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Diverse effects of endocrine-disruptive chemicals on Leydig and adrenocortical cell steroidogenesis in rodents and humans

Iