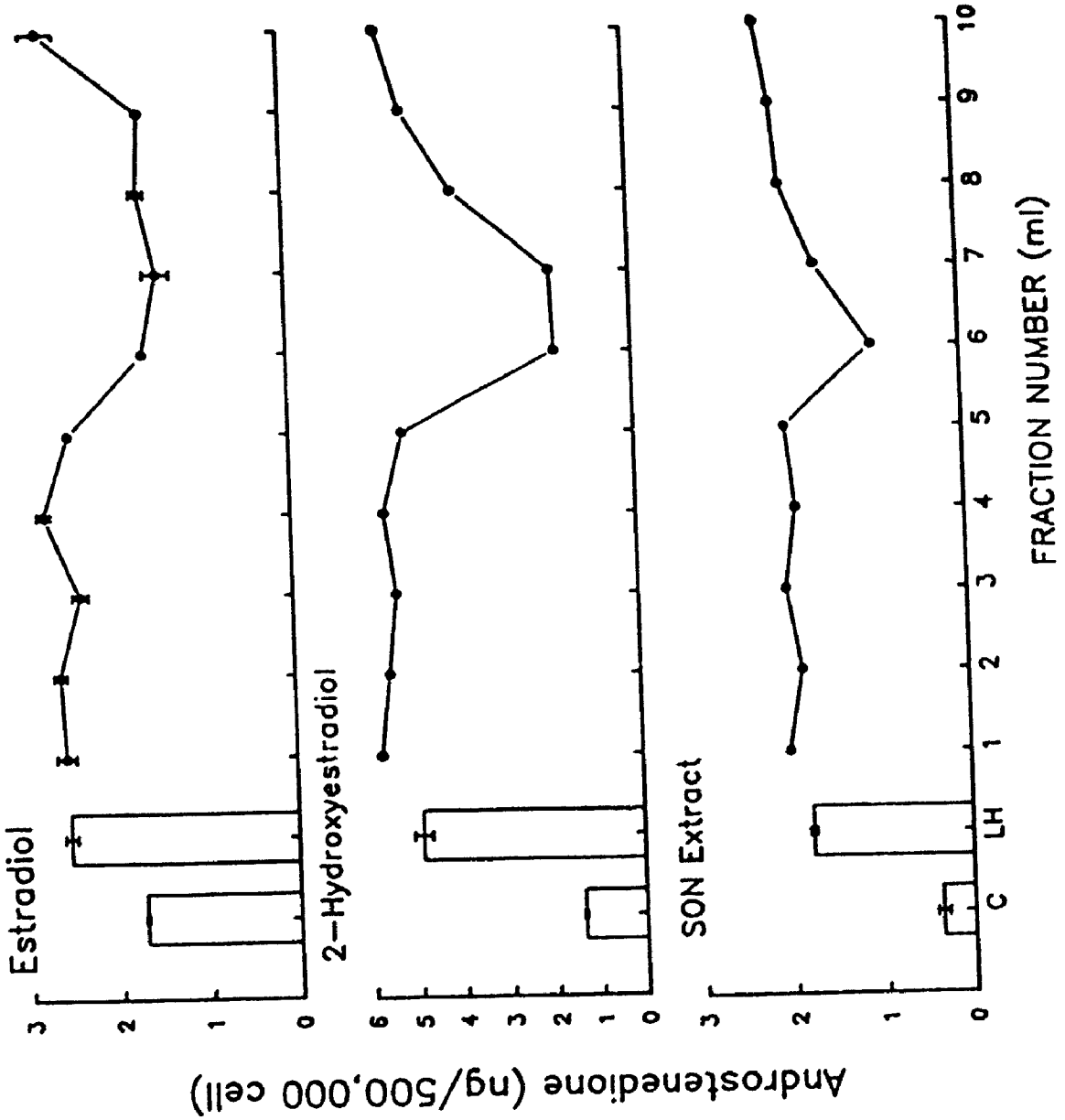
cultures, the 5-8 ml fractions of the standard estradiol and catecholestrogen showed activity inhibitory to thecal androstenedione accumulation (Figure 34). The SON extracts' inhibitory activity was found only in the 6 ml fraction.

9.3 Discussion

These initial characterization studies have provided some insight on the nature of the inhibitory activity of the SON extract and the stimulatory activity of the SCN extract. The activities of the SON and SCN extracts, E_2 and 4-OH-E_2 influencing androstenedione accumulation were removed by charcoal treatment. Charcoal treatment is a non-specific means of removing relatively small molecules from solutions. For example, charcoal treatment is used in radioimmunoassay procedures to separate the antibody bound and free fractions of insulin, which is a 51 amino acid peptide with a molecular weight of 5808 daltons (Brown et al., 1975). In addition to removing all of the activity inhibitory to thecal androstenedione accumulation, charcoal treatment removed nearly all of the immunoreactive estradiol from the SON extract.

The non-specific protease chymotrypsin splits secondary amide and peptide bonds and carboxylic and phenolic ester bonds. Treatment of the SON extract with chymotrypsin, or deproteination, both failed to remove the inhibitory effect of the SON extract on thecal androstenedione accumulation, suggesting that the inhibitory factor was not a large peptide or protein. Although it has not been tested, E_2 and 4-OH-E_2 should be insensitive to protease digestion. The SCN extract was not digested with chymotrypsin; however, the complete loss of its stimulatory activity following deproteination suggests the active factor is a protein or large

Figure 34. Inhibition of thecal androstenedione accumulation by Sephadex LH-20 column fractions. Samples of estradiol (50 μg), 2-hydroxyestradiol (50 μg) and SON extract (320 mg starting tissue) were run on Sephadex LH-20 columns and 1 ml fractions were collected, dried under nitrogen and resuspended in culture media as described in Section 9.2.5. Aliquots of each fraction were added to LH-stimulated (250 ng/ml; LH) theca cells cultured for 48 h. Values represent the mean $\pm$ S.E.M. of triplicate cultures from a single experiment which was repeated one further time. Fractions of each sample tested, from 6-8 ml possessed activity inhibitory to thecal androstenedione accumulation.


Estradiol
2-Hydroxyestradiol
SON Extract
Androstenedione (ng/500,000 cell)
C
LH
FRACTION NUMBER (ml)

peptide. Since chymotrypsin treatment and deproteination presumably did not remove small peptides from the SON extract, the possibility that the inhibitory factor is a small peptide cannot be excluded. If the inhibitory factor is a small peptide, it would have to be hydrophobic in nature, as it is soluble in diethyl ether and methylene chloride. However, as described in Chapter 6, the inhibitory actions of the SON extract were not mimicked by any of the putative ovarian neurotransmitters tested in theca cell cultures.

Boiling the raw SON and SCN extracts for 30 min caused them to lose all of their activity which influenced thecal androstenedione accumulation. These observations suggest the active factors were heat labile peptides or proteins. Boiling the raw extracts also caused the precipitation of proteins in the extracts. If the nerve extracts contained binding proteins that had a high affinity for the active factors, then the active factors could be removed when the proteins in the extract were precipitated during boiling. This was shown to be correct by extracting the inhibitory factor of the SON extract from the precipitated protein pellet with diethyl ether. Boiling the raw SON extract also caused the loss of nearly all of the immunoreactive estradiol in the extract. The SCN extracts stimulatory factor was not extractable from the protein pellet with diethyl ether.

If there are binding proteins for the SON extracts inhibitory factor in the extract, then the active factor must have a greater affinity for the solvents used in the deproteination procedure than for the binding proteins. There was no loss of the SON extracts' inhibitory activity when the deproteinated SON extract was boiled, suggesting that the inhibitory factor is stable to boiling. The inhibitory factor in the SON extract is also stable to lyophilization (data not shown).

Extraction of the deproteinated SON extract with organic solvents showed that the activity of each inhibitor to thecal androstenedione accumulation was only found in the solvent phases. In addition, nearly all of the SON extracts' immunoreactive estradiol was found in the solvent phase. The presence of specific binding proteins in the extract could effect the extraction of the inhibitory factor(s), depending upon the binding affinity of the protein for the factor(s) and the extent to which binding is affected by the extracting solvent.

Chromatography of the SON extract on Sephadex LH-20 columns showed the SON's immunoreactive estradiol and activity inhibitory to androstenedione accumulation ran in the same fractions as standard estradiol and 2-hydroxyestradiol on Sephadex LH-20 columns. It is not known why fraction 7 of the SON extract, which contained a large amount of immunoreactive estradiol activity had only a small inhibitory effect on thecal androstenedione accumulation. The recovery of estradiol and 2-hydroxyestradiol from the columns was very poor, probably due to an overloading of the column. This problem would have to be addressed prior to using LH-20 columns for the purification of the SON extracts immunoreactive estradiol and inhibitory activities.

These data are consistent with the possibility that estrogen or an estrogen-like molecule is present in the SON extract. Estradiol and catecholestrogens are both avidly bound by the sex hormone binding globulin in plasma (Dunn et al., 1981). Although there have been no reports of similar binding proteins in nerves, sex steroids have specific uptake and retention mechanisms related to the presence of specific intracellular receptors in nerve cells (Muldoon, 1980). Estradiol and

catecholestrogens are also known to be stable to boiling, and extractable with organic solvents and charcoal.

Future experiments will require the use of physicochemical methods to separate and identify the inhibitory factor in the SON extract. Several problems in methodology will have to be overcome. First, an improved extraction procedure will be necessary to maximize the yield of the active factor extracted from suspensory ligaments. Second, the inhibitory factor will have to be purified. The deproteination and LH-20 column chromatography steps may provide sufficient purification to allow the extract to be applied to HPLC to determine if the immunoreactive estradiol and inhibitory activities of the SON extract are the same or different compounds. This will require collecting fractions from the HPLC and testing them for estradiol immunoreactivity and biological activity at each step. When the inhibitory factor has been purified by HPLC, gas chromatographic and mass spectrometric techniques can be used to identify the active factor. Problems may arise if the inhibitory factor is not stable, and therefore loses its inhibitory activity during the purification or chromatographic procedures. In addition, these physicochemical methods require a substantial amount of material for identification purposes, and it may be difficult to obtain sufficient sample for these procedures.

If a substance is isolated from the SON extract, antibodies could be raised against the inhibitory factor. The antibody would allow the use of immunocytochemical techniques to determine the normal distribution of the inhibitory factor in the ovary, ovarian nerves and their ganglia. The antibody could also be used to reverse the effects of the SON extract, or

the antibody alone could be supplied to the ovary to assess the effects of removing the inhibitory factor on ovarian function.

In summary, the studies in this chapter have shown the biochemical properties of the factor in the SON extract inhibitory to thecal androstenedione accumulation. However, the present studies were unable to determine if the factor inhibitory to theca cell androstenedione accumulation and a factor that cross reacts with the estradiol antiserum were the same or distinct compounds. Because the SON extract may contain multiple types of inhibitors and stimulators, the contribution of the putative estrogen-like compound to the inhibition of theca cell steroidogenesis could be studied following neutralization of this compound with an estradiol antibody or antiestrogens. Studies mentioned previously were unable to show the ability of the antiestrogens, tamoxifen, nafoxidine, or CI-628, to reverse the inhibitory effects of standard estradiol or catecholestrogens on porcine thecal androstenedione accumulation (data not shown) and therefore they were not tested for their ability to reverse the SON extracts' inhibitory effects. Alternatively, the purified IgG fraction of the estradiol antiserum could be used to immunoneutralize the immunoreactive estradiol activity in the SON extract. If the estradiol IgG blocked the inhibitory action of the SON extract, then the antibody could also be used in the purification of the putative factor. In the next chapter the possible source of the SON extracts activity inhibitory to thecal androgen accumulation and estradiol immunoreactivity will be examined.

CHAPTER 10-SOURCE OF THE INHIBITORY ACTIVITY AND IMMUNOREACTIVE ESTRADIOL IN THE SON EXTRACT

10.1 Introduction

Data presented previously in this thesis have suggested that the immunoreactive estradiol and activity inhibitory to thecal androstenedione accumulation are present in the nerve fibers of the SON and not in some other component of the ligament. Assuming these factors are localized in the nerve fibers, there are at least two possible sources of the inhibitory activity and immunoreactive estradiol in the SON extract. They could be produced locally in the ovary, taken up by the nerve terminals and transported retrogradely by the axons of the SON to the CNS. Alternatively, the inhibitory factor and the estradiol immunoreactivity in the SON extract could be transported anterogradely from the CNS to the ovary. A procedure for ligating peripheral nerves and studying the buildup of molecules on either side of the ligature has been described for the determination of the direction of transport of neural factors (Bisby, 1982; Fried et al., 1985).

Axoplasmic transport is the movement of materials in nerve fibers. The nerve cell body is the principle site of synthesis of molecules in nerve cells, and the axon and nerve terminals rely on this source for a continual supply of supporting factors by axoplasmic transport (Forman, 1987). In typical axons, molecules move in both anterograde and retrograde directions. Anterograde transport moves soluble proteins, membranous components and various organelles as well as neurotransmitters and transmitter-related components to the nerve terminals for release (Ochs, 1987). Retrogradely transported organelles are typically multilamellar

bodies, multivesicular bodies or other prelysosomal organelles carrying membranous material destined to be degraded by lysosomes in the cell body (Forman, 1987). Axoplasmic transport occurs at velocities ranging from 0.5-410 mm/day (Ochs, 1972). In addition, exogenous substances are known to be taken up by nerve terminals, either by endocytosis or after binding to membrane carriers, and transported retrogradely. For example, the uptake by nerve terminals and retrograde transport of horse radish peroxidase to the perikarya is used as a means of tracing neural pathways in the CNS (Kristansson, 1977).

The purpose of the studies presented in this chapter is to attempt to determine the source of the SON extracts' inhibitory activity and immunoreactive estradiol. In addition, since central neuroendocrine tissues are able to aromatize androgens to estrogens (Naftolin et al., 1975b), studies were done to determine if the axons of the SON have the ability to aromatize androgens to estrogens.

10.2 Methods and Results

10.2.1 Effect of SON Extracts Prepared From Hypophysectomized Rats

To determine if the ovary is the source of the inhibitory activity and immunoreactive estradiol in the SON extracts, SON extracts were prepared from suspensory ligaments obtained from adult female rats which had been hypophysectomized at least 1 week prior to nerve collection. Follicles in the ovaries of hypophysectomized rats develop to the preantral stage only (Nakano et al., 1975). Since estrogen production in the follicle requires granulosa cell stimulation by FSH (Armstrong and Papkoff, 1976), the ovaries of hypophysectomized rats should not contain estradiol. If the

source of the SON extract's estradiol immunoreactivity and activity inhibitory to androgen accumulation is the ovary, then SON extracts prepared from the ovaries of hypophysectomized rats should not contain activity inhibitory to androstenedione accumulation or estradiol immunoreactivity.

Hypophysectomized rat SON extracts were prepared in the usual manner and fractions taken for the determination of estradiol immunoreactivity and biological activity. Hypophysectomized rat SON extracts were added to LH-stimulated (250 ng/ml) theca cell cultures. Androstenedione accumulation in the presence of the SON extract (2.00 $\pm$ 0.56 ng/ml) was significantly ($p < 0.05$) less than in cultures incubated with LH alone (5.57 $\pm$ 0.35 ng/ml). The hypophysectomized rat SON extract contained 270 pg/ml immunoreactive estradiol, which is in the same range as the immunoreactive estradiol reported previously in extracts from intact SON's (522 $\pm$ 174 pg/ml; range 42-1980 pg/ml; n=10).

10.2.2 Source of the SON Extract's Immunoreactive Estradiol and Activity Inhibitory to Thecal Androstenedione Accumulation

Nerve ligation studies were performed to determine the direction the immunoreactive estradiol and activity inhibitory to androstenedione accumulation are transported in the axons of the SON. 3-0 silk sutures were tied bilaterally around the suspensory ligaments of adult female rats under Avertin anaesthesia, midway between the kidney and ovary. After 72 h, a small area of bulging of the nerve fiber a few mm above the level of the ligation was observed. Segments of suspensory ligaments proximal and distal to the ligation were excised and pooled. Extracts of the distal and proximal SON segments were prepared in the normal manner and added to

control and LH-stimulated (250 ng/ml) theca cell cultures to assess their biological activity and aliquots were taken for the determination of their immunoreactive estradiol concentration.

