ABSTRACT

A common sequential pathway for steroidogenesis, designed to produce steroid hormones, is expressed in the adrenal cortex, testes and ovaries. In this process, cholesterol is converted to all known steroids by specific enzymes in a tissue-specific manner. In addition to the maintenance of reproduction, fertility and normal sexual development by androgens and estrogens, mineralocorticoids and glucocorticoids, other steroids produced by the adrenal cortex, play important roles in the regulation of salt balance and responses to stress, respectively. Steroidogenesis is controlled by interactions between the hypothalamus and pituitary in the form of a negative feedback loop, and, disruption of any step in this loop exerts an impact on the entire axis.

In recent years, numerous anthropogenic or naturally occurring compounds in the environment have been reported to influence the homeostasis of endocrine systems. These xenobiotics, referred to as endocrine disrupting chemicals (EDCs), may disrupt the development and/or functioning of various physiological systems, including reproduction, fertility, sexual differentiation and stress responses, both in wildlife and humans. Such disruption may be involved in the increased incidences of testicular cancer, hypospadias and cryptorchidism, as well as the decline in sperm count observed in humans during the past decades. Accordingly, the aim of the present thesis was to explore the effects of several xenobiotics (i.e., the anti-androgen procymidone, the phytoestrogens genistein and resveratrol, and sesquiterpene lactone helenalin and phthalates) on the function of the pituitary-gonadal and pituitary-adrenal axes and the biosynthesis of sex steroids and glucocorticoids, employing *in vitro* and *ex vivo* cultures of rat Leydig and adrenocortical cells as a model system.

Dietary administration of procymidone to male rats resulted in elevated serum levels of luteinizing hormone (LH) and testosterone. Furthermore, Leydig cells isolated from these animals displayed an enhanced capacity for the production of testosterone in response to stimulation by human chorionic gonadotropin (hCG) or $(Bu)_2$cAMP, as well as elevated expression of steroidogenic acute regulatory (StAR) protein, cytochrome P450scc (P450scc) and cytochrome P450c17 (P450c17). In contrast, diets containing genistein inhibited the *ex vivo* steroidogenic response of Leydig cells to hCG or $(Bu)_2$cAMP by down-regulating their expression of P450scc. In a similar manner, phytoestrogen resveratrol suppressed corticosterone production by primary cultures of adrenocortical cells both *in vitro* and *ex vivo* in association with a decrease in the expression of cytochrome P450 c21-hydroxylase. Helenalin, a sesquiterpene lactone produced by several species of the Asteracea family of plants, inhibited both adrenocorticotropic hormone (ACTH) - and hCG-activated steroidogenesis in primary cultures of rat adrenocortical and Leydig cells as a result of

attenuated expression of the StAR protein, which mediates cholesterol transport into mitochondria.

Further experiments demonstrated that administration of di-2-ethylhexyl phthalate (DEHP) to male rats causes age-dependent alterations in their pituitary-adrenocortical axis *in vivo* and adrenocortical steroidogenesis *ex vivo.* Thus oral exposure to this phthalate ester elevated serum levels of ACTH and corticosterone in rats 20 and 40 days of age, but not in adult, 60-day-old animals. In addition, primary cultures of adrenocortical cells isolated from the two younger groups of rats treated with DEHP exhibited enhanced production of corticosterone in response to stimulators, as well as elevated ACTH-stimulated transport of endogenous cholesterol into mitochondria.

Together, these finding reveal that the EDCs examined can influence the function and regulation of the pituitary-gonadal and pituitary-adrenal axes in rats, as well as impair their adrenocortical and Leydig cell steroidogenesis by suppressing the expression of several proteins involved in steroidogenesis (e.g., StAR, P450c21 and P450scc). Such effects might disrupt reproductive potential, fertility and homeostasis in both humans and wildlife.

Keywords: Endocrine disrupting chemicals (EDCs), steroidogenesis, Leydig cells, adrenocortical cells, steroidogenic acute regulatory protein (StAR) and steroidogenic enzymes

LIST OF PUBLICATIONS

The present thesis is based on the following publications, which will be referred to by their Roman numerals:

I. Svechnikov K, **Supornsilchai V**, Strand ML, Wahlgren A, Seidlova-Wuttke D, Wuttke W, Söder O. Influence of long-term dietary administration of procymidone, a fungicide with anti-androgenic effects, or the phytoestrogen genistein to rats on the pituitary-gonadal axis and Leydig cell steroidogenesis. *J Endocrinol, 2005,187:117-24.*

II. **Supornsilchai V**, Svechnikov K, Seidlova-Wuttke D, Wuttke W, Söder O. Phytoestrogen resveratrol suppresses steroidogenesis by rat adrenocortical cells by inhibiting cytochrome P450c21-hydroxylase. *Horm Res,2005,64:280-6.*

III. **Supornsilchai V**, Söder O, Svechnikov K. Sesquiterpene lactone helenalin suppresses adrenocortical and testicular steroidogenesis by inhibiting steroidogenic acute regulatory protein (StAR) in vitro. *Reprod Toxicol,2006,22:631-5.*

IV. **Supornsilchai V**, Söder O, Svechnikov K. Stimulation of the pituitary-adrenal axis and of adrenocortical steroidogenesis ex vivo by administration of di-2-ethylhexyl phthalate to prepubertal male rats. *J Endocrinol, 2007,192:33-9.*

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LIST OF ABBREVIATIONS

SER	Smooth endoplasmic reticulum
AMH	Anti-Müllerian hormone
DHT	Dihydrotestosterone
ACTH	Adrenocorticotropic hormone
FSH/LH	Follicular stimulating hormone/ Luteinizing hormone
hCG	Human Choriotropin gonadotropin
SCP2	Sterol carrier protein 2
SAP	Steroidogenesis activator polypeptide
DBI	Diazepam binding inhibitor
PBR	Peripheral benzodiazepine receptor
MBR	Mitochondrial DBI receptor
StAR	Steroidogenic acute regulatory protein
P450scc	Side chain cleavage enzyme
P450c17	17α-hydroxylase
P450c11	11β- hydroxylase
P450c21	21α-hydroxylase
P450aldo	Aldosterone synthase
P450aro	Aromatase
3β-HSD	3β-hydroxysteroid dehydrogenase
17β-HSD	17β- hydroxysteroid dehydrogenase
DHEA	Dehydroepiandrosterone
HPA/HPG	Hypothalamic-pituitary-adrenal/gonadal axes
GnRH	Gonadotropin releasing hormone
CRH	Corticotropin releasing hormone
22R-OHC	22R-hydroxycholesterol
ER	Estrogen receptor
DEHP	Di-2-ethylhexyl phthalate
MEHP	Mone-2- ethylhexyl phthalate
PPAR	Peroxisome proliferators activator receptor
HRP	Horseradish peroxidase
RIA	Radioimmunoassay

ELISA	Enzyme linked immunosorbant assay
(BU)2cAMP	Dibutyryl cyclic adenosine monophosphate
IGF-I	Insulin-like growth factor-I
Cox-1	Cyclooxygenase-1
TNF	Tumor necrosis factor
LIF	Leukemia inhibitory factor
TGF-α	Transforming growth factor α
TGF-β	Transforming growth factor β
SCF	Stem cell factor
TBT	Tributylin
DDE/DDT	Dichlorodiphenyltrichloroethane/ dichlorodiphenyldichloroethylene
PCBs	Polychlorinated biphenyls
EDCs	Endocrine disrupting chemicals
CDC	Center of disease control
AR	Androgen receptor
PTK	Protein tyrosine kinase
CREB	Cyclic AMP responsive element binding protein
3α-HSD	3α-hydroxysteroid dehydrogenase
TMB	Tetramethylbenzidine

1 INTRODUCTION

1.1 The adrenal cortex

1.1.1 Structure and cell types

The two main regions of the adrenal gland have different embryonic origins, the cortex arising from the mesoderm and the medulla from the neuroectoderm. In humans, after 4-5 weeks of gestation, the primitive cortex has developed from the coelomic epithelium, to later be covered by the permanent cortex. Following 8 weeks of gestation, the steroidogenic cells from the genital ridge have migrated retroperitoneally to the permanent cortex, where they subsequently form the fetal cortex (Migeon *et al.*, 2003). At the time of birth, the outer region of the fetal adrenal cortex has differentiated into a zona fasciculata and zona glomerulosa and at 1 year of age, the fetal cortex has disappeared and the zona reticularis formed, which however, is not morphologically distinguishable until 3 years of age (Miller *et al.*, 2002).

In the average adult human each adrenal gland located on top of the upper pole of each kidney weighs approximately 4 gm and has a triangular form with the dimensions 2 x 4 x 1 cm. In mature rats, each adrenal accounts for about 0.02% of the total body weight (Sapirstein *et al.*, 1959). The adrenal cortex accounts for 80-90% of the mature gland, the remainder being the medulla. The cortex can be divided morphologically and functionally into three distinctive zones: the outer zona glomerulosa, which secretes mineralocorticoids; the middle zona fasciculata, which secretes glucocorticoids; and the inner zona reticularis, that secretes androgens. These zones constitute approximately 15, 75 and 10% of the total weight of the cortex, respectively.

The cells of each zone exhibit characteristic densities, diameters and ultrastructural features (i.e., lipid droplets in the cytoplasm, nuclear shapes, abundance of mitochondria and smooth endoplasmic reticulum (SER) (Table 1)) that can be used for their identification (Rosol *et al*., 2001). The cells of all three zones contain prominent mitochondria, but with cristae of different shapes. The cells of the zona glomerulosa are characterized by extensive Golgi apparatus, whereas those of the zona fasciculata contain many cytoplasmic lipid droplets and abundant SER. The zona reticularis is distinguished by the presence of large numbers of lysosomes.
The adrenal gland receives its blood via the aorta and renal and phrenic arteries,

which form a network of arteriole anastomosis in the subcortical area that distributes the blood into cortical sinuses (Hinson *et al*., 1996). This organ is highly vascularized, e.g., approximately 0.14% of the cardiac output passes through the rat adrenal, which only accounts for 0.02% of the body weight (Sapirstein *et al*., 1959). The system of vessels associated with the left adrenal vein drains into the left renal vein, while the right adrenal vein empties directly into the vena cava.

Parameter	Z. glomerulosa	Z. fasciculata	Z. reticularis
Size	11-13×8-10 μm	18-22×10-15 μm	Intermediate
Density	1.072 ± 0.04	1.040 ± 0.001	Intermediate
Lipid droplets	++	++++	++
Mitochondrial cristae	Tubular	Vesicular	Tubulovesicular
SER	+	++++	++++
Golgi apparatus	++++	++	++
Lysosomes	-	++	++++

Table 1 *Characteristic features and representative illustrations of the cells located in the three different zones of the adrenal cortex*

1.1.2 Functions and regulation

The activities of adrenocortical cells are regulated by the HPA axis (Fig 1). The hypothalamus secretes CRH, which is transported to the anterior pituitary, where it stimulates cells to secrete adrenocorticotropic hormone (ACTH), which, in turn, stimulates cells in the adrenal glands to synthesise and secrete glucocorticoids (Morohashi *et al*., 1997). All mammalian species, except the dog (Dallman *et al*., 1987), exhibit a distinct circadian pattern of secretion of adrenocortical steroids in response to peak serum levels of ACTH between 6 and 9 AM and lowest levels in the evening. In the rat, which is a nocturnal animal, this rhythm is reversed with the peak levels occurring in the evening (Hinson, 1996).

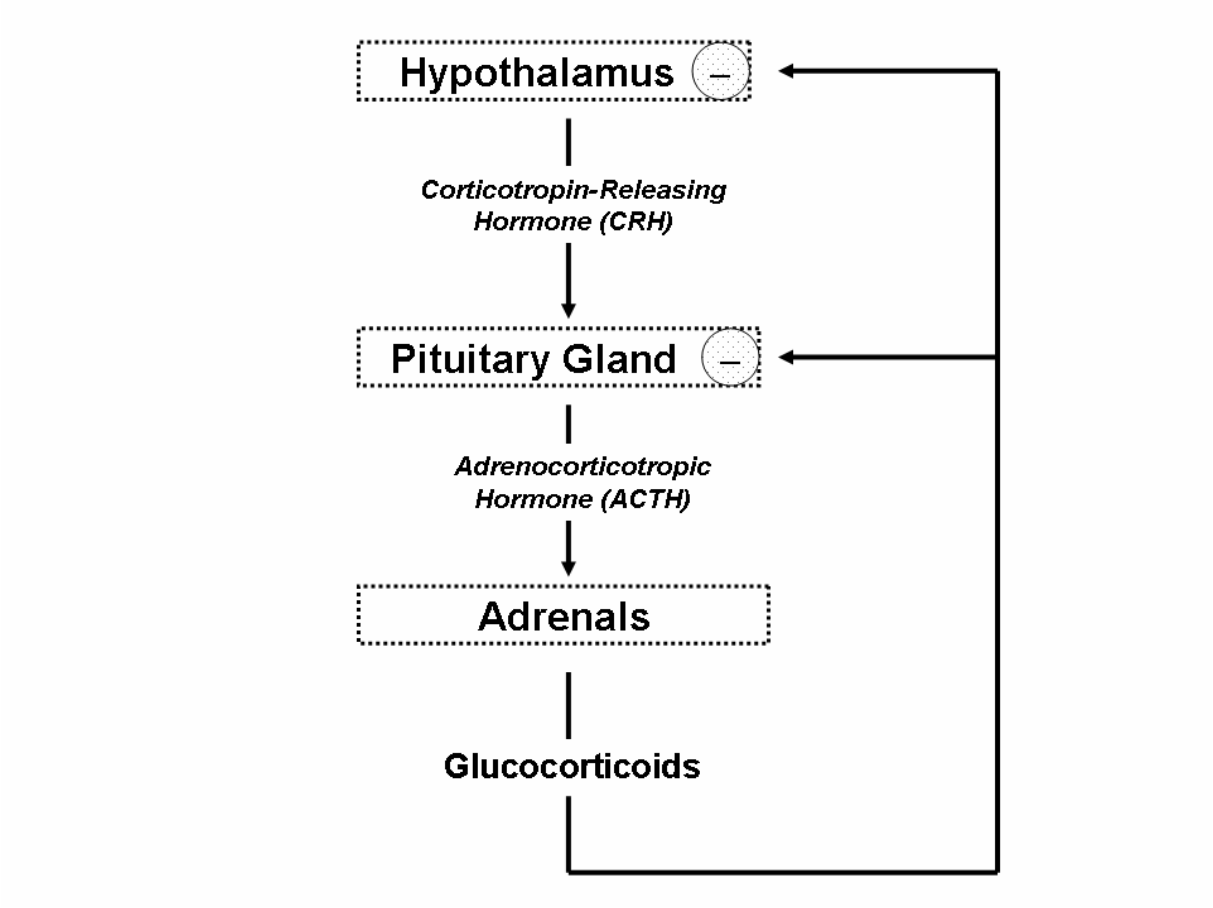

Figure 2 *Feedback regulation of glucocorticoid synthesis via the hypothalamic-pituitary-adrenal axis (HPA).*

Although, adrenocortical steroidogenesis is regulated primarily by ACTH and by the levels of glucocorticoids secreted, certain evidence indicates that other factors may also be involved in this regulation. Accordingly the integrated control of adrenocortical function involves cortico-medullary interactions, the vascular supply and neural input of the gland, the immune system, growth factors, and the intra-glandular renin-angiotensin and CRH-ACTH systems (Monika *et al*., 1998 (Fig 3).

1.2 The testis

1.2.1 Structure and cell types

The testis contains two primary functional elements, namely, the seminiferous tubules where sperm develop and are transported to the ejaculatory ducts and, the Leydig cells in the interstitial tissue, which contain the enzymes that produce androgenic hormones (Fig.4). The seminiferous tubules contain both germ cells and Sertoli cells which are

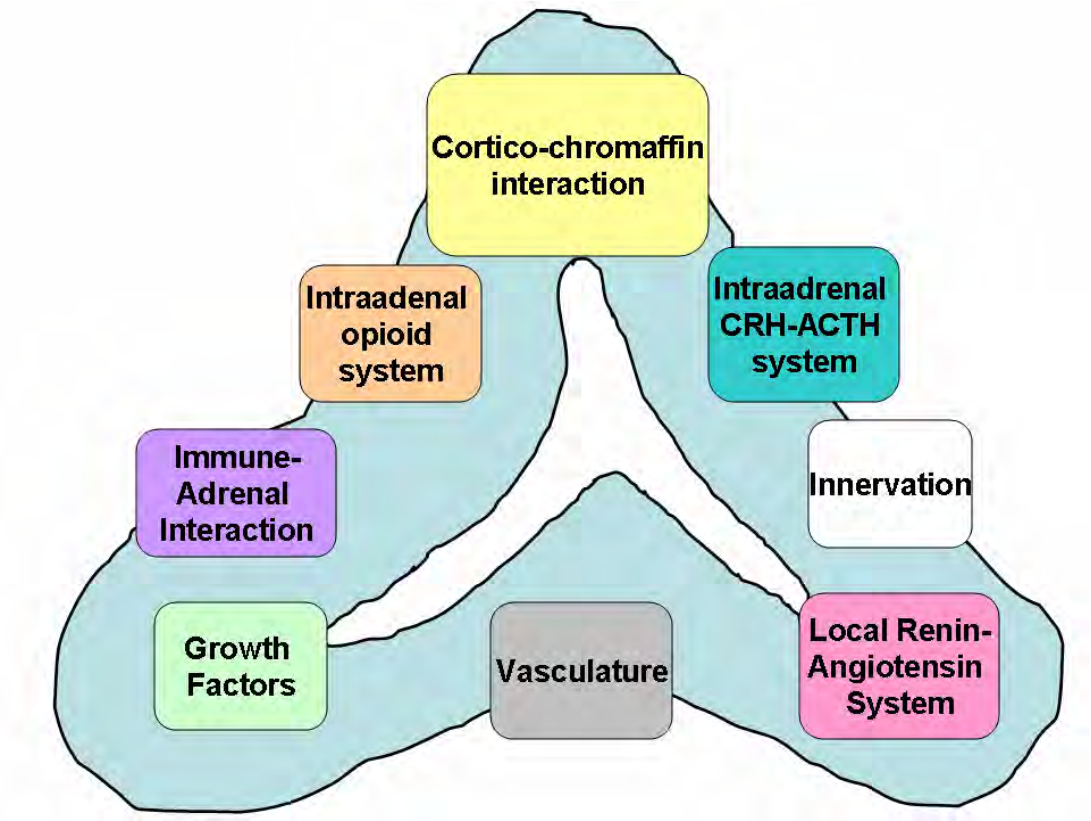

Figure 3 *Schematic illustration of paracrine and autocrine regulation of the adrenal gland (adapted from* Monika *et al.)*

separated into two functional compartments by the structure referred to as the blood-testis barrier and composed of peritubular cells, connected by tight junctions and a basal membrane. The basal compartment of the testis consists of the Leydig cells, peritubular myoid cells and spermatogonia; while the adluminal compartment containing the inner two-thirds of the tubules consists of primary spermatocytes and cells at more advanced stages of spermatogenesis.

Mammals develop at least two generations of Leydig cells, i.e., fetal and adult-type Leydig cells (Habert *et al*., 2001). During the fetal period, Leydig cells present secrete testosterone and other androgens, which regulate not only the masculinization of internal and external genitalia, but also neuroendocrine function. These fetal cells remain for at least 90 days after birth in the rat (Kerr and Knell, 1988, Ariyarate and Mendis-Handagama, 2000). The second generation of adult-type Leydig cells appears during puberty and produces the testosterone required for the onset of spermatogenesis and maintenance of male reproductive function.

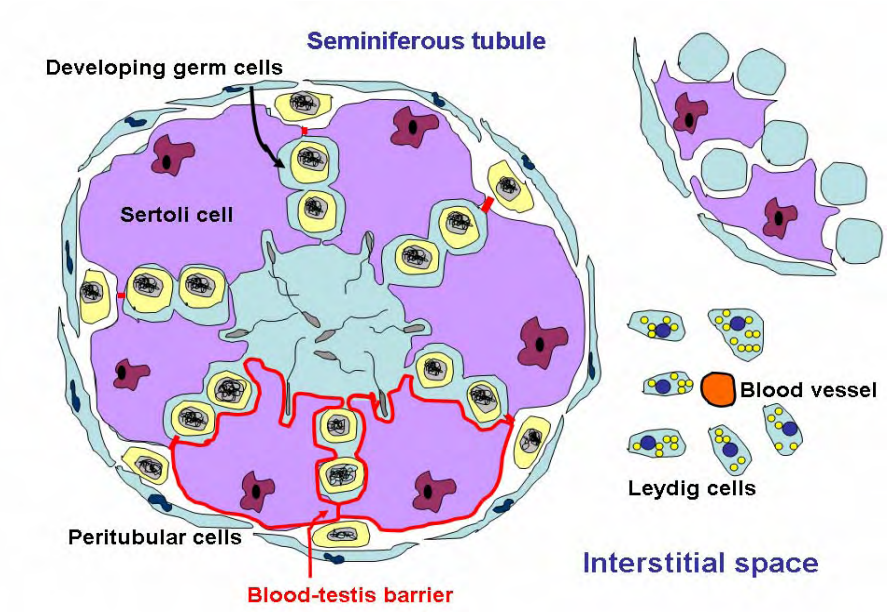

Figure 4. *The functional organization of the testis, illustrating the seminiferous tubules and interstitial tissue, as well as the basal and adluminal compartments*

Fetal Leydig cells

In rats, the fetal Leydig cells that first appear in the interstitial tissue arise by differentiation of mesenchymal-like stem cells and begin producing testosterone after the seminiferous cords have been formed, i.e., 15 days post-conception (Habert and Picon, 1984). The signal(s) that triggers this differentiation is not yet known, but a normal Y chromosome containing an intact Sry region is absolutely required. The process must be independent of gonadotropins, since pituitary LH is first detected 16.5-17 days post-conception (El-Gehani *et al.*, 1998, Aubert *et al.*, 1985, Tougard *et al.*, 1977) and the paracrine action of the Sertoli cells may be involved (Jost *et al.*, 1973, Byskow, 1986). The number of these cells reach its maximum 21.5 days post-partum and remains unaltered thereafter (Kerr and Knell, 1988, Migrenne et al., 2001). The maintenance of functionality differentiated cells during late gestation requires LH and paracine IGF-1 subsequently, the fetal Leydig cells start to regress in response to TGF-β in the presence of high levels of LH.

Adult-type Leydig cells

Postnatally adult-type Leydig cells are not derived from pre-existing fetal Leydig

cells, but from stem cells (Ge *et al.,* 1996) that double in number approximately once every 7 days to produce a cluster of spindle-shaped undifferentiated cells (Hardy *et al.,* 1989, Vergowen *et al.,* 1991, Ariyarate and Mendis-Handagama, 2000). By 11-12 days post-partum, these cells develop into progenitor Leydig cells which already express characteristic marker proteins, i.e., 3β-hydroxysteroid dehydrogenase, P450scc, P450c17, LH and androgen receptors (Hardy *et al.,* 1990, Shan and Hardy, 1992, Ariyarate *et al.,* 2000a). By day 28, these progenitor cells have rounded up and contain numerous lipid droplets and abundant SER (Shan and Hardy, 1992).

The enzymatic activities of 3β-hydroxysteroid dehydrogenase, P450scc and P450c17 in immature Leydig cells are elevated dramatically from day 28 to 56 post-partum, while 17β-hydroxysteroid dehydrogenase begins to increase only after day 35. The immature Leydig cells express testosterone-metabolizing enzymes (e.g., 5α-reductase and 3α–hydroxysteroid dehydrogenase) at high levels and secrete predominantly 5α-androstane-3α, 17β-diol. In contrast, mature Leydig cells express higher numbers of LH receptors, together with elevated levels of the enzymes that synthesize testosterone and attenuated activities of testosterone-metabolizing enzymes and these cells produce primarily testosterone (Shan *et al., 1993*).

1.2.2. Functions and regulation of the Leydig cells

The primary function of Leydig cells is to produce testosterone and this function is regulated mainly by the HPG axis (Fig. 5). The hypothalamus secretes GnRH, which stimulates the anterior pituitary, to secret the gonadotropins FSH and LH, following which these hormones stimulate the gonads to synthesise and secrete sex steroids (Morohashi *et al*., 1997). Located in the interstitial space, Leydig cells are separated from the avascular seminiferous tubules by the blood-testis barrier.

In addition to LH or hCG, local interactions between factors produced within the testis, which provide the only means of communication between compartments, is also required to obtain complete Leydig cell functions. Some of the local paracrine factors presently known to modulate steroidogenesis, and other functions of these cells are IGF-I, TGF-α,TGF-β, IL-1, TNF, interferon, leukemia inhibitory factor (LIF) and stem cell factor (SCF) (Saez *et al*., 1994) (Fig. 6)

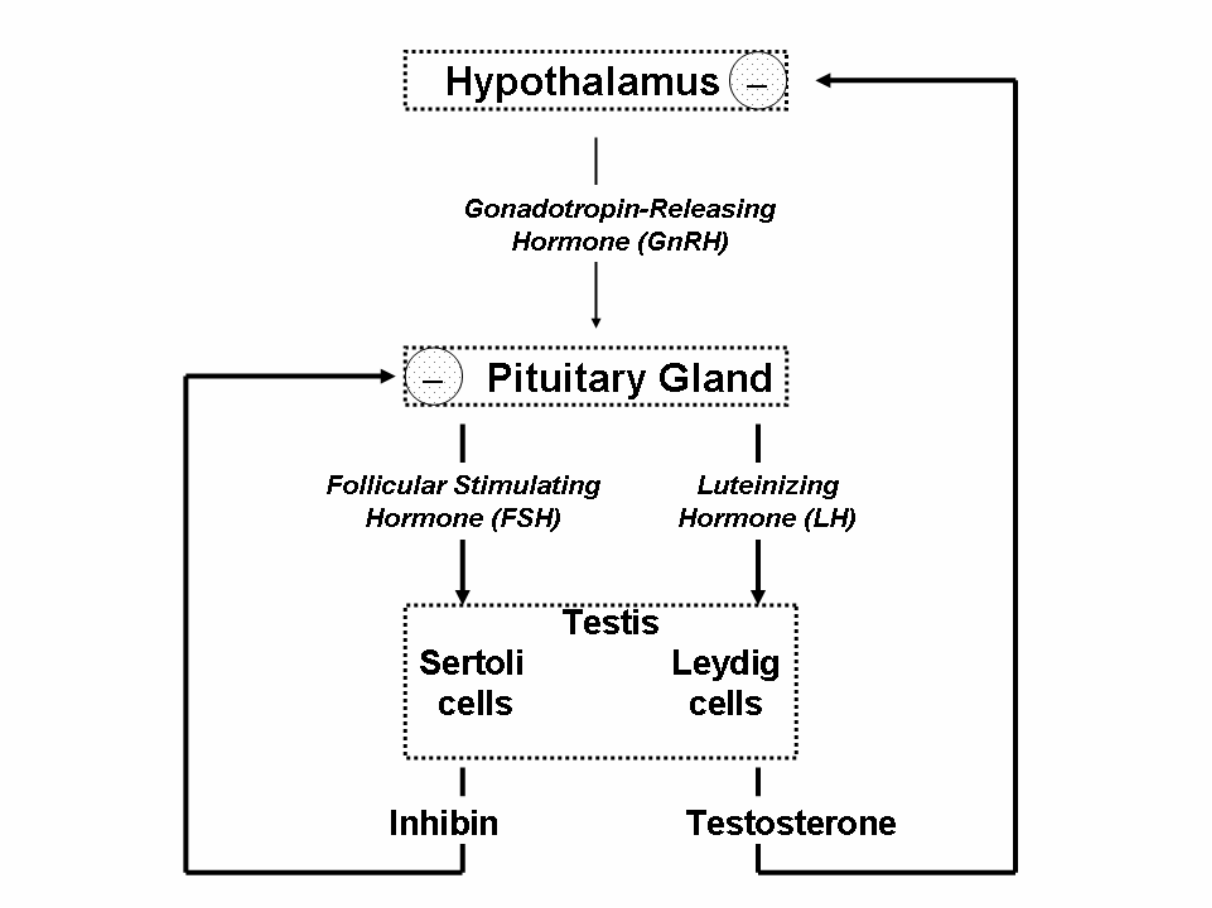

Figure 5 *Feedback regulation of testosterone production by Leydig cells via the hypothalamic-pituitary-gonadal axis (HPG).*

1.3 Steroidogenesis by Leydig and adrenocortical cells

Steroid hormones synthesized in specialized steroidogenic cells located in the adrenal gland, ovary, testis, placenta and brain are required for normal reproduction and maintenance of physiological homeostasis. These hormones can be classified in five groups on the basis of their physiological actions: mineralocorticoids regulate salt balance and blood pressure; glucocorticoids regulate carbohydrate metabolism and responses to stress; progesterone and estrogens regulate reproductive functions and secondary sex characteristics in females; and androgens are essential for successful fertility and development of secondary sex characteristics in males.

Alteration in steroidogenesis occurs both within minutes and over the course of hours and are regulated predominantly by trophic hormones. The acute response is initiated by mobilization and delivery of cholesterol, the precursor for all steroid hormones, from the outer to the inner mitochondrial membrane, where it is metabolized to

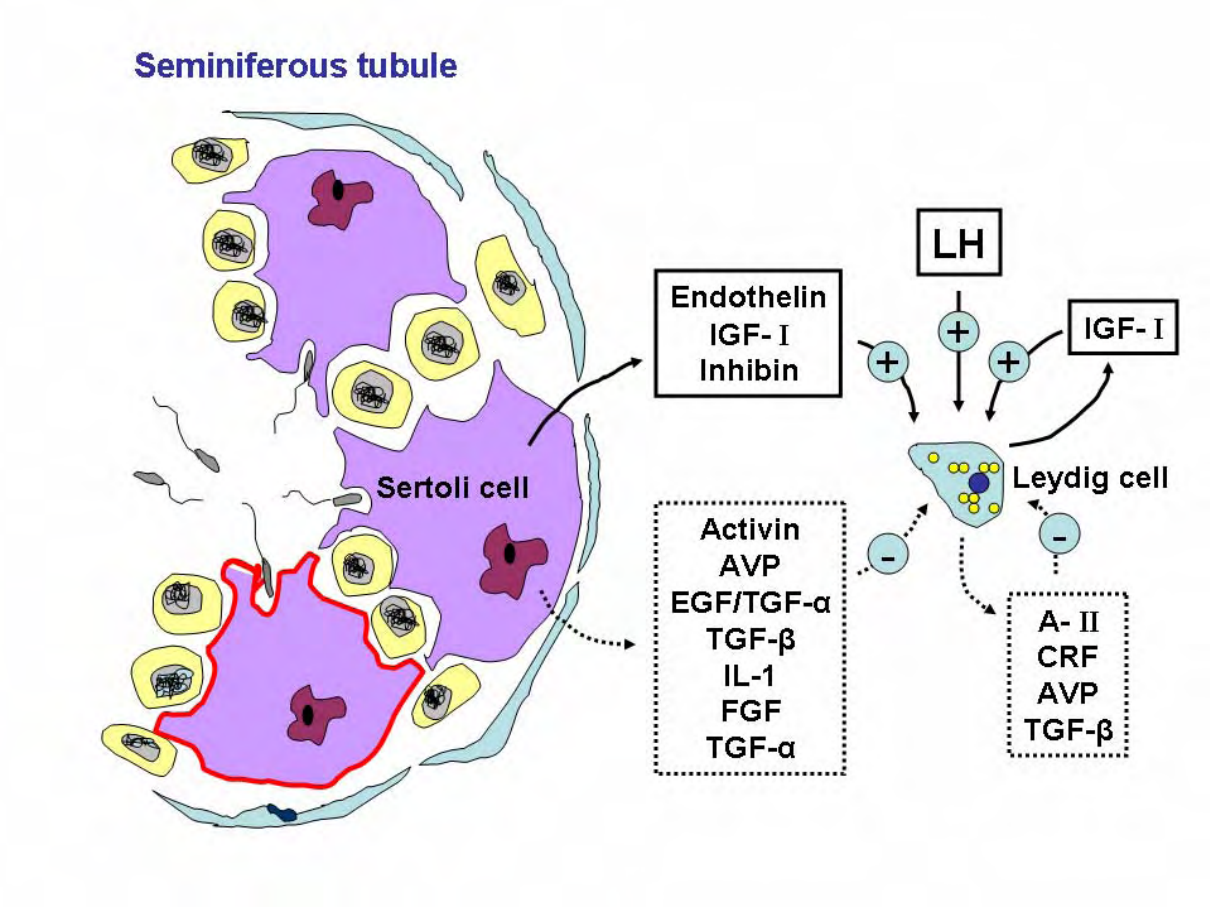

Figure 6 *Schematic illustration of endocrine, paracrine and autocrine regulation of Leydig cells*

pregnenolone by cytochrome P450 side-chain cleavage (P450scc) (Stocco et al., 2005). The slower, chronic responses involve increased transcription of genes encoding steroidogenic enzymes, as well as enhanced translation of the corresponding mRNA species (Simpson et al., 1988).

The cholesterol required for the production of steroid hormones can either be synthesized *de novo* from acetate, primarily on the endoplasmic reticulum of steroidogenic cells; mobilized from cholesterol esters stored in intracellular lipid droplets; and/or taken up from cholesterol-containing low-density plasma lipoproteins. Rapid increases in the production of steroids in response to trophic hormones involve the transfer of cholesterol from the outer to the inner mitochondrial membrane, which is the primary rate-limiting and regulatory step in steroidogenesis (Stocco *et al*., 1996; 2005). The rapidly synthesized protein that mediated this transfer

was identified in the middle of the 1990's and designated steroidogenic acute regulatory protein (StAR) (Clark et al., 1994).
The mechanism by which StAR facilitates the transport of cholesterol into mitochondria is still unclear. This protein is expressed predominantly in steroid-producing tissues and is synthesized as a 37-kDa precursor containing an N-terminal mitochondrial targeting sequence, which is subsequently transformed into several forms of the 30-kDa mature protein (Stocco, 2001). Studies on patients suffering from congenital lipoid adrenal hyperplasia, who produce virtually no steroids, as well as on StAR-null mice, illustrate clearly the indispensable role played by StAR in regulating steroidogenesis (Lin et al., 1995).
The outer mitochondrial membrane contains a specific receptor for the precursor form of StAR and at least three cleavage processes are involved in producing the proteins that mediate cholesterol transport to the inner membrane (Stocco *et al*., 1996). Employing preparations of adrenal mitochondria, at least three intermediate metabolites, i.e., 22R-OH-cholesterol, 22α-OH-cholesterol and 20α,22R-dihydrocholesterol, have been shown to be formed in connection with the conversion of cholesterol to pregnenolone. However, these metabolites are present in very low amounts, due to the high affinity and catalytic efficiency of P450scc (Dixon *et al*., 1970; Bursten *et al*., 1976; Orme-Johnson *et al*., 1979).

In addition to StAR, intra-mitochondrial transfer of cholesterol appears to require several other factors. For example, SCP2 (Sterol carrier protein-2), a 13-kDa protein, purified from the liver, enhances transfer of cholesterol from lipid droplets to mitochondria (Vahouny *et al*., 1985), a prerequisite for the intramitochondrial transport of this compound (Xu *et al*., 1991). The SAP (steroidogenesis activator polypeptide) expressed in all steroidogenic tissues has been implicated in cholesterol transfer within mitochondria. Moreover, DBI (diazepam-binding inhibitor or endozepine), a protein which binds to peripheral or mitochondrial benzodiazepine receptors (PBR) or to be specific, mitochondrial receptor that is a multidrug resistance (MDR) protein is also an important element of the mitochondrial machinery involved in cholesterol transferring (Hauet et al., 2005).

Most of the enzymes that catalyze the sequence of reactions involved in

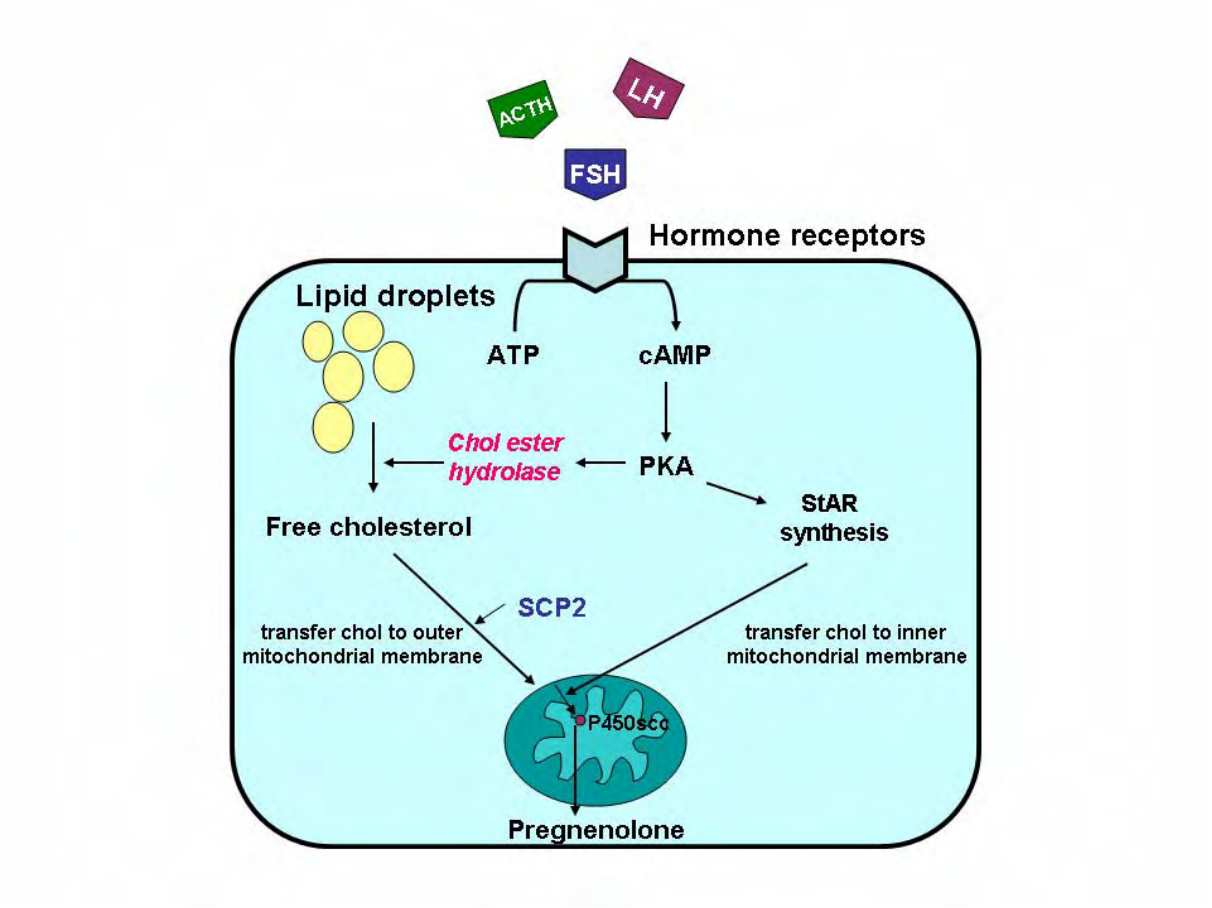

Figure 7 *Regulation of cholesterol transport into the mitochondria of steroidogenetic cells in response to tropic hormones.*

steroidogenesis are cytochrome P450-associated monooxygenases. Thus, adrenal steroidogenesis requires P450scc (side chain cleavage) and P450c11 (11β-hydroxylase), located in the mitochondria, as well as P450c17 (17α-hydroxylase), P450c21 (21α-hydroxylase) and P450aldo (aldosterone synthase), that are present in the endoplasmic reticulum. In the case of the gonads, in addition to these same reactions, aromatization of androgens to estrogens by P450arom also occurs (Fig. 8).

Cytochrome P450scc, situated on the matrix side of the inner mitochondrial membrane, catalyzes the first step in steroidogenesis. The single active site of this enzyme hydroxylates cholesterol at the 22R and 20S positions and subsequently cleaves the side-chain between C_{20} and C_{22} to yield pregnenolone. (Lambeth 1986). Pregnenolone is then converted to progesterone by 3β-HSD, localized both on the smooth endoplasmatic reticulum and in the mitochondria (Pelletier, Li et al. 2001).

The bifunctional enzyme cytochrome P450 17α-hydroxylase/17-20 lyase (P450c17 or CYP17) is not expressed by adrenocortical cells, but is present in abundant amounts on the endoplasmic reticulum of Leydig cells, where it metabolizes progesterone to androstendione (in rodents) or pregnenolone to dehydroepiandrostendione (DHEA) (in humans). Both 17α-hydroxylation and cleavage of the 17,20 carbon-carbon bond are catalyzed by the same, single active site on this enzyme, which is encoded by a single gene. Its activity is regulated by cAMP-dependent phosphorylation of serine and threonine residues (Zhang, Rodriguez et al. 1995; Pandey, Mellon et al. 2003). Furthermore, this enzyme involved in both the Δ5- and Δ4 pathways, i.e., utilizes both pregnenolone and progesterone as substrates. The Δ5-pathway predominates human Leydig cells (Fluck, Miller et al. 2003); whereas in the rat, both pathways are catalyzed, although the Δ4 is preferred (Fevold, Lorence et al. 1989).

In the case of adrenocortical cells, following the synthesis of progesterone (in rodents) or 17-hydroxyprogesterone (in humans), cytochrome P450c21 hydroxylates these steroids at the 21 position, to yield deoxycorticosterone or 11-deoxycortisol, respectively. The final step in the synthesis of adrenal mineralocorticoids and glucocorticoids is catalyzed by P450c11, which also performs the final steps in the synthesis of aldosterone from deoxycorticosterone. Conversion of androstenedione to testosterone by Leydig cells is mediated by 17β-hydroxysteroid dehydrogenase, type 3 (Geissler *et al*., 1994; Payne *et al*., 2004).

The capacity of Leydig cells to metabolize testosterone further varies with age. Accordingly, immature Leydig cells express high levels of 5α-reductase and 3α-hydroxysteroid dehydrogenase (3α-HSD), which convert testosterone to DHT and the 3α-diol, respectively (Ge and Hardy 1998). In a similar manner, type I 5α-reductase, the most abundant isoform in the testis (Killian, Pratis et al. 2003), is more highly expressed and more active in immature than in adult rat Leydig cells (Viger and Robaire 1995) and moreover, 3α-HSD activity exhibits age-dependent variation as well (Ge, Hardy et al. 1998).