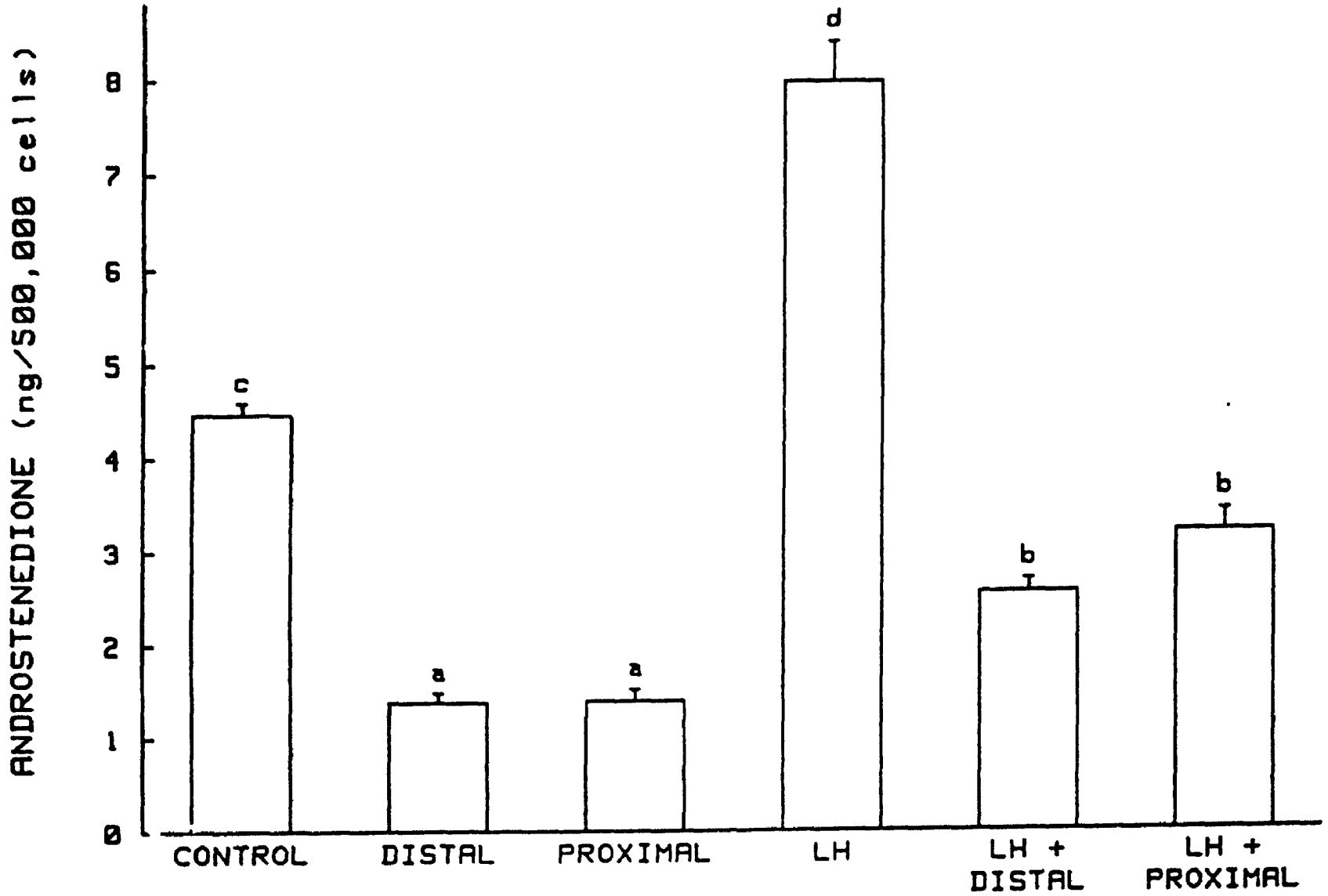
Both the proximal and distal SON extracts significantly ($p < 0.05$) inhibited thecal androstenedione accumulation (Figure 35). The degree of inhibition of androstenedione accumulation by the two extracts was not significantly different. Measurement of the immunoreactive estradiol concentration in the proximal and distal SON extracts showed the distal extract contained 1.29 ng/ml and the proximal extract 6.06 ng/ml immunoreactive estradiol. In repeat experiments, similar results were observed, ie. both proximal and distal SON extracts caused similar inhibitory effects on androstenedione accumulation and the proximal segments contained up to 17-fold greater immunoreactive estradiol concentration than distal segments.

In previous ligation experiments, carried out for 21 days, degeneration of the SON occurred distal to the ligation. To ensure that the three day ligation did not cause degeneration of the SON, ovaries from control and 3-day ligated rats were obtained for the determination of ovarian noradrenaline concentration. Control ovaries contained 226 $\pm$ 24 ng/g tissue (n=6) and 3-day ligated ovaries contained 252 $\pm$ 13 ng/g tissue (n=6).

10.2.3 Can Superior Ovarian Nerves Aromatize Androgens to Estrogens?

Since neurons in the brain contain the enzymes for the aromatization of androgens to estrogens (Naftolin et al., 1975b) and since there appears to be an estrogenic molecule in the SON extracts, studies were done to

Figure 35. Source of the SON extracts' immunoreactive estradiol and activity inhibitory to androstenedione accumulation. Suspensory ligaments of adult female rats were bilaterally ligated midway between the kidney and ovary. After 72 h, the segments of suspensory ligaments proximal and distal to the ligation were excised and pooled. Extracts of the distal and proximal SON segments were prepared and added to control and LH-stimulated (250 ng/ml) theca cells cultured for 48 h. Values represent the mean ± S.E.M. of quadruplicate cultures from a single experiment which was replicated two further times. Values with different superscripts are significantly ($p < 0.05$) different. Segments of SON from proximal and distal to the ligation caused similar inhibitory effects on control and LH-stimulated thecal androstenedione accumulation; however, the immunoreactive estradiol concentration of proximal segments was significantly greater than in distal segments.


ANDROSTENEDIONE (ng/500,000 cells)
0
1
2
3
4
5
6
7
8
c
a
a
d
b
b
CONTROL
DISTAL
PROXIMAL
LH
LH + DISTAL
LH + PROXIMAL

determine if the axons of the SON were able to aromatize exogenous androgens to estrogens.

Freshly excised and frozen superior ovarian and sciatic nerve segments (approximately 30 mg each) were incubated in culture media with androstenedione (0-1 μg/ml) at 37°C for 48 h, after which the media were collected and assayed for estradiol by radioimmunoassay. Culture media from SON and SCN incubations with androstenedione had no detectable estradiol immunoreactivity (data not shown). The sensitivity of the estradiol radioimmunoassay was 5 pg.

10.3 Discussion

Receptor mediated uptake and retrograde axoplasmic transport are general and physiologically important mechanisms for cellular communication in the nervous system. It is possible that the inhibitory factor and estradiol immunoreactivity of the SON extract are produced in the ovary and are transported up the nerves to the CNS. Hypophysectomy, which inhibits ovarian follicular development by preventing the normal transition from preantral follicles to antral stages, may decrease the production of these factors in the ovary, which could lead to a decreased uptake by the nerves and a loss of the SON extracts' inhibitory activity and estradiol immunoreactivity. Since the hypophysectomized rat SON extract had significant levels of immunoreactive estradiol and inhibited androstenedione accumulation, it suggests that the ovary is not the source of these factors. Alternatively, production of the inhibitory factor in the ovary may not be affected by hypophysectomy. No speculation on the production of these factors by the ovary can be made until they are identified and a procedure is developed to measure them.

To further investigate the ovary as a possible source of the immunoreactive estradiol and activity inhibitory to thecal androstenedione accumulation, rats could be ovariectomized leaving the suspensory ligaments intact. Following an appropriate time period, extracts could be prepared from the suspensory ligaments of both intact control and ovariectomized ovaries for the comparison of immunoreactive estradiol and activity inhibitory to thecal androstenedione accumulation. If these factors are produced by the ovary and are travelling retrogradely to the CNS, then removing their source by ovariectomy should remove the extracts' immunoreactive estradiol and inhibitory activity. If the source of these factors is not the ovary, as suggested by the SON extracts prepared from hypophysectomized animals, then the SON extracts from ovariectomized animals should have normal levels of immunoreactive estradiol and activity inhibitory to thecal androgen accumulation.

Following acute axonal injury, such as occurs in the ligation experiments, axoplasmic transport is interrupted and compounds moving up and down the axons accumulate proximal and distal to the ligation (Bisby, 1982). The buildup of molecules at the distal or proximal side of the ligation determines if anterograde or retrograde transport is involved. The bulging of the nerve fiber at the ligation is, in other systems, due to the accumulation of vesicles, mitochondria, soluble proteins and other components within the nerve fibers (Ochs, 1981). In adrenergic nerves this includes the buildup of dense-cored vesicles containing the transmitter noradrenaline (Ochs, 1981). The buildup of estradiol immunoreactivity on the proximal side of the ligature suggests that the estradiol immunoreactivity is being transported from the CNS to the ovary. These data need to be interpreted cautiously, since injury to neurons can cause

the breakdown of the normal neural permeability barrier and may permit local uptake of blood borne materials into the nerve (Bisby, 1982). In addition, molecules may be degraded or undergo local synthesis at the site of the ligation during the ligation procedure (Bisby, 1982). The observation that extracts of both proximal and distal SON segments cause similar inhibitory effects on androgen accumulation, while having significantly different immunoreactive estradiol levels, suggests that the immunoreactive estradiol and activity inhibitory to thecal androstenedione accumulation may be distinct compounds.

Although I am not aware of any reports of the transport of steroid hormones in nerve fibers, the endocrine neurons specialized to produce, axonally transport and secrete the peptide hormones oxytocin and vasopressin have been well described (Pickering, 1978).

The observation that the estradiol immunoreactivity builds up on the proximal side of the ligature suggests that the source of the estradiol immunoreactivity is the cell body of the neurons. This idea is supported by observations of the localization of labelled estradiol injected into mice (Carr and Williams, 1969). Following the subcutaneous injection of ^{3}H-estradiol (50-200 μC; 0.4-110 μg) to mice, tissues were fixed, sectioned and autoradiographs were prepared. Small amounts of estradiol were located in the liver, spleen and uterus. In addition to estrogens being taken up by certain areas of the CNS, especially the hypothalamus, intense estradiol uptake was also found in autonomic ganglia near the splenic artery and alongside the aorta at the level of the kidneys (Carr and Williams, 1969). The estradiol was located in the cytoplasm of large cells which were thought to be neurons; however, the neurons were not identified as sympathetic, parasympathetic or sensory. Previous studies have shown

that the cell bodies of nerves bound for the ovary are located in the ovarian ganglion and celiac and renal plexuses (Crosby et al., 1962) which are located in the same regions as the ganglia reported to contain labelled estradiol by Carr and Williams (1969). The functional significance of estradiol in the autonomic ganglia is unknown.

Initial studies were unable to show that the axons of the SON were capable of aromatizing androgens to estrogens. Future studies will determine if the nerve segments can convert exogenously supplied estradiol to catecholestrogens by incubating nerve fibers in the presence of labelled estradiol followed by the isolation of catecholestrogens by HPLC. A potential problem with these studies is that the majority of molecular synthesis occurs in the perikaryon and not in the axons. It may be necessary, and indeed useful, to examine the ganglia where the cell bodies of these neurons are located to determine if they contain aromatase activity for the conversion of androgens to estrogens or hydroxylase activity for the conversion of estradiol to catecholestrogens. Since the estradiol antibody used for radioimmunoassays appears to bind to something in the SON extract, it could be used for immunocytochemistry to confirm the localization of the immunoreactive estradiol in the nerve fibers. If the immunoreactive estradiol is present in the nerve fibers of the SON, then immunocytochemistry could be used to trace the distribution and source of the immunoreactive estradiol in intact and ligated SON fibers.

The results of these studies suggest that the estradiol immunoreactivity of the SON extract is transported from the CNS to the ovary, raising the possibility that the immunoreactive estradiol is produced in the cell bodies of the axons of the SON. Since SON extracts prepared from segments proximal and distal to the ligation had similar

inhibitory effects on androstenedione accumulation, these studies do not provide information on the source of this activity. These data, however, strongly suggest that the SON extracts' activity inhibitory to thecal androstenedione accumulation and immunoreactive estradiol are distinct compounds. The final chapter will examine the site(s) of action of the inhibitory factor in the SON extract and compare the site of action to that of estradiol and the catecholestrogens.

CHAPTER 11-SITE OF ACTION OF THE INHIBITORY ACTIVITY OF THE SUPERIOR OVARIAN NERVE EXTRACT

11.1 Introduction

In the literature review, I discussed several factors known to inhibit theca cell androstenedione accumulation. These included estrogen, GnRH, prolactin and EGF. In addition, data have been presented in this thesis to show that catecholamines (Chapter 6) and catecholestrogens (Chapter 8) also inhibit thecal androstenedione accumulation *in vitro*.

The site of action of estradiol, catecholestrogens and GnRH appears to be the same, at the level of the 17α-hydroxylase:C_{17-20} lyase enzyme complex (Leung and Armstrong, 1979b; Magoffin and Erickson, 1981; Magoffin et al., 1981; Magoffin and Erickson, 1982 a,b). The inhibition does not involve effects on LH receptor number and/or affinity or the generation of cyclic AMP (Magoffin and Erickson, 1982d). The inhibition is very rapid and irreversible. As a result of the inhibition of the 17α-hydroxylase:$C_{17\,20}$ lyase enzymes, each of these agents causes an accumulation of progesterone and pregnenolone, while inhibiting the formation of 17α-hydroxyprogesterone, androgens and estrogens.

Prolactin (Magoffin and Erickson, 1982b), EGF (Erickson and Case, 1983) and catecholamines (Chapter 6) have similar inhibitory effects on thecal steroidogeneis. Each of these agents causes a generalized inhibition of thecal steroidogenesis, without affecting the generation of cyclic AMP. This suggests that their inhibitory effects are expressed at a site distal to the adenylate cyclase enzyme.

Comparison of the site and mechanism of action of the inhibitory factor in the SON extract with those of other factors known to inhibit theca cell androstenedione accumulation will be used to provide information on the possible identity of the active factor in the SON extract.

11.2 Methods and Results

11.2.1 SON Extract Inhibition of Thecal Androstenedione Accumulation

To examine the ability of the SON extract to inhibit thecal androstenedione accumulation in the presence of increasing concentrations of LH, theca cells were cultured in the absence or presence of increasing concentrations of LH (31.25-500 ng/ml) with or without the SON extract. Treatment with increasing concentrations of LH resulted in dose-dependent increments in androstenedione accumulation in the absence and presence of the SON extract (Figure 36). However, at all doses of LH studied, androstenedione accumulation in the presence of the SON extract was significantly ($p < 0.05$) less than in cells cultured in the absence of the SON extract.

To study the time dependence of the inhibitory effect of the SON extract on thecal androstenedione accumulation, theca cells were cultured in the presence of LH (250 ng/ml), with or without SON extracts for different incubation times (2-48 h). Time-dependent increments in androstenedione accumulation were observed in LH-stimulated cultures in the absence and presence of the SON extract (Figure 37). Treatment of LH-stimulated cultures with the SON extract caused a significant ($p < 0.05$) inhibition of androstenedione accumulation by 6 h and at all subsequent times studied.

Figure 36. SON extract inhibition of thecal androstenedione accumulation: LH dose-dependence. Theca cells were cultured in the absence and presence of increasing concentrations of LH (31.25-500 ng/ml) with or without SON extracts for 48 h. Data are the mean ± S.E.M. of quadruplicate cultures from a single experiment which was replicated one further time. Values with different superscripts are significantly ($p < 0.05$) different. The SON extract inhibited androstenedione accumulation at all doses of LH tested.