The end-point for conversion of cholesterol to steroid hormones is species-specific and, in the case of the adrenal, zone-specific as well. The most abundant

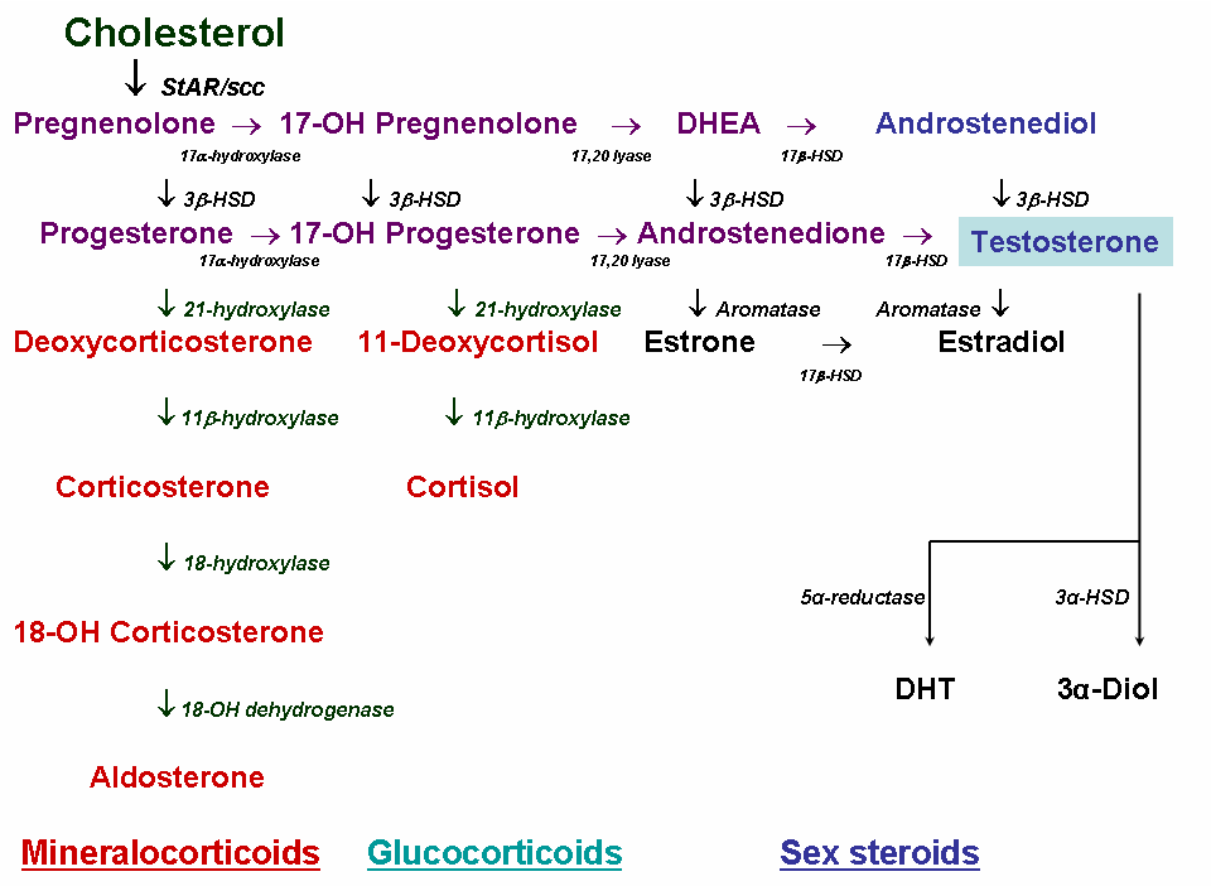

Figure 8 *The principle pathways of steroid hormone synthesis in humans.*

glucocorticoid produced by rats and mice is corticosterone, because these animals lack 17α-hydroxylase activity; whereas the major glucocorticoid in the guinea pig, dog and man is cortisol. In the inner zones of the adrenal cortex of humans the 17α-hydroxy pathway predominates, with the zona fasciculata producing primarily cortisol and the zona reticularis producing mainly androgens and sulphated steroids (Hinson *et al*., 1996).

1.4 Endocrine disrupting chemicals (EDCs)

In recent years, growing evidence links xenobiotics in the environment to adverse changes in the reproductive health of both animals and humans. Such changes induce regional changes in wildlife populations, e.g., masculinisation of female marine snails by tributyltin (TBT) in anti-fouling paints, all over Europe and in the open North Sea (Gooding *et al*., 2001) and severe declines in bird populations in Europe and North America as a result of eggshell thinning caused by DDE (Lundholm *et al*., 1997). Moreover, PCBs cause reproductive problems in seals in the Baltic and Wadden Seas

(Bernt *et al.*, 1999) and DDT induces disruptions in the development and function of sex organs in alligators in the lakes of Florida (Vonier *et al*., 1996). One advantage of studying the effects of these xenobiotics on wild fauna is that the shorter gestation and life cycles of these animals allow the effects of exposure to be seen clearly, although there are, of course, difficulties involved in applying this information to humans (Preziosi *et al*., 1998). Endocrine disruptors are believed to be involved in the increasing incidences of a number of anomalies in man, including testicular and breast cancer, hypospadias, cryptorchidism and declining sperm counts, particularly in industrialized countries. Of course, the situation is complicated by the fact that we are exposed to many environmental xenobiotics simultaneously (Fisher *et al*., 2004), of which any given one may exert numerous effects some of which may take time to develop, arising e.g., only after the development of puberty (Preziosi *et al*., 1998).

Endocrine disrupting chemicals (EDCs) have been defined by many different organizations. In general, EDCs are exogenous substances, either anthropogenic or naturally occurring, that alter endocrine functions via a variety of different mechanisms, e.g.,

1. by acting as either agonists or antagonists; for the receptors for estrogens and androgens;
2. by altering the synthesis, transport and/or catabolism of endogenous hormones; and/or
3. by modifying the levels of expression and/or functioning of hormone receptors.

EDCs are present in the general environment (air, water, soil), as well as in food products (e.g., soybeans, legumes) and plants (phytoestrogens in fruits, vegetables and grasses). Some arise from household products (e.g., breakdown products of detergents and associated surfactants, including nonylphenol and octylphenol), while others are agricultural (e.g., the pesticide o'p-DDT) or industrial chemicals (e.g., phthalate plasticizers). Exposure can occur directly in the workplace or via consumer products such as food items, plastics and paints, or indirectly via air, water and soil. The toxicity of such chemicals towards different species of living organisms is determined by their individual chemical properties; the duration and/or frequency, of exposure; and the pharmacokinetics (absorption, distribution, transformation and elimination). The adverse effects of exposure at a developmental stage are irreversible, whereas

most post-developmental effects are reversible (McLachlan *et al*., 2001; Colborn *et al*., 1998).

Centers for disease control and prevention (CDC) have classified the hormone-modulating effects of EDCs as having estrogenic (approximately 48%), anti-androgenic (19%), anti-estrogenic (15%), androgenic (4%), and those with effects on thyroid hormone status (42%) and others (Choi *et al*., 2004). McLachlan (2001) has also reported that for EDCs exhibiting estrogenic and anti-androgenic activities are more common than those with anti-estrogenic and anti-androgenic activities. The estrogenic and anti-estrogenic activities of EDCs arise from competition with endogenous estrogens for binding to their receptors (ER), thereby functioning as a less potent agonist or inhibiting endogenous estrogenic activities. Such activity can be screened for by assaying hormone receptor activation and comparison of the results with the biological potency of the natural agonist (Fig 9).

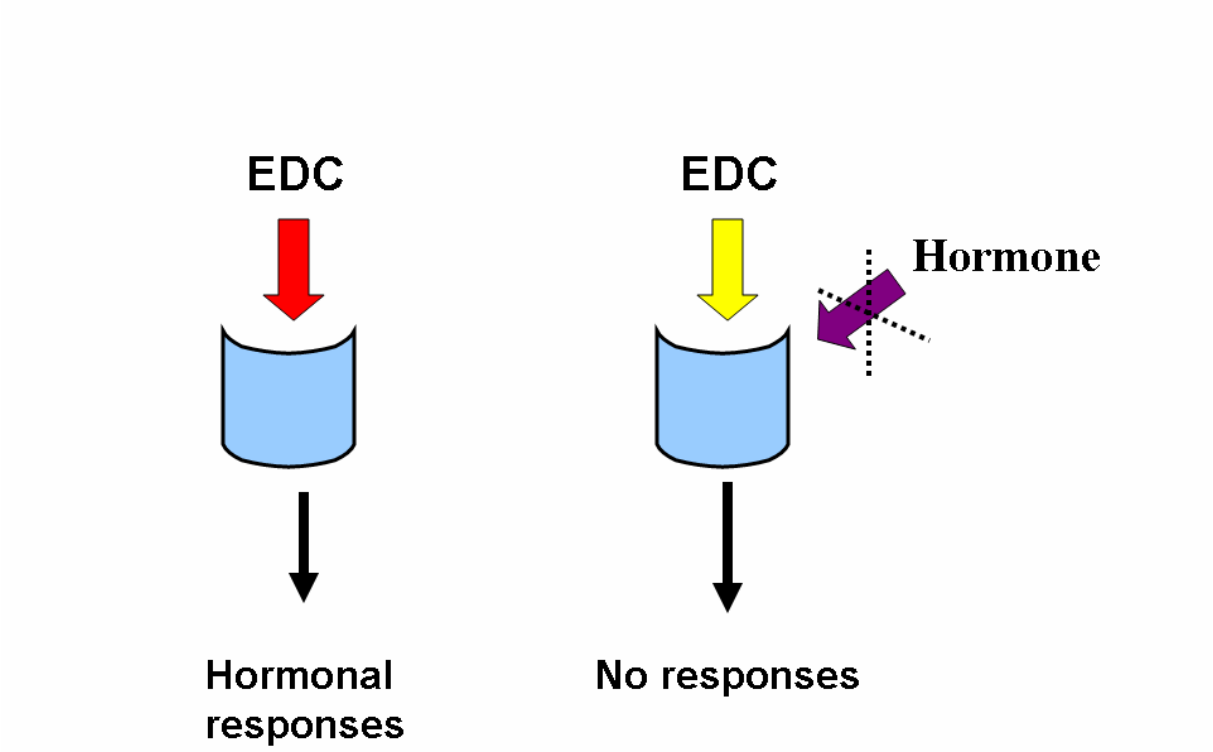

Figure 9 *The basic principles of action of EDCs with hormone agonistic and antagonistic actions*

In contrast, anti-androgenic EDCs can interfere with the androgen function in two different ways, both by preventing androgens from binding to their receptors in peripheral tissues and/or by interfering centrally with androgen binding to their receptors in the pituitary gland or hypothalamus (Svechnikov et al., 2006) (Fig 10).

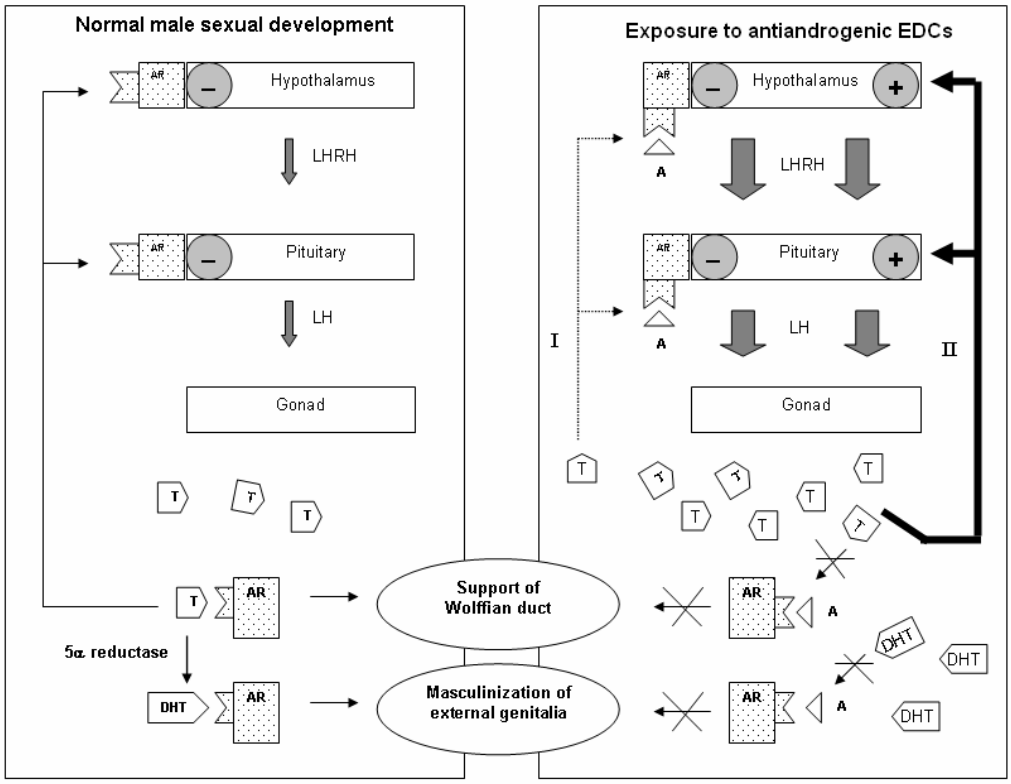

Figure 10 *The mechanisms of action of EDCs exhibiting anti-androgenic activity.*

1.5 The influence of EDCs on the male reproductive system and adrenal function

Germ, Sertoli and Leydig cells are all highly sensitive to the toxic effects of a variety of xenobiotics. Attenuation of fetal Leydig cell steroidogenesis is associated with development of congenital abnormalities, i.e., cryptorchidism and hypospadias, as a result of incomplete masculinization. Epidemiological studies have revealed an elevated risk for cryptorchidism among sons of women who work with pesticides (Weinder *et al*., 1998). Furthermore, significantly higher concentrations of hexachlorbenzene and heptachlorepoxide have been detected in the adipose tissue of boys with testicular maldescent, in comparison to healthy boys (Hosie *et al*., 2000). Moreover, exposure of fathers to dioxins in connection with the Seveso accident in

1976 resulted in an increased incidence of hypospadias among their sons (Baskin *et al*., 1976).

The adrenal cortex, and in particular the zona fasciculata (Rosol *et al*., 2001), has been reported to be among the most common of site lesions in the endocrine system (Ribelin *et al*., 1984; Thomas *et al*., 1993). The factors which predispose this organ to such lesions include its disproportionately large blood supply per unit mass; its high content of lipids and the susceptibility of its unsaturated fatty acids to peroxidation damage; and its high levels of cytochrome P450 which metabolize xenobiotics to reactive intermediates, as well as producing reactive species of oxygen (Hornsby *et al*., 1983; Hallberg *et al*., 1990). In addition to its unique role in stress management, the adrenal expresses several of the pathways for steroid production present in the testes and ovaries. Therefore, toxic chemicals can affect the adrenal or its axis directly or indirectly in a manner similar to the testes and ovaries.

Adrenal toxicity can be divided into three classes:
Primary toxicity (type 1): In this case the adrenal gland is a target organ for the toxic chemical and the severity of toxicity can be predicted, at least in part from its pharmacokinetic properties (Colby *et al*., 1992; Atterwill *et al*., 1992). A adrenocortical destruction induced by Mitotane (o,p-DODD) is one example of this type of toxicity.
Secondary toxicity (type 2): This form of toxicity originates elsewhere in the endocrine axis or is due to chemically-induced stress (Walker *et al*., 1992; Hadley *et al*., 1990; Spindel *et al*., 1984). For example testicular atrophy induced by phthalates gives rise to secondary alterations in the production of pituitary gonadotropins.
Indirect toxicity (type 3): Here, a specific effect exerted on a non-endocrine organ influences the hypothalamic-pituitary-adrenal axis (***type 3a***) (Rehulka *et al*., 1987) e.g., the hepatic toxicity induced by phenobarbital. Moreover, the increased levels of cortisol arising from chemical stress can potentiate the toxic effects of certain chemicals (e.g., aflatoxin) on the adrenal gland (***type 3b***)(Harvey et al., 1994; Vogel *et al*., 1993).

1.6 The compounds examined here

In the present investigation, we have tested the endocrine disruptive effects of the

chemicals whose structures are shown in Figure 11.

1.6.1 Procymidone

Procymidone (N-(3,5-dichlorophenyl)-1,2-dimethyl-cyclopropane-1,2-dicarboximide) is widely used as a fungicide for the control of plant diseases. The weak anti-androgenic activity of this compound is due to low-affinity binding to the androgen receptor in the prostate (Hosokawa *et al*., 1993). A feasible hypothesis concerning the effects of long-term administration of procymidone is that this compound inhibits the negative feedback exerted by androgens on the hypothalamus and/or the pituitary, thereby giving rise to hypergonadotropism in rats. The attenuated binding of testosterone to the androgen receptor under these circumstances suppresses feedback inhibition of LH production by this steroid, resulting in hypersecretion of LH with concomitant elevation in serum levels of testosterone (Fig 10). Long-term hypergonadotropism and hyperstimulation of Leydig cells induced by procymidone give rise to interstitial cell tumors in male rats (Murakami *et al*., 1995).

1.6.2 Genistein

The phytoestrogen genistein has a structure similar that of estrogen (Fig 11) and can thereby produce estrogenic effects (Knight *et al.*,1996). Many phytoestrogens act both as agonists and antagonists of estrogen, depending on the tissue involved.
The phytoestrogens are divided into lignans and isoflavonoids, with the latter being further subdivided into isoflavones, isoflavans and coumestans. These compounds are present at high levels in a variety of food items, particularly those on soy, such as soy infant formula, tofu and soy flour. Thus, serum concentrations of phytoestrogens are higher in Japanese than in Finnish men (Adlercreutz *et al*., 1993) and are also elevated in infants who consume large quantities of food products derived from soy beans (Setchell *et al*., 1997).

Treatment of rats with genistein 40 mg/kg once daily for 3 weeks tends to reduce plasma levels of testosterone, while increasing the concentration of LH in serum. These effects are thought to be due to alterations in the feedback inhibition by testosterone of LH production by the pituitary (Ohno *et al*., 2003). Furthermore, genistein (5-50 μM) causes dose-dependent inhibition of both basal and LH-

stimulated testosterone production by cultured Leydig cells isolated from roosters (*Allus gallus domestics*) via a mechanism that does not involve tyrosine protein kinase (Opalka *et al*., 2004). In the case of the adrenal gland, genistein inhibits cytochrome P450 c21-hydroxylase and thus enhances androgen production (Mesiano *et al*., 1999), thereby producing effects similar to those associated with deficiencies in 21-hydroxylase. In addition, Ohno and colleagues (2003) have demonstrated that genistein reduces serum levels of corticosterone in rats by inhibiting 3β-hydroxysteroid dehydrogenase (3β-HSD) and, to a lesser extent, the activity of cytochrome P450 c21-hydroxylase.

1.6.3 Resveratrol

Resveratrol (3,4,5-trihydroxystilbene Fig 11) is a naturally occurring phytoalexin polyphenol found in various plants, including grapes, berries and peanuts (de la Lastra *et al*., 2005). In certain of these plants this compound is produced in response to ultraviolet injury or fungal attack (Fremont *et al*., 2000). Resveratrol exists as cis- and trans- isomers but only the trans-form has been identified in grape extracts (Orallo, 1991).

Resveratrol has been reported to be estrogenic (i.e, to be a phytoestrogen), acting as an ER agonist (Gehm *et al*.,1997). The levels of this polyphenol in red wine and grape juices (Gehm *et al*., 1997) range between 0.84-7.33 mg/ l and 0.12-6 mg/l, respectively (Di *et al*., 2004). Relatively high doses of this phytoestrogen have also been employed in chemotherapy against cancer (Jang *et al*., 1997) and aging (de la Lastra *et al*.,2005), and anti-inflammatory agent that inhibits cyclooxygenase-1 (Alarcón et al. 2005) as well as a cardioprotectant (Frankel *et al*., 1993; Bertelli *et al*., 1995; Pace *et al*., 1995; Goldberg *et al*., 1995). Thus resveratrol has multiple effects on physiological and metabolic processes, and at least one company in the United States now sells resveratrol in tablet form with 7-50 mg as the suggested daily intake.

1.6.4 Helenalin

The sesquiterpene lactones helenalin (Fig 11), produced by several species of the Asteracea family of plants, exhibits a variety of biological activities including anti-neoplastic (Huang *et al*., 2005), anti-inflammatory (Lyss *et al*., 1997) and anti-

parasitic properties (Francois et al., 2004; Jemenez *et al*., 2005). Consequently, this compound and its derivatives may prove to be of pharmacotherapeutic value. For example, in the rat helenalin alleviates carageenan-induced edema as well as arthritis produced by chronic administration of Freund's adjuvant (Hall *et al*., 1979) Moreover, both helenalin itself and certain of its derivatives inhibit the migration and chemotaxis of human neutrophils (Hall *et al*., 1980), as well as the activities of 5-lipoxygenase and leukotriene C4 synthase (Tornhamre *et al*., 2001). In addition, this substance selectively alkylates two specific cysteinyl residues in the DNA-binding region of the p65 subunit of $NF_{\kappa}B$, thereby preventing this factor from recognizing and binding to its target DNA sequences (Lyss *et al*., 1998). Accordingly, the anti-inflammatory and, possibly, the anti-neoplastic effects of helenalin appear to involve a common chemical mechanism, i.e., formation of covalent bonds with free cysteinyl residues (Lyss *et al*.,1998).

1.8.5 Phthalates

Phthalates are widely used as plasticizers (to soften plastics), as well as solvents in inks, and are present, among other places, in food packaging and certain cosmetics (Thomas *et al*., 1973). Although both diethylhexyl phthalate (DEHP) and its major metabolite, monoethylhexyl phthalate (MEHP) (Fig 11), disturb reproductive development in an anti-androgenic fashion, neither of these compounds binds to the AR (Parks *et al*., 2000). These phthalates inhibit Leydig cell steroidogenesis at different stages of fetal development and the Sertoli and Leydig cells of young rats appear to be more sensitive to their action than the corresponding cell types in adult rats, as shown in both *in vitro* and *in vivo* experiments (Jones *et al*., 1993, Dostal *et al*., 1988, Li *et al*., 2000, Akingbemi *et al*., 2004).

The exact biochemical or molecular mechanism(s) underlying the effects of DEHP on the testis remains unclear. The mechanisms that have been proposed include alterations in testicular levels of zinc and zinc-dependent enzymatic activities; elevated oxidative stress in the testis, modulation of estrogenic activity via interactions with ERs; and changes in pathways involving peroxisome proliferator-activated receptors PPARs or other cellular molecular events pathways (e.g., alterations in interactions between Sertoli and germ cells or changes in the expression

of genes critical to germ cell survival or the functioning of Sertoli and/or Leydig cells).

Furthermore, in male rats inhibition of steroidogenesis in fetal Leydig cells by phthalates gives rise to numerous malformations of androgen-dependent tissues, including a reduction in the anogenital distance, hypospadia, epididymal agenesis and testicular atrophy (Gray *et al*., 2001). These abnormalities are suggestive of sub-optimal virilisation of the Wolffian duct and urogenital sinus. Finally, recent investigations have revealed that treatment with phthalates attenuates the transcription of several key genes whose protein products are involved both in cholesterol transport and the biosynthesis of testosterone (Barlow *et al*., 2003; Thompson *et al*., 2004).

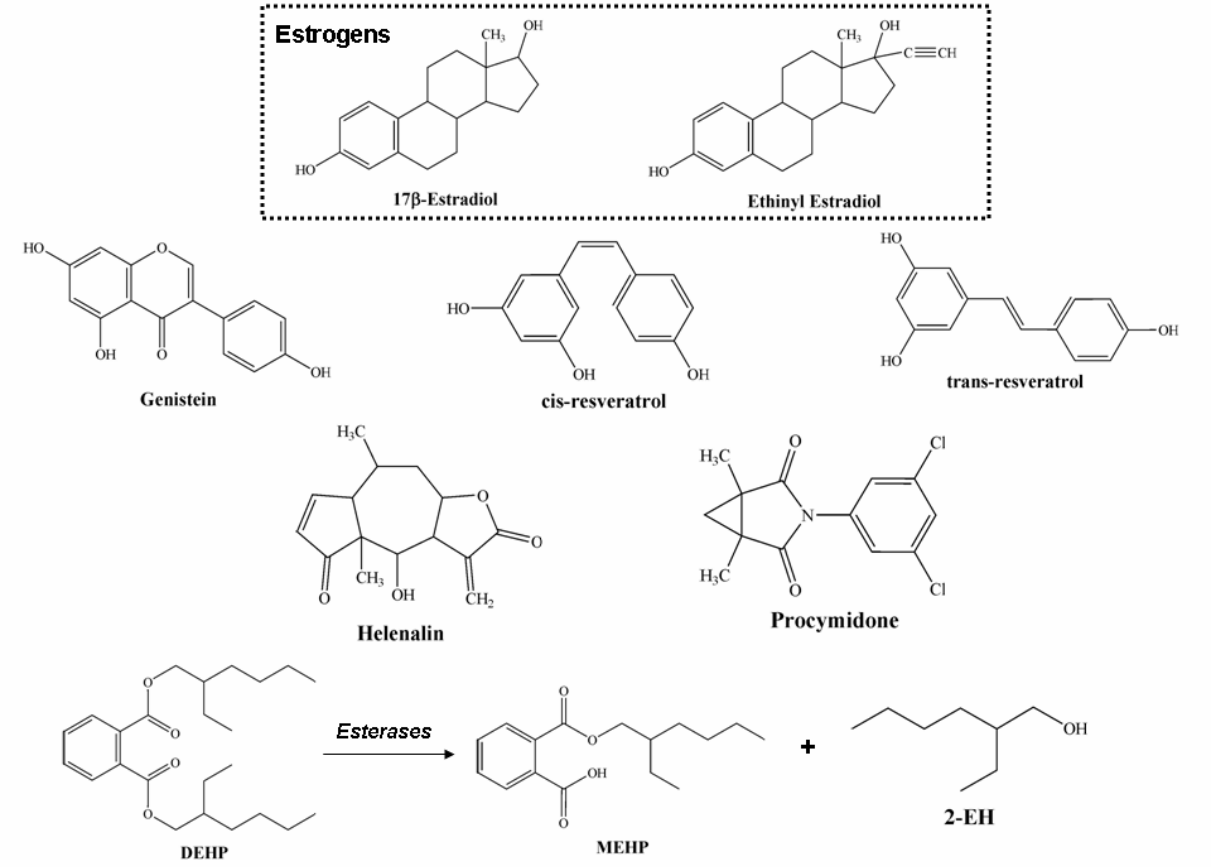

Figure 11 *The chemical structures of the compounds tested here and of estrogen derivates for comparison.*

2 AIMS

The primary aims of this study have been the following:

I. to investigate the effects of long-term dietary intake of a relatively high level of procymidone or genistein by rats on the functions of their pituitary-gonadal axis *in vivo*;

II. to characterize the influence of resveratrol on rat adrenal steroidogenesis using an *in vitro* system and to determine whether this effect is mediated by interaction with the ER and/or via direct effects on steroidogenic enzymes;

III. to examine the effects of sesquiterpene lactone helenalin on testicular and adrenal steroidogenesis using *in vitro* systems and; and

IV. to study the acute effects of DEHP on *in vivo* adrenocortical steroidogenesis in rats at different developmental stages.

3 METHODOLOGICAL CONSIDERATIONS

Detailed information concerning the methodology employed is present in the individual publications. Accordingly, only a brief presentation is given here.

3.1 EXPERIMENTAL ANIMALS

Sprague-Dawley (SD) rats were used for both the *in vitro* and *in vivo* experiments.

In vivo (I, II)

Animals, 3 months of age were allowed to consume rat chow containing genistein (1g/kg, I), procymidone (1.25 gm/kg, I) or resveratrol (0.84 g/kg, II) for 12 weeks, while the control animals received a soy-free diet supplement with potato protein. In study IV, three groups of rats 20 days (prepubertal) 40 days (undergoing puberty) or 60 days of age (adult) received DEHP dissolved in corn oil by gavage (500 mg/kg for the youngest group and 750 mg/kg for the other two) once daily for 4 days, while the control animals received the same volume at the vehicle (corn oil) alone in the same manner.