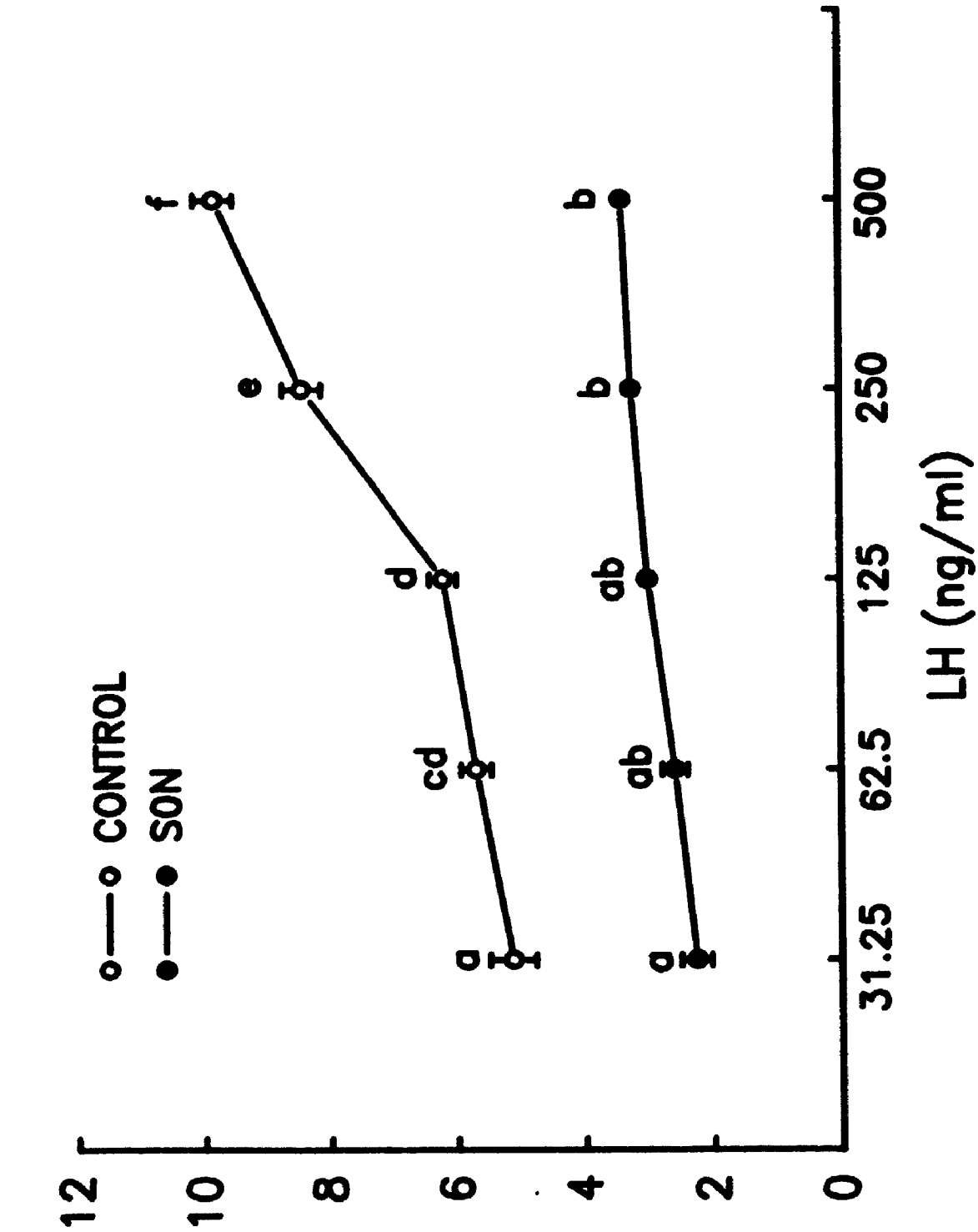
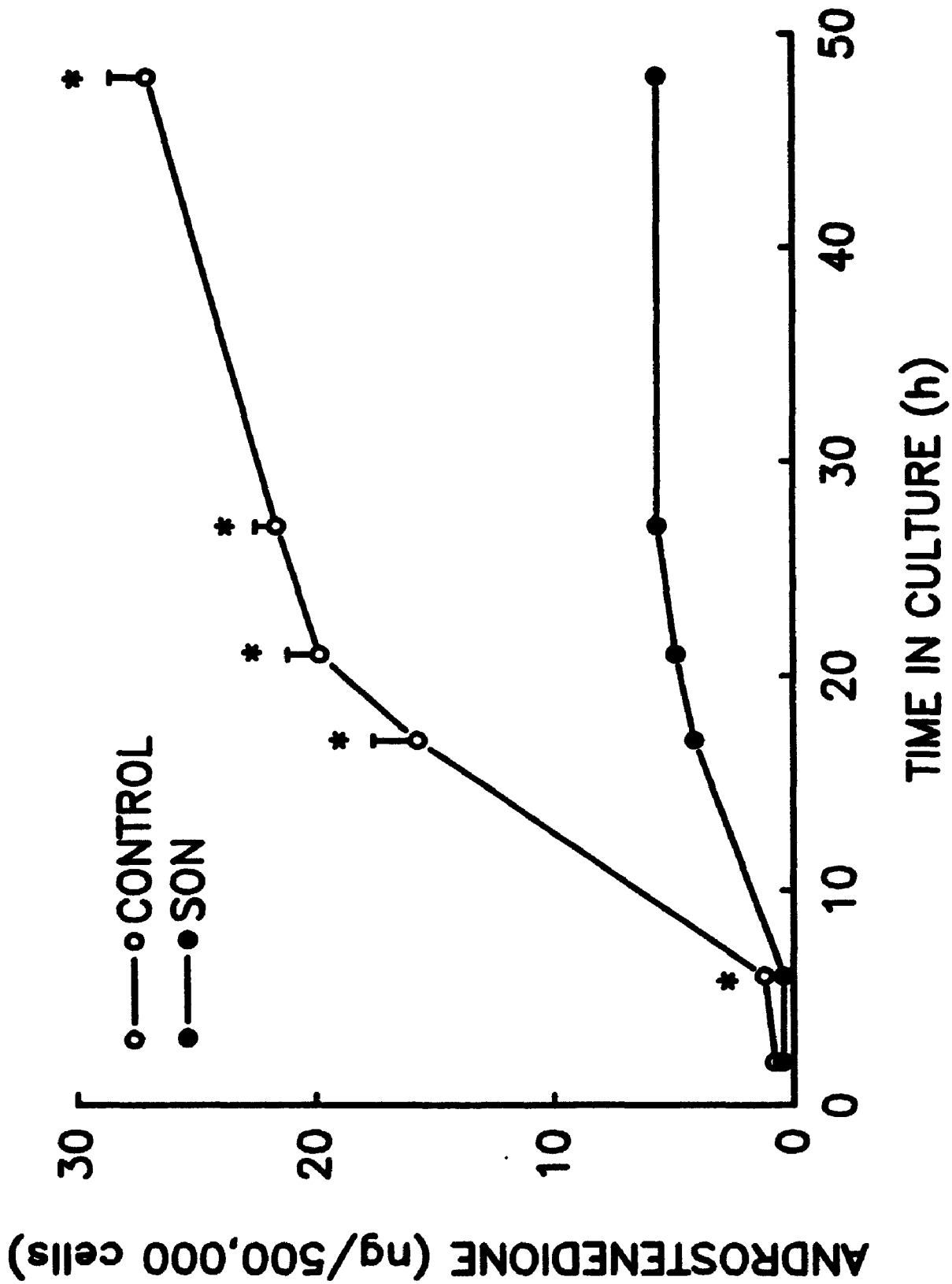

ANDROSTENEDIONE (ng/500,000 cells)
12
10
8
6
4
2
0
o—o CONTROL
●—● SON
a
cd
d
e
f
a
ab
ab
b
b
31.25
62.5
125
250
500
LH (ng/ml)

Figure 37. SON extract inhibition of thecal androstenedione accumulation: Time course. LH-stimulated (250 ng/ml) theca cells were cultured with or without SON extracts and the media was collected at varying times (2-48 h) after plating. Values are the mean ± S.E.M. of quadruplicate cultures from a single experiment. Values with asterisks are significantly ($p < 0.05$) different between cultures incubated with and without the SON extract. The SON extract inhibited androstenedione accumulation by 6 h and at all subsequent times studied.


ANDROSTENEDIONE (ng/500,000 cells)
30
20
10
0
0
10
20
30
40
50
TIME IN CULTURE (h)
o—o CONTROL
●—● SON
*

11.2.2 Site of the SON Extracts' Inhibition of Thecal Androstenedione Accumulation

To determine the site at which the SON extract inhibits thecal androstenedione accumulation, the accumulation of extracellular cyclic AMP in theca cells incubated in the absence and presence of LH (250 ng/ml), with or without the SON, extract was determined. Extracellular cyclic AMP and androstenedione accumulation were measured at the end of 48 h of culture. LH significantly ($p < 0.05$) stimulated the accumulation of extracellular cyclic AMP and androstenedione over control levels (Table 16). Addition of the SON extract significantly ($p < 0.05$) inhibited both basal and LH-stimulated androstenedione accumulation without significantly affecting the accumulation of extracellular cyclic AMP.

To study the ability of the SON extract to inhibit the action of other physiological and pharmacological agents for which cyclic AMP is a second messenger and dibutyryl cyclic AMP, theca cells were cultured in the presence of LH (250 ng/ml), dibutyryl cyclic AMP (0.5 mg/ml; 1 mM), PGE_2 (10 μg/ml), cholera toxin (1 μg/ml) and forskolin (10^{-4} M) in the presence and absence of SON extracts. Dibutyryl cyclic AMP-stimulated cultures also contained the phosphodiesterase inhibitor IBMX (89 μg/ml; 400 μM). All agents caused significant ($p < 0.05$) increases in androstenedione accumulation over control levels and the levels produced were comparable to those stimulated by LH (Figure 38). The SON extract significantly ($p < 0.05$) inhibited LH-, dibutyryl cyclic AMP-, PGE_2, cholera toxin- and forskolin-stimulated androstenedione production by 57, 57, 54, 54 and 49% respectively.

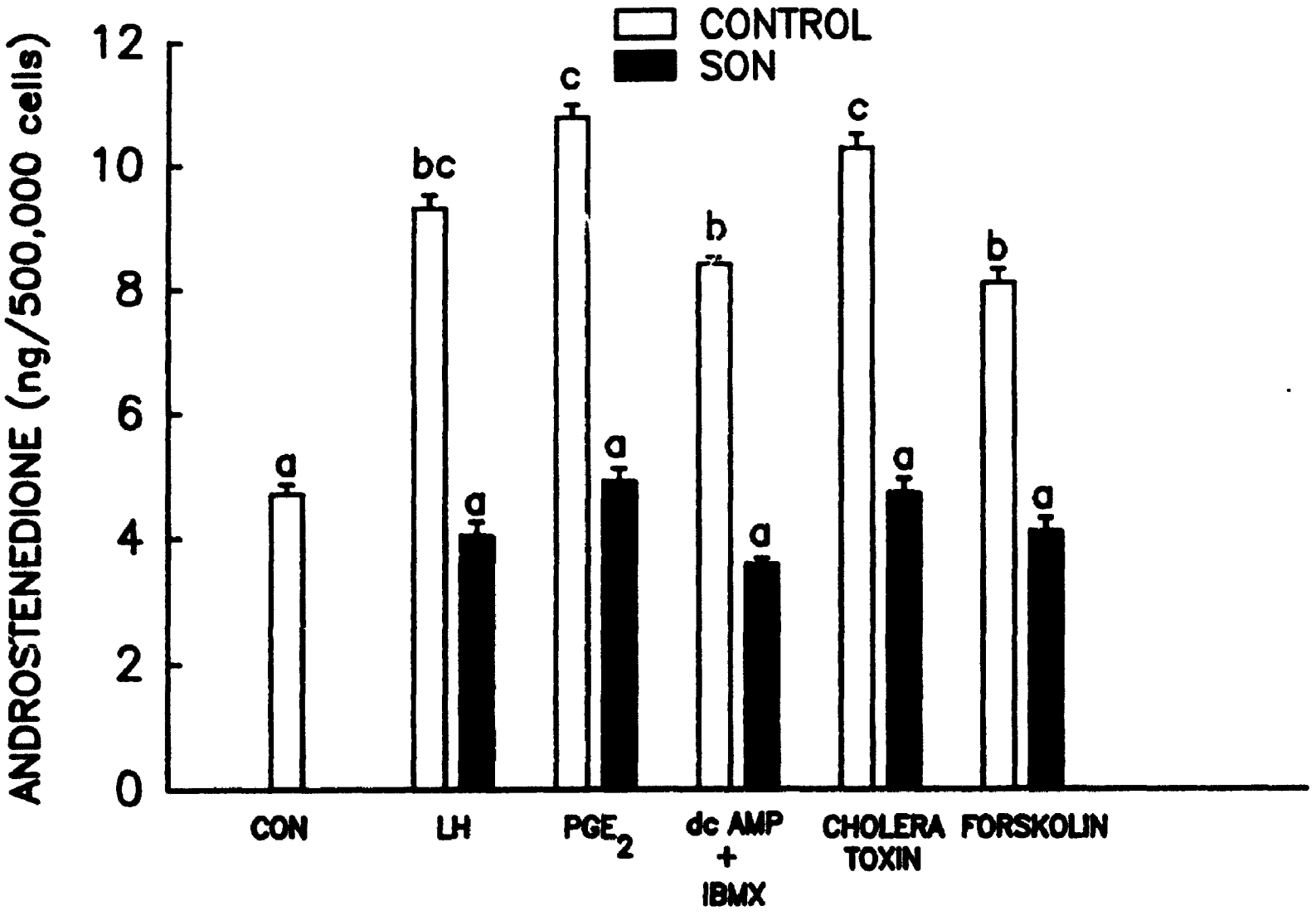
To investigate the site(s) in the steroidogenic pathway at which the SON and SCN extracts exerted their inhibitory and stimulatory effects,

Table 16. Effect of SON extracts on extracellular cyclic AMP and androstenedione accumulation by porcine theca cells.

Treatment[1]	Cyclic AMP (pmol/500,000 cells)	Androstenedione (ng/500,000 cells)
Control	1.65 ± 0.16[a]	4.96 ± 0.08[b]
SON	1.74 ± 0.25[a]	3.12 ± 0.13[a]
LH	6.73 ± 0.30[b]	11.07 ± 0.27[c]
LH + SON	6.99 ± 0.22[b]	3.32 ± 0.16[a]

[1]Theca cells were incubated in the absence (Control) or presence (LH) of LH (250 ng/ml) with or without SON extracts (SON) for 48 h. Values represent the mean ± S.E.M. of quadruplicate cultures from a single experiment which was replicated one further time. Values with different superscripts are significantly ($p < 0.05$) different. The SON extract inhibited basal and LH-stimulated thecal androstenedione accumulation without effecting the accumulation of extracellular cyclic AMP.

Figure 38. Effect of the SON extract on LH-, PGE_2-, dibutyryl cyclic AMP-, cholera to¬- and forskolin-stimulated androstenedione accumulation by porcine theca cells. Theca cells were cultured in the absence (CON) or presence of 250 ng/ml LH, dibutyryl cyclic AMP (db cAMP; 1 mM) and IBMX (400 μM), PGE_2 (10 μg/ml), cholera toxin (1 μg/ml) or forskolin (10^{-4} M), with or without SON extract for 48 h. Values represent the mean $\pm$ S.E.M. of quadruplicate cultures from a single experiment which was replicated one further time. Values with different superscripts are significantly ($p < 0.05$) different. The SON extract caused a significant inhibition of androstenedione accumulation stimulated by all agents.


CONTROL
SON
ANDROSTENEDIONE (ng/500,000 cells)
12
10
8
6
4
2
0
a
bc
a
c
a
b
a
c
a
b
a
CON
LH
PGE2
dc AMP + IBMX
CHOLERA TOXIN
FORSKOLIN

respectively, theca cells were cultured in the absence or presence of LH (250 ng/ml), with or without SON or SCN extracts. As shown previously, LH significantly ($p < 0.05$) increased 17α-hydroxyprogesterone, androstenedione, testosterone, estradiol-17β and estrone concentrations over control levels (Table 17). In the presence of LH, progesterone levels were increased over control levels, but not significantly, and pregnenolone levels were at or below the sensitivity of the assay for both control and LH-stimulated cultures. Following SON treatment, the levels of 17α-hydroxyprogesterone, androstenedione, testosterone, estradiol-17β and estrone in control and LH-stimulated cultures were reduced compared to cultures incubated in the absence of the SON extract. Pregnenolone and progesterone accumulation in both control and LH-stimulated cultures in the presence of the SON extract showed small but not statistically significant increases over cells cultured in the absence of the SON extracts. In the presence of the SCN extract, the accumulation of all steroids measured was increased in both control and LH-stimulated cultures.

To determine if the inhibitory effect of the SON extract was associated with alterations in cell number, cell viability or the number of differentiating theca cells, theca cells were cultured in the absence (Control) and presence of LH (250 ng/ml) with or without the SON extract. To determine cell number, culture media were removed after 48 h, the cells scraped from the culture well and counted in a Coulter Electronics Inc, Model ZB1 Coulter Counter. As shown in Table 18 there was no significant difference between theca cell number in control and LH-stimulated cultures. However, in the presence of the SON extract theca cell number

Table 17. Effect of nerve extracts on porcine theca cell steroid accumulation.

Steroid Accumulation (ng/500,000 cells)	Treatment[1]					
	Control	SON	SCN	LH	LH + SON	LH + SCN
Pregnenolone	0 ± 0^{a}	0.06 ± 0.03^{a}	0.38 ± 0.03^{a}	0 ± 0^{a}	0.38 ± 0.26^{a}	0.60 ± 0.19^{a}
Progesterone	2.90 ± 1.41^{a}	6.72 ± 0.92^{ab}	6.68 ± 0.38^{ab}	5.88 ± 1.02^{ab}	8.63 ± 0.82^{bc}	12.25 ± 0.94^{c}
17α-Hydroxy-progesterone	5.03 ± 0.72^{a}	4.05 ± 0.07^{a}	6.66 ± 1.89^{ab}	7.11 ± 1.51^{ab}	5.89 ± 0.31^{ab}	8.73 ± 0.88^{b}
Androstenedione	5.75 ± 0.47^{b}	4.16 ± 0.06^{a}	8.72 ± 0.77^{c}	13.38 ± 1.66^{d}	8.12 ± 0.62^{c}	18.57 ± 0.76^{e}
Testosterone	0.17 ± 0.03^{b}	0 ± 0^{a}	0.32 ± 0.05^{c}	0.96 ± 0.05^{d}	0.25 ± 0.06^{bc}	1.27 ± 0.05^{e}
Estradiol-17ß	0.56 ± 0.01^{b}	0.27 ± 0.02^{a}	0.89 ± 0.10^{cd}	0.78 ± 0.03^{c}	0.39 ± 0.02^{ab}	1.04 ± 0.08^{d}
Estrone	0.12 ± 0.01^{ab}	0.08 ± 0.01^{a}	0.16 ± 0.02^{b}	0.23 ± 0.01^{c}	0.12 ± 0.01^{ab}	0.37 ± 0.04^{d}

[1]Theca cells were cultured with (LH) or without (Control) LH (250 ng/ml) in the absence or presence of SON and SCN extracts (SON and SCN; 100 µl/ml). At the end of 48 h of culture the media was removed for radioimmunoassay of secreted steroids. Values represent the mean ± S.E.M. of quadruplicate cultures from a single experiment which was replicated two further times. Values with different superscripts are significantly different ($p < 0.05$) for each steroid. Treatment of LH-stimulated theca cells with SON extracts caused a decrease in 17α-hydroxyprogesterone, androstenedione, testosterone, estradiol and estrone accumulation, and small but not statistically significant increases in pregnenolone and progesterone accumulation. In contrast, the SCN extract increased the accumulation of all steroids measured.