In vitro (II, III)

Rats, 40 or 60 days of age, were given access to a standard pellet diet and tap water ad libitum. All of the animal experiments were pre-approved by the Northern Stockholm Ethical Committee for Animal Experiment (registration numbers 192/03 and N

218/05) and by the German Bezirksregierung Braunschweig (AZ 509.42502/01-13.00,17.07.2000).

3.2. Cell cultures (I-IV)

3.2.1 Primary cultures of adrenocortical cells (II-IV)

Utilization of primary cultures of steroidogenic cells for our investigations offered several important advantages. Such cultures retain their steroidogenesis and signaling pathways involved in the regulation of this process, thereby providing information of relevance to the situation. In addition, experimental conditions can be easily controlled. However, preparation of primary cultures of steroidogenic cells is time-consuming, requires the sacrifice of many animals and provides only a limited number of cells that are not entirely of one type. Many years ago, unit gravity sedimentation (Hyatt *et al*., 1974, 1983) was applied to prepare adrenal cells, but since then, the use of Percoll gradients for this purpose has further been developed extensively (Hyatte *et al*., 1983). This approach offers several advantages, such as a high degree of cell purity, absence of toxic effects and preservation of characteristic morphological features and functional activities (Hyatt *et al*., 1974, 1983). Therefore, we utilized this method to isolate adrenocortical cells from SD rats.

On Percoll gradients adrenocortical cells are separated from other adrenal cells on the basis of differences in density. For this purpose, a crude suspension of adrenal cells is loaded onto a discontinuous gradient consisting of layers of 20, 40, 60 and 90% Percoll and subsequently centrifuged at 800×g for 20 minutes. The adrenocortical cells are recovered in the fractions with a density of 1.030-1.060 g/ml (as indicated by the marker density beads) (Tait *et al*., 1974), and are subsequently washed to remove the Percoll. In order to confirm that the cells we obtained in this manner were adrenocortical cells that produce corticosterone, their ability to produce this steroid upon stimulation by different concentrations of ACTH was observed (Fig 12A). In addition, the cells were stained with a specific antibody directed towards 11α-hydroxylase, a marker for adrenocortical cells (Fig 12B), which demonstrated that they were approximately 90% pure. The cells were also 90% viable, as judged by Trypan blue exclusion.

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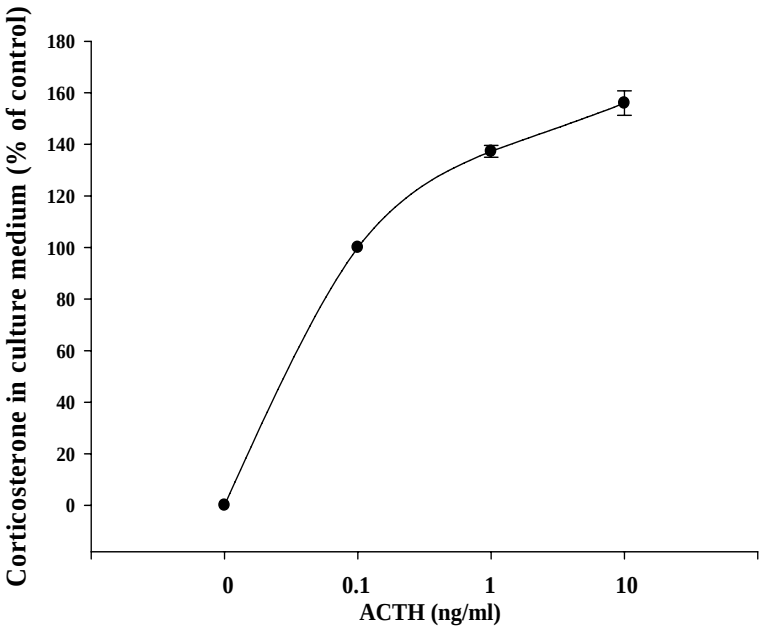

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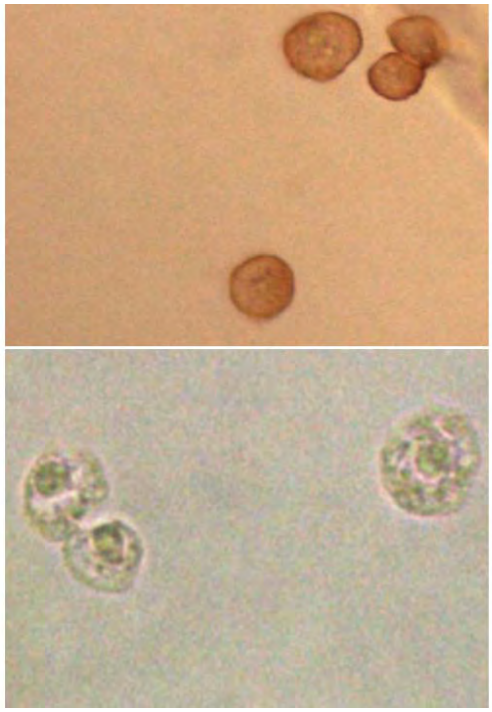

Cell stained for 11α-hydroxylase

Unstained cells

Figure 12 *(***A***) Corticosterone production by primary adrenocortical cells from rats in response to different concentrations of ACTH (***B***) Immunohistochemical staining of the cells for 11α-hydroxylase*

3.2.2. Primary cultures of Leydig cells (I, III)

Leydig cells comprise only 5-10% of the total number of cells in the testis and must therefore be purified in order to prepare primary cultures. For this purpose, we again employed density centrifugation on a Percoll gradient. As in the case of isolation of adrenocortical cells, a crude suspension of testicular cells was loaded onto a discontinuous gradient consisting of layers of 20, 40, 60 and 90% Percoll and subsequently centrifuged at 800×g for 20 minutes. The fractions thus enriched in Leydig cells then further purified in a continuous, self-generating density gradient obtained by starting with a 60% solution of Percoll and centrifuging at 20,000×g for 30 minutes at 4°C.

The purity of the Leydig cell preparations obtained in this manner was examined by histochemical staining for 3β-hydroxysteroid dehydrogenase (Payne *et al*., 1980), an enzyme expressed only in steroidogenic cells. The procedure employed involves 3β-HSD-catalyzed formation of blue granules of formazan upon incubation with nitroblue tetrazolium. This approach revealed that, the Leydig cells obtained here were approximately 85% pure. Trypan blue exclusion demonstrated that these Leydig cells were 90% viable. Isolated Leydig cells were cultured on 96-well plates for 2 hours prior to the experimental incubation. In *ex vivo* experiments, these cells were isolated from rats treated with endocrine disruptors (Fig 13A). In order to ascertain the direct effects of the xenobiotics of interest on Leydig cell steroidogenesis, the cells were isolated from untreated animals and then exposed to the compound *in vitro* (Fig 13B).

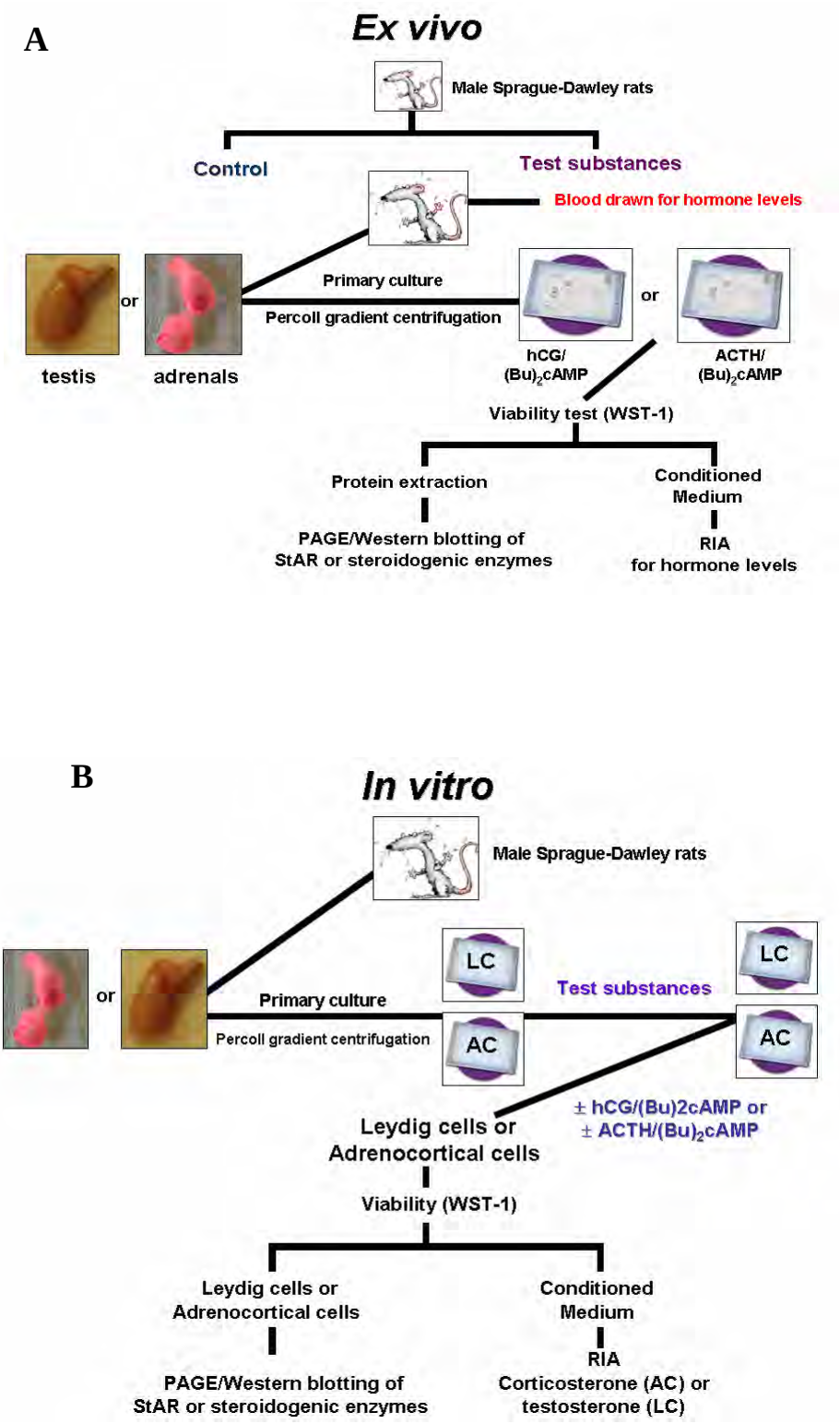

Figure 13. *The treatment diagrams of adrenocortical and Leydig cells, ex vivo (A) and in vitro(B).*

3.3 Quantitation of testosterone, corticosterone and pregnenolone by radioimmunoassay (I-IV)

Steroid production by adrenocortical and Leydig cells was determined by radio immunoassay (RIA). This method based on competition between the sample and radioactive steroid for binding to a specific antibody is specific, sensitive and very accurate. In this context several ready-made kits are commercially available and here we used the Coat-a-Count RIA kit (Diagnostic products Corp., Los Angeles, CA, USA) to assay testosterone and corticosterone. For measurement of pregnenolone levels, specific antisera (Fitzgerald Industries Inc., MA, USA) and tracer amounts of 7-^{3}H(N)-pregnenolone (NEN Life science Products, Boston, MA, USA) were employed.

3.4 Determination of the levels of specific proteins by enzyme-linked immunosorbent assay (ELISA) (I, IV)

In this so-called sandwich immunoassay, monoclonal antibodies directed against the peptide of interest, and labeled with biotin or horseradish peroxidase (HRP) (for detection) are employed. Standards and samples are incubated separately with both of these antibody preparations in a microplate well coated with streptavidin. At the end of the incubation, the microwell is washed and the biotinylated enzyme-protein of interest – HRP-antibody sandwich bound to the solid-phase is incubated with tetramethylbenzidine (TMB), a substrate for HRP, and the color that develops quantitated.

3.5 Quantitation of StAR and cytochromes P450scc, c17 and c21 by Western blotting (I-III)

PAGE/Western blotting was used to assay the contents of StAR, cytochrome P450scc, and cytochromes P450c17 and P450c21 in Leydig and adrenocortical cell lysates, as well as in protein extracts of whole adrenal glands. Sample protein, measured by the Bradford procedure, was subjected to SDS-PAGE and then transferred electrophoretically to a PVDF membrane. Immunoblotting was performed employing primary antibodies directed against the protein of interest followed by incubation with HRP-conjugated secondary antibodies, addition of substrate for HRP and detection of the resulting signals by chemiluminescence.

4. RESULTS AND DISCUSSION

4.1 Influence of procymidone and genistein on the pituitary-gonadal axis and Leydig cell steroidogenesis (I)

Long-term dietary exposure of rats to procymidone increased the plasma levels of LH and testosterone in the treated animals. Leydig cells isolated from these exposed rats displayed an enhanced capacity for producing testosterone in response to stimulation by hCG or $(Bu)_2$cAMP, as well as elevated expression of StAR, P450scc and P450c17. The explanation for these observations might involve the fact that this fungicide exerts anti-androgenic activity by binding directly to the androgen receptor in the prostate (Hosokawa *et al*, 1993). In this manner, procymidone might inhibit the negative feedback by androgen on the hypothalamus and/or the pituitary, thereby giving rise to hypergonadotropism. Increased secretion of LH by the pituitaries of procymidone-treated rats stimulated the expression of steroidogenic enzymes in Leydig cells and, thereby, the biosynthesis of androgens, resulting in an elevation in the plasma level of testosterone.

In contrast, treatment of rats with genistein suppresses the *ex vivo* steroidogenic response of their Leydig cells to hCG or $(Bu)_2$cAMP by down-regulating their expression of P450scc, while plasma levels of testosterone and LH were not significantly altered. These findings could mean that, the inhibition of P450scc was not severe enough to affect testosterone production; that compensatory mechanisms designed to counteract the effect of a decrease in this enzyme activity were activated *in vivo*; or that this phytoestrogen inhibits the activity of 5α-reductase in Leydig cells, as it does in peripheral tissues (Kellis Vickery 1984, Evans *et al*.1995), thereby decreasing the rate of testosterone catabolism and maintaining a normal level of circulating androgen despite the suppression of P450scc activity.