Table 18. Effect of SON extracts on theca cell number, viability and alkaline phosphatase activity.

Treatment[1]	Cell Number (10^3)	Cell Viability (%)	ALP Activity (nmol substrate hydrolyzed/30 min)
Control	749±6.3^{ab}	84.7±2.9^{a}	1474±117^{a}
SON	805±8.1^{c}	83.9±2.3^{a}	1382±28^{a}
LH	717±10.5^{a}	81.5±1.7^{a}	1276±60^{a}
LH + SON	776±3.3^{bc}	84.9±2.8^{a}	1303±59^{a}

[1]Theca cells were cultured in the absence (Control) and presence of LH (250 ng/ml) with or without SON extracts (SON). Cell number was measured by direct counting in a Coulter Counter, cell viability was measured by trypan blue exclusion and alkaline phosphatase activity was measured by the spectrofluorometric assay described in the Materials and Methods. For cell number and viability, data are the mean ± S.E.M. of five cultures from a single experiment and for alkaline phosphatase activity data represent the mean ± S.E.M. of triplicate cultures from a single experiment. Values with different superscripts are significantly ($p < 0.05$) different for each experiment. Control and LH-stimulated theca cell viability and alkaline phosphatase activity after 48 h of culture were not significantly different in the absence and presence of the SON extract. In the presence of the SON extract both control and LH-stimulated theca cell number was significantly increased.

showed small, but statistically significant ($p < 0.05$), increases in both control and LH-stimulated cultures.

Control and LH-stimulated theca cell viability in the presence and absence of the SON extract was determined by trypan blue exclusion after 48 h of culture. As shown in Table 20, there was no significant effect of the addition of the SON extract on control or LH-stimulated theca cell viability.

Since alkaline phosphatase is an index of theca cell differentiation (Erickson et al., 1985), alkaline phosphatase activity in the presence and absence of the SON extract was determined. As shown in Table 20, there was no significant effect of the addition of the SON extract on control or LH-stimulated theca cell alkaline phosphatase activity.

11.3 Discussion

The site of the inhibitory effect of extracts of the SON of rats on porcine theca cell steroidogenesis was investigated using cultured porcine theca cells. These findings indicate that the SON extracts effects are time-dependent, but independent of the LH dose employed. The inhibitory effect of the SON extract, which had a very rapid onset, was not associated with alterations in cell number, cell viability or theca cell alkaline phosphatase activity, suggesting the inhibitory effect was not due to a decreased theca cell survival.

The failure of the SON extract to prevent the LH-stimulated increase in cyclic AMP accumulation indicates that the inhibitory influence of the SON extract occurs distal to the generation of cyclic AMP. This suggests that the SON extract does not affect LH receptor number and/or affinity or the LH sensitive adenylate cyclase system. These conclusions are

supported by the findings that in cultures containing the SON extract in which androstenedione accumulation was inhibited, there was no significant inhibition of theca cell cyclic AMP accumulation. In addition, androstenedione accumulation in response to a variety of physiological and pharmacological agents for which cyclic AMP is a second messenger, was also inhibited.

To determine the site in the steroid biosynthetic pathway at which the SON extract exerts its inhibitory effect the steroid metabolic profile in the presence and absence of the SON extract was examined. The results suggest the antagonistic effects of the SON extract on ovarian androgen biosynthesis are expressed through a selective inhibition of the 17α-hydroxylase:$C_{17,20}$ lyase enzyme complex, thereby preventing the conversion of C_{21} steroids to C_{19} steroids. Inhibition of this complex results in an accumulation of pregnenolone and progesterone and an inhibition of the accumulation of 17α-hydroxyprogesterone, androstenedione, testosterone, estradiol and estrone. The large increase in pregnenolone accumulation seen in theca cells cultured with estradiol (Chapter 8) was not evident in cells cultured with the SON extract. Instead, the steroid profile in the presence of the SON extract more closely resembled that of theca cells incubated with catecholestrogens. Direct enzyme assays of 17α-hydroxylase and the $C_{17,20}$ lyase are necessary to confirm this conclusion. Since all of the metabolites of androstenedione have not been measured in this study, the possibility that the SON extracts were decreasing androstenedione accumulation by increasing androstenedione metabolism cannot be ruled out.

The site of action of the SON extract could be investigated further by incubating theca cells with ^{14}C-4-pregnenolone in the presence of

estradiol, catecholestrogens and the SON extract, followed by isolation of the radioactive metabolites formed. The radioactive metabolites can be extracted, separated by thin layer chromatography and the labelled products located by autoradiography. The R_f values of the metabolites can then be compared with appropriate standards, the radioactive spots scraped from the chromatograms and the amount of radioactivity determined by scintillation counting. Samples of each metabolite can be run in two different HPLC systems to provide an identification of each metabolite. This study should confirm the radioimmunoassay results that estradiol, catecholestrogens and the SON extract each act through an inhibition of the 17α-hydroxylase:C_{17-20} lyase enzyme complex. Comparison of the metabolites formed in the presence of estradiol and catecholestrogens may provide evidence as to why pregnenolone accumulation is much greater in cultures incubated with estradiol than with catecholestrogens. It would also be possible to compare the pregnenolone metabolic profile in the presence of the SON extract with that of estradiol and the catecholestrogens as a means of providing more information on the site of action and the possible identity of the SON extracts' active factor.

The site in the steroid biosynthetic pathway at which the SON extract exerts its inhibitory action is similar to that previously described for estrogen (Leung and Armstrong, 1979 a,b; Magoffin and Erickson, 1981) and GnRH (Magoffin et al., 1981; Magoffin and Erickson, 1982a). It is unlikely that the active factor is GnRH since it has been demonstrated that the pig ovary does not possess GnRH receptors (Brown and Reeves, 1983). Prolactin (Magoffin and Erickson, 1982b), EGF (Erickson and Case, 1983) and catecholamines (Chapter 6) although inhibitory to thecal androstenedione accumulation can be ruled out as being the active factor

since these factors all cause a generalized inhibition of thecal steroidogenesis not seen in the presence of the SON extract.

The small increase in theca cell number in the presence of the SON extract is an interesting observation. In muscle tissue, peripheral nerves have a well described trophic action. As described in the literature review, theca cells undergo a very rapid growth during follicular development; however, it is not known if the innervation of the ovary plays a role in this process. Using the in vitro culture model for porcine theca cells, the effect of nerve factors on cell growth could be studied by examining the effects of nerve extracts on theca cell number, DNA and protein content, the incorporation of ^{3}H-thymidine into DNA and the incorporation of labelled amino acids into cellular protein.

CHAPTER 12 - GENERAL DISCUSSION

Androgens are important intraovarian regulators and play important roles in controlling progesterone biosynthesis, follicle growth and atresia and oocyte maturation. Their importance is demonstrated by the severe reproductive abnormalities that occur when androgen production is abnormal. While the control of ovarian theca cell androgen production by the pituitary gonadotropins has been well established the neural control of ovarian steroidogenesis has received little attention, despite the demonstration of the innervation of these cells over 100 years ago. These studies were initiated to investigate the potential regulatory role of ovarian nerves on thecal steroidogenesis.

A serum-free culture system for porcine theca cells was developed to study the direct effects of ovarian nerve factors and putative ovarian neurotransmitters on thecal steroidogenesis under defined conditions. In culture, porcine theca cells responded to LH with increases in cyclic AMP and androgen accumulation in a manner comparable to that observed in vivo.

The presence of noradrenergic and a variety of peptidergic nerve fibers innervating the ovary have been demonstrated by histochemical and immunocytochemical methods. Initial studies investigated the effects of catecholamines and putative ovarian neurotransmitters on thecal steroidogenesis in vitro. These studies demonstrated that β-, but not α-adrenergic stimulation inhibited porcine thecal androgen accumulation in a dose- and time-dependent manner through a generalized suppression of steroidogenesis. The site of their inhibitory effects were distal to adenylate cyclase since catecholamines did not affect extracellular cyclic AMP accumulation. It was not established if the inhibitory effects on

steroidogenesis were due to a inhibition of each step in the steroidogenic pathway or due to inhibition at an early step in the pathway. Enhancement of the inhibitory effects of catecholamines in the presence of an inhibitor of the COMT enzyme, suggests the presence of an active COMT enzyme system in porcine theca cells.