We also found that genistein inhibits the stimulation of steroidogenesis in adrenocortical cells by ACTH, giving rise to an accumulation of pregnenolone and reduction in corticosterone production by these cells *in vitro*. Perhaps part of this accumulated pregnenolone is metabolized further into testosterone by testicular Leydig cells. However, it should be remembered in this connection that the mechanisms underlying the biological actions of genistein are complex, since in

addition to its estrogen-like effects (Murkies, 1998), this compound also inhibits tyrosine kinase (Akiyama *et al*, 1987). Therefore, biological responses to genistein will be determined both by enhanced expression of specific estrogen-dependent genes and by alterations in signalling pathways in the target cells.

We conclude that long-term dietary administration of procymidone or genistein to rats exerts different effects on the pituitary-gonadal axis *in vivo* and on Leydig cell steroidogenesis *ex vivo*. Possibly as a result of disruption of hormonal feedback control as a secondary consequence of its anti-androgenic action, procymidone activates this endocrine axis, thereby causing hypergonadotropic activation of testicular steroidogenesis. In contrast, genistein influences spermatogenesis and significantly inhibits Leydig cell steroidogenesis *ex vivo* without altering the serum level of either LH or testosterone.

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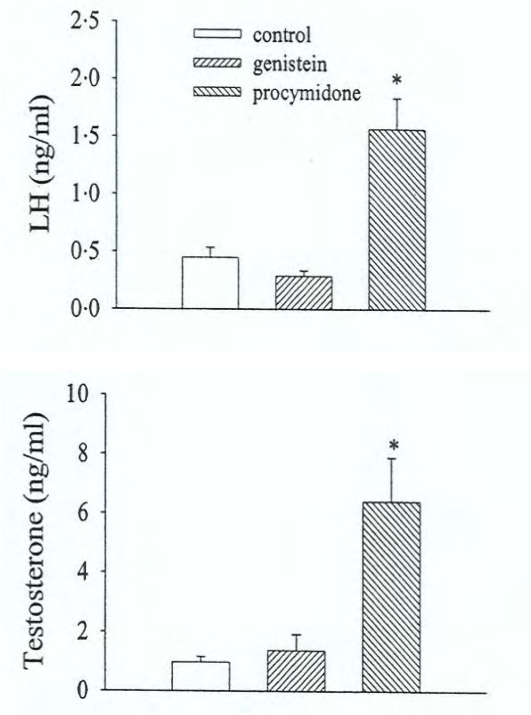

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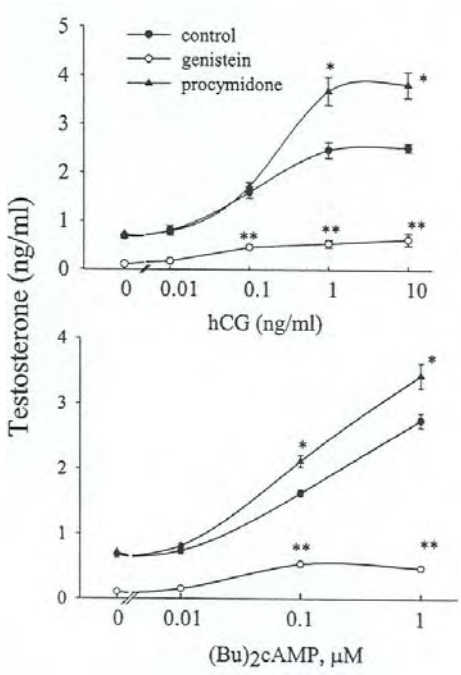

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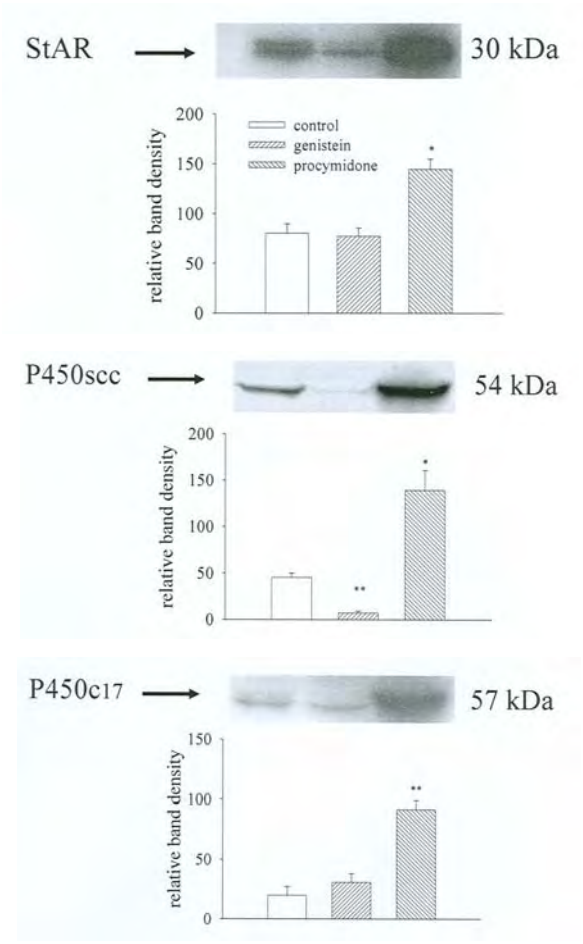

Figure 14

(A) Serum levels of LH and testosterone. (B) Testosterone production by isolated Leydig cells in response to different concentrations of hCG or dibuturyl cAMP. (C) Levels of StAR, and P450c17 in isolated Leydig cells.

4.2 Effects of resveratrol on steroidogenesis by rat adrenocortical cells (II)

The mechanism(s) by which resveratrol inhibits adrenocortical steroidogenesis is still unclear. Here we examined this effect, both *ex vivo* and *in vitro*. Resveratrol was found to inhibit *in vitro* corticosterone production in adrenocortical cells isolated from untreated rats by 47%. In addition, *in vivo* treatment with this phytoestrogen markedly suppressed steroidogenesis in adrenocortical cells isolated from treated animals. These alterations in glucocorticoid production were associated with enhanced accumulation of progesterone and reduced expression of cytochrome P450 c21-hydroxylase (Fig. 15).

This phytoestrogen may cause such effects via estrogen-independent pathways. This is indicated by the observation that ICI 182,780, (which binds to the ER with an affinity similar to that of estradiol and thereby blocks its actions), cannot abolish these effects of resveratrol. Thus, this compound may itself inhibit cytochrome P450c21-hydroxylase directly and/or disturbing some signaling pathway(s) involved in the regulation of this enzyme.

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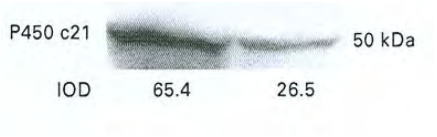

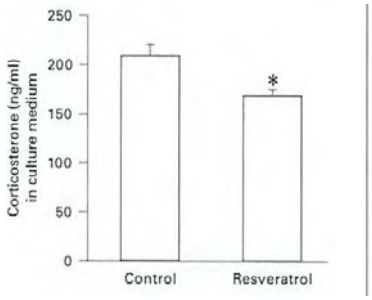

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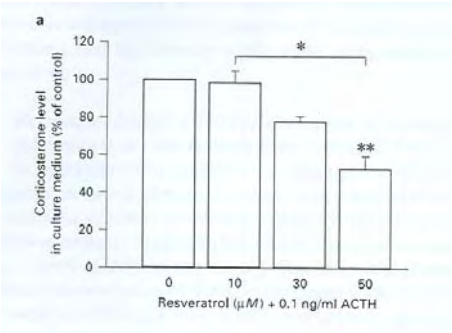
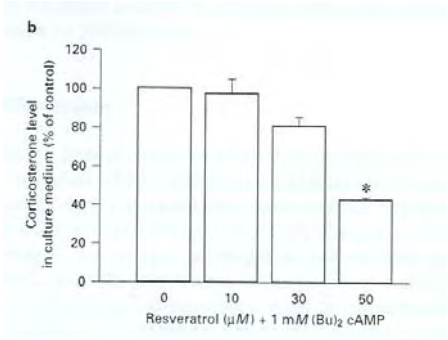
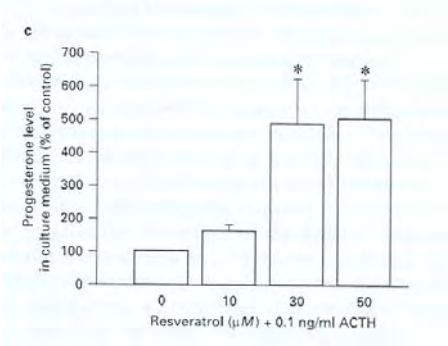






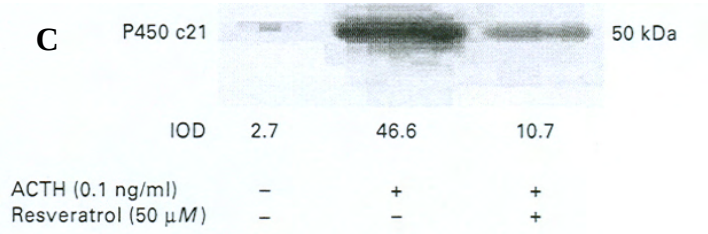


Figure 15

*(A) Effects of dietary treatment with resveratrol (0.84 g/kg) for 12 weeks on the expression of cytochrome P450c21, as analyzed by Western blo,t and in vitro corticosterone production in response to ACTH (0.1 ng/ml). (B) Effects of exposure of Leydig cells isolated from untreated rats to different concentrations of resveratrol on stimulation of corticosterone and progesterone synthesis by ACTH (0.1 ng/ml) and of corticosterone synthesis by $(Bu)_2$cAMP. (C) Effects of resveratrol (50 μM) on in vitro ACTH (0.1 ng/ml) -stimulated expression of cytochrome P450c21 by Leydig cells isolated from untreated rats, *p<0.05. **p<0.01 compared to the corresponding control value.*

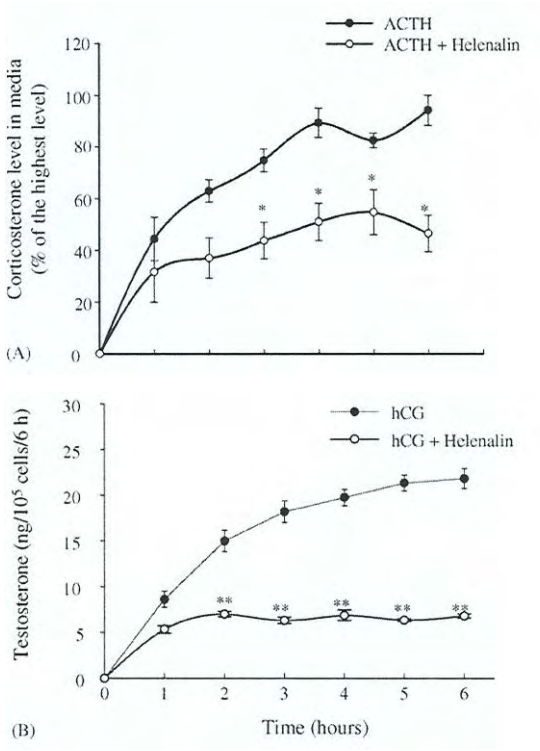
4.3 Effects of helenalin on steroidogenesis by Leydig and adrenocortical cells (III)

We found here that helenalin inhibits stimulation of steroidogenesis in primary cultures of adrenocortical and Leydig cells isolated from untreated male rats by ACTH and hCG, respectively. These effects were already evident within 2-3 hours after initiation of exposure to helenalin and could be reversed by 22R-hydroxycholesterol, a form of cholesterol that freely penetrates membranes. This latter observation indicates that helenalin disturbs cholesterol trafficking between mitochondrial membranes, rather than the activities of down-stream steroidogenic enzymes. Indeed, further experiments revealed that helenalin does attenuate the up-regulation of StAR, induced by ACTH and hCG in adrenocortical and Leydig cells, respectively. We conclude that the sesquiterpene lactone helenalin is a potent inhibitor of the expression of the StAR protein and, thereby, of steroidogenesis in cultured rat adrenocortical and Leydig cells. This novel discovery should be given careful consideration in connection with the further development of anti-inflammatory and anti-cancer drugs related structurally to the family of sesquiterpene lactones.

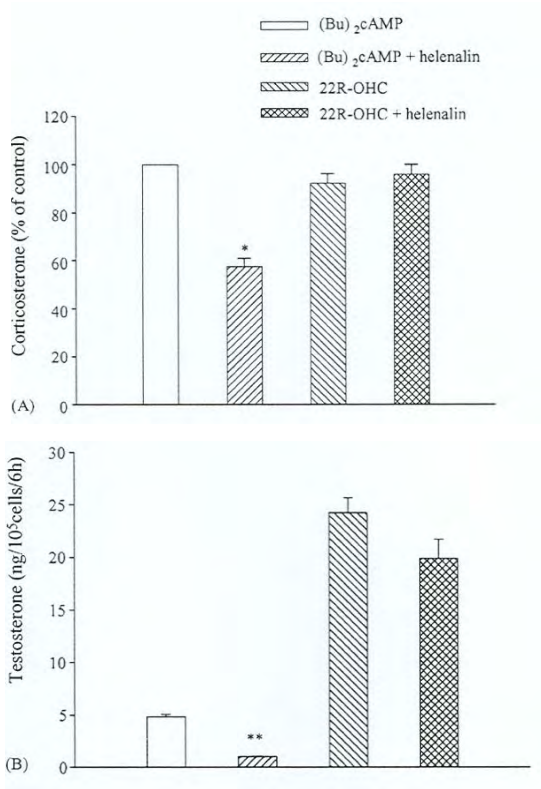
One possible mechanism underlying this inhibition of StAR expression by helenalin may be selective alkylation of the sulfhydryl groups of specific cysteine residues in proteins that participate in the regulation of the corresponding gene in steroidogenic cells. Such alkylation might reduce the affinity of these transcription factors for their target DNA sequences, thereby attenuating expression of StAR. Several different transcription factors, including SF-1, CREB/CREM, C/EBP and GATA, bind to the promoter of the gene encoding StAR and may be involved in positive regulation of the expression of this protein (Manna *et al.*, 2003).