The physiological importance of the inhibitory effect of catecholamines on thecal androgen accumulation was not established, nor did these studies demonstrate that the catecholamines were from ovarian nerves. Catecholamines of adrenal origin could reach the ovary via the circulation. In addition, no effects of other putative ovarian neurotransmitters could be demonstrated.

In order to look directly at the regulatory role of potential ovarian nerve factors on thecal androgen production, extracts of superior ovarian nerves were prepared and added to theca cell cultures. Thecal androstenedione accumulation was decreased by extracts of rat superior ovarian nerves while non-ovarian sciatic nerve controls increased androstenedione accumulation. Control studies attempted to establish that the superior ovarian nerves' inhibitory activity was of neural origin. The inhibitory activity was not present in ligamentous, connective, muscle or adipose tissues that were associated with the suspensory ligament. By testing the effect of the rat SON extract in rat theca cell cultures the effects of the extract were shown not to be species specific.

The SON, but not the SCN extract, contained estradiol immunoreactivity, suggesting that there is an estradiol-like molecule in these peripheral nerves. This observation was particularly interesting since estradiol is a well described inhibitor of thecal androgen production; however, the amount of immunoreactive estradiol in the SON extract was far less than

had previously been shown to be necessary to inhibit porcine thecal androstenedione accumulation in vitro. These studies also showed that the estrogen metabolites, the catecholestrogens, had similar inhibitory effects on thecal androgen accumulation as estradiol.

The site of the inhibitory action of the SON extract on thecal steroidogenesis was investigated to compare the site of action to the site of action of other known inhibitors of thecal androgen accumulation. Radioimmunoassay studies of the steroid metabolic profile suggested that the SON extract inhibits androgen production through an inhibition of the 17α-hydroxylase:C_{17-20} lyase enzyme complex. This was concluded based on the observation that in the presence of the SON extract extracellular cyclic AMP concentrations were not affected, 17α-hydroxyprogesterone and androgen accumulation were inhibited and progesterone and pregnenolone accumulation were increased. The possibility that the SON extract also enhanced the metabolism of androstenedione was not investigated. This site of action is similar to that previously reported for estradiol and GnRH, however the porcine ovary has been reported not to contain GnRH receptors, so it is unlikely that the inhibitory activity is related to GnRH.

Biochemical tests were attempted to determine the characteristics of the inhibitory factor in the SON extract. The nerve extract was extractable with charcoal and organic solvents and stable to boiling and chymotrypsin treatment. In addition, both the inhibitory activity and estradiol immunoreactivity of the SON extract eluted in similar fractions as standard estradiol and catecholestrogens on Sephadex LH-20 columns.

Preliminary attempts to determine the source of the inhibitory factor and estrogen immunoreactivity suggested that the immunoreactive estradiol activity is transported down the axons of the SON to the ovary. This

observation is based on the increase in the amount of estradiol immunoreactivity found proximal to a ligature tied around the suspensory ligament. However, the observation that extracts prepared from segments of the nerve both proximal and distal to the ligature caused a similar degree of inhibition of thecal androgen production, suggested that the inhibitory activity and estradiol immunoreactivity are distinct compounds.

These studies have shown the direct effects of an ovarian nerve factor on porcine thecal androgen accumulation in vitro. These studies provide a small step toward the long range goal of determining the possible regulatory role of ovarian nerves in controlling ovarian function.

Future experiments should initially make use of physicochemical methods to establish the identity of both the factor inhibitory to thecal androgen accumulation and the estradiol immunoreactivity. The studies should also determine if the estradiol immunoreactivity and the inhibitory activity of the extract are the same or distinct compounds.

Once the identity of the inhibitory factor is established an assay could be developed to measure quantitative changes in the levels of the active factor during various reproductive states. This would allow the association of changes in neural activity with changes in physiological states. Immunocytochemical studies would also be useful to establish that the estradiol antibody is binding to a factor in the fibers of the SON. This procedure could also be used to trace the origin of the fibers of the SON to the CNS.

Additional studies should be done to look at the interactions of catecholamines, ovarian nerve factors and the catecholestrogens in the control of thecal steroidogenesis. These interactions may be very important to the physiological control of ovarian function given the

recent observations of the importance of cotransmission and the interaction of synaptic transmitters.

CHAPTER 13 - SUMMARY AND CONCLUSIONS

The results presented in this thesis can be summarized as follows:

1) Freshly isolated porcine theca cells from prepubertal gilts cultured in defined medium respond to LH with increased cyclic AMP and androstenedione accumulation. This *in vitro* system provides a model in which to study the direct effects of putative ovarian neurotransmitters and ovarian nerve factors on thecal androgen production without the confounding effects on blood flow present in *in vivo* studies.

2) Catecholamines inhibit thecal androstenedione accumulation via β- but not α-, adrenergic receptors. The catecholamine effect is dose- and time-dependent and is enhanced in the presence of the catechol-o-methyl transferase inhibitor U-0521. Catecholamines produce a generalized inhibition of thecal steroidogenesis at a site distal to the formation of cyclic AMP and at or before the cholesterol side chain cleavage enzyme.

3) Other putative ovarian neurotransmitters (GABA, neuropeptide Y, dopamine, vasoactive intestinal peptide, oxytocin and the acetylcholine agonist carbachol) do not effect thecal androstenedione accumulation *in vitro*.

4) Extracts of rat superior ovarian nerves inhibit both rat and porcine thecal androstenedione accumulation in a dose-dependent manner. Extracts of rat sciatic nerves, used as non-ovarian nerve controls, stimulate thecal androstenedione accumulation.

5) The inhibitory effects of the superior ovarian nerve extract are not due to contamination by factors from other tissues in the suspensory ligament, or to the presence of catecholamines in the extract.

6) Estradiol and catecholestrogens inhibit thecal androstenedione accumulation in vitro in a similar manner as the superior ovarian nerve extract. All of these agents cause a rapid (2-6 h) and specific inhibition of the 17α-hydroxylase:C_{17-20} lyase enzyme complex, resulting in an inhibition of androgen accumulation and an increase in progesterone and pregnenolone accumulation.

7) The SON extract and catecholestrogens both cross react in the estradiol radioimmunoassay.

8) The superior ovarian nerve extract's activity inhibitory to thecal androstenedione accumulation and estradiol immunoreactivity are extracted by charcoal and organic solvents, but are stable to heat and protease digestion.

9) The superior ovarian nerve extract's inhibitory activity and estradiol immunoreactivity elute in the same fractions as estradiol and catecholestrogens on Sephadex LH-20 columns.

10) When the suspensory ligament, and hence the superior ovarian nerve, is ligated the proximal segment contains significantly more immunoreactive estradiol than the distal segment, however, both inhibit thecal androstenedione accumulation to a similar degree.

11) Freshly isolated superior ovarian nerves are unable to aromatize androstenedione to estrogens *in vitro*.

The results presented in this thesis suggest that the superior ovarian nerve of rats contains a factor inhibitory to theca cell androstenedione accumulation and a factor that cross reacts with an estradiol antibody. These studies were unable to determine if the two factors were the same or distinct compounds. Although few clear answers have resulted from these studies, the results suggest that in addition to the well described hypothalamic-pituitary-ovarian hormonal system, neural connections between the CNS and the ovary may play a role in controlling ovarian theca cell androgen production. Future experiments to determine the function of the ovarian nerves need to look at physiological ovarian responses such as follicular growth, atresia and the control of follicular progesterone biosynthesis in which androgens are known to play important roles. Since the superior ovarian nerve is a mixed nerve, with fibers also innervating the uterus, specific chronic stimulation studies to measure ovarian androgen secretion or immunoreactive estradiol accumulation in the ovary are not possible.

It is not known how nerves exert their effects on the ovarian steroidogenic cells. From studies in other visceral organs, the nerves could provide a fine tuning of the hormonal responses or they could act

as a rapid communication system between the CNS and the ovary, in addition to the slower hormonal system. In vivo the actual androgen production may depend on the balance of inhibitory neural inputs and stimulatory gonadotropin inputs to the theca cells. It is not known how the neural inputs to the ovary are controlled.

Little consideration has been given to the possibility of afferent versus efferent nerves. In attempting to assess the function of these nerves, it is of primary importance to establish whether the nerves are sensory or motor in type. The lack of specific sensory organs and the presence of catecholamines in these nerves argue in favour of a motor innervation.

Ovarian nerve physiology is still at an early stage of development. Many important questions remain to be answered to determine the role of the ovarian innervation in normal and pathological ovarian function.

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Gregory F Erickson

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