One specific possibility is that helenalin disrupts the interaction of the cyclic AMP-responsive element-binding (CREB) protein, which plays a central role in regulation of the StAR gene (Manna *et al.*, 2003), with its binding site on DNA. The DNA-binding domain of CREB contains two cysteinyl residues that are critical to its activation of gene expression (Abramovitch *et al.*, 2004). However, inhibition of other transcription factors in adrenocortical and Leydig cells by helenalin cannot, of course, be ruled out at the present time. The observation that the inhibitory effect of helenalin on steroidogenesis by adrenocortical and Leydig cells is abolished in the presence of 22R-OHC indicates that these cells remain viable under these conditions.

A


ACTH
ACTH + Helenalin
Corticosterone level in media
(% of the highest level)
(A)
hCG
hCG + Helenalin
Testosterone (ng/10⁵ cells/6 h)
Time (hours)
(B)

B


(Bu) ₂cAMP
(Bu) ₂cAMP + helenalin
22R-OHC
22R-OHC + helenalin
Corticosterone (% of control)
(A)
Testosterone (ng/10⁵cells/6h)
(B)

C

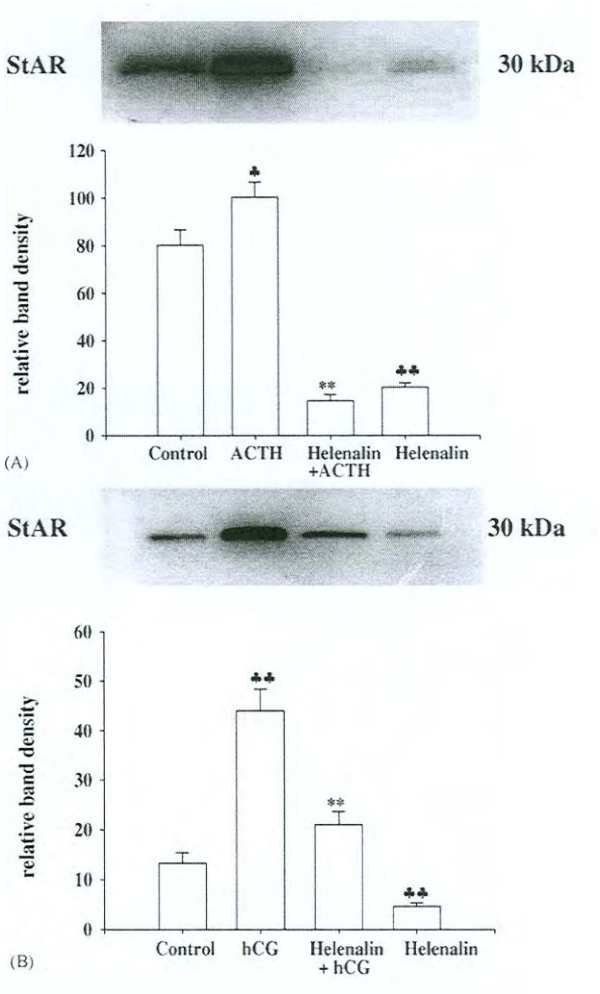


Figure 16. *In vitro responses of adrenocortical and Leydig cells isolated from untreated male rats to helenalin. (A) Time-course of the influence of helenalin pretreatment on the stimulation of steroidogenesis in adrenocortical (upper) and Leydig cells (lower). (B) 22R-hydroxy-cholesterol (22R-OHC) prevents inhibition of steroidogenesis in cultured rat adrenocortical (upper) and Leydig cells (lower) by helenalin (c). Helenalin inhibits the expression of StAR by cultures of hormonally stimulated rat adrenocortical (upper) and Leydig cells (lower).*

4.4 Di-2-ethylhexyl phthalate (DEHP) stimulates the pituitary-adrenal axis of prepubertal rats as well as *ex vivo* steroidogenesis by their adrenocortical cells (IV)

We observed that treatment of prepubertal, developing male rats with DEHP

stimulates the pituitary gland to produce ACTH, which in turn activates adrenocortical steroidogenesis. Accordingly, adrenocortical cells isolated from these treated rats exhibit an enhanced capacity to produce corticosterone in response to ACTH, $(Bu)_2$ cAMP or 22R-OHC, as well as an elevated rate of transport of cholesterol into their mitochondria. In attempt to determine whether DEHP or its major metabolite, MEHP can directly stimulate steroidogenesis by adrenocortical cells, cultures of such cells isolated from untreated rats were incubated with these compounds. The lack of any significant effect on either ACTH- or $(Bu)_2$cAMP-induced steroidogenesis indicates that DEHP and/or its major metabolite act directly on the pituitary-adrenal axis.

The hormonal response of the adrenal glands of young rats to treatment with DEHP is similar to that characteristic of stress, suggesting that DEHP can be considered to be a chemical stressor acting on the pituitary-adrenal axis. Exposure to stressors is known to activate the hypothalamic-pituitary-adrenal (HPA) axis (Dunn 1990) by stimulating a cascade of events involving the release of corticotrophin-releasing factor (CRF) and subsequent release of ACTH. One possible mechanism by which DEHP could activate the HPA axis in young animals is via activation of cytokine expression in the hypothalamus.

In contrast treatment of mature rats with DEHP does not influence the pituitary-adrenal axis or adrenocortical steroidogenesis. Differences in the levels and/or nature of hepatic metabolites of DEHP might explain, at least in part, the higher susceptibility of the hypothalamus and/or pituitary gland of younger male rats to the effects of this phthalate. A mixture of DEHP metabolites might directly stimulate the hypothalamus and/or pituitary of developing rats, thereby resulting in hypercorticotropism. This proposal is consistent with observations from numerous other studies.

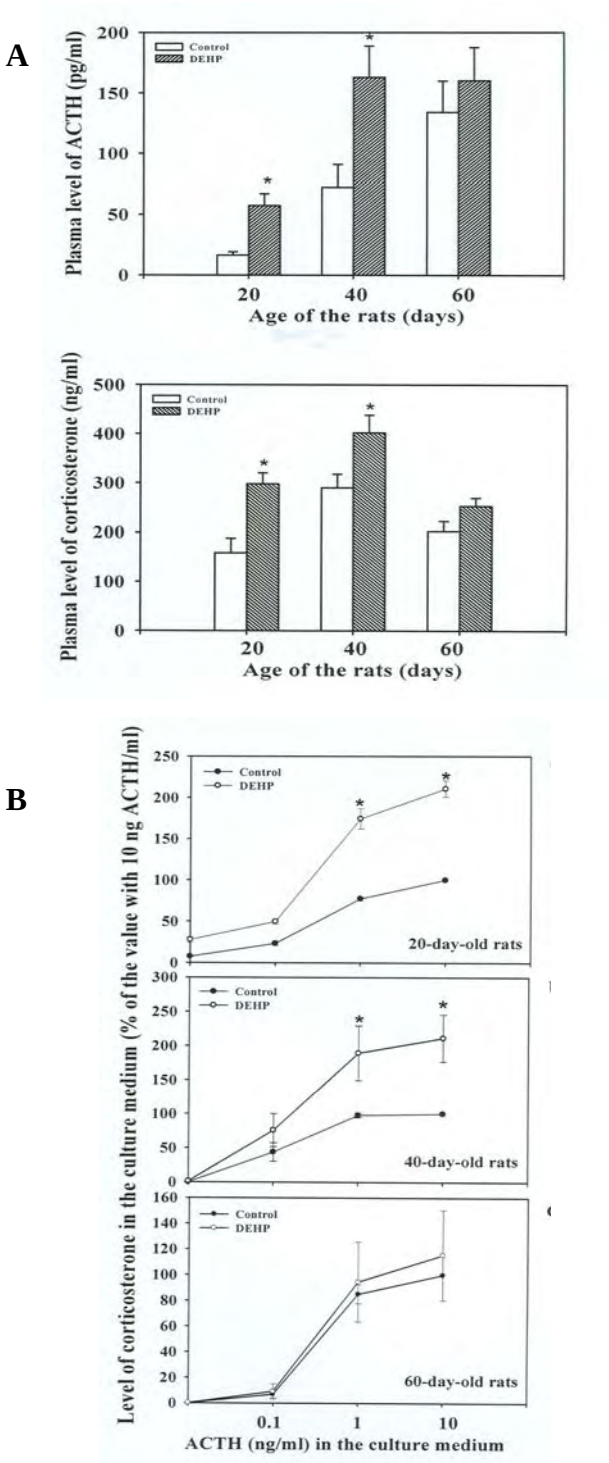


Figure 17. *(A) Plasma levels of ACTH and corticosterone in male rats of different ages exposed to DEHP (500 mg/kg at 20 days of age and 750 mg/kg at the ages of 40 and 60 days) by oral gavage once daily for 4 days. The control animals received corn oil (vehicle) alone in the same manner. (B) Corticosterone production by cultures of adrenocortical cells isolated from DEHP-treated and control male rats of different ages in response to ACTH.*

From a clinical perspective, we suggest that those xenobiotics (e.g., resveratrol and helenalin) that repress the expression of StAR and/or some steroidogenic enzyme(s) may give rise to a hormonal pattern similar to that associated with certain types of autosomal recessive congenital adrenal hyperplasia. Thus, if the human embryo is exposed to these chemicals at a gestational age of 7-8 weeks, which is a critical period in the differentiation of the internal and external genitalia, the risk for adverse effects on this differentiation cannot be neglected. Furthermore, when high doses of these chemicals are used in treating some diseases, adrenal and/or testicular functions should be monitored for serious side-effects, especially among individuals in whom these functions are already suboptimal.

5 CONCLUSIONS

1. The anti-androgen procymidone, disrupts hormonal feedback in the pituitary-gonadal axis of male rats and, consequently after *ex vivo* steroidogenesis *ex vivo* by their Leydig cells.
2. The phytoestrogen genistein inhibits *ex vivo* sterodiogenesis by the Leydig cells of male rats without altering their serum level of either LH or testosterone.
3. Resveratrol suppresses *in vivo*, *in vitro* and *ex vivo* corticosterone production by male rat adrenocortical cells by attenuating expression of cytochrome P450c21-hydroxylase.
4. In male rat adrenocortical and Leydig cells cultured *in vitro*, the sesquiterpene lactone helenalin is a potent inhibitor of the expression of the StAR protein and of steroidogenesis.
5. Treatment of male rats with DEHP exerts an age-dependent stimulatory influence on their pituitary-adrenocortical axis *in vivo*, as well as on adrenocortical steroidogenesis *ex vivo*.

Based on these findings presented here, the diagram below summarizes the fact that the ECDs examined exert their effects on different steps involved in steroidogenesis.

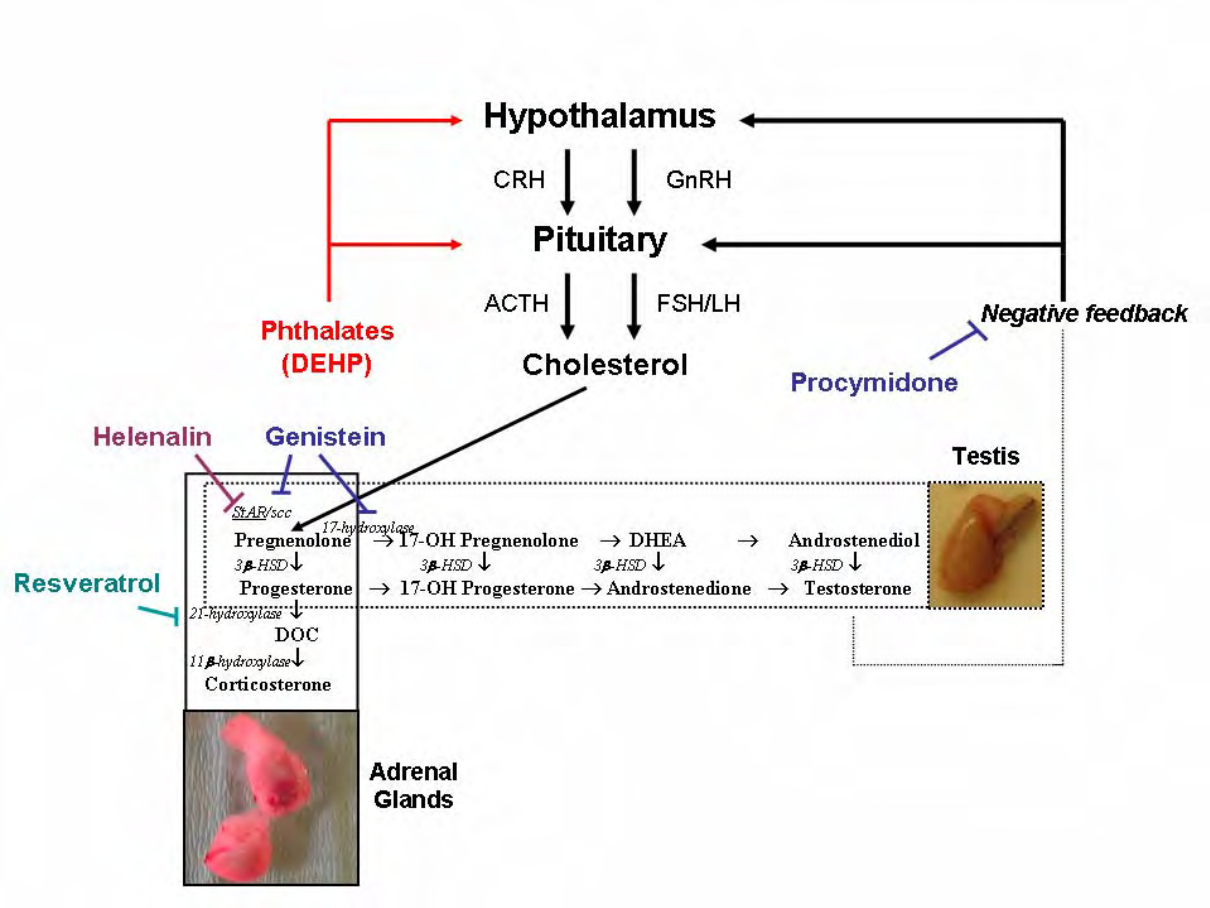


Figure 18. *Summary of the proposed effects of the studied EDCs on HPG/HPA axes and/or steroidogenesis.*

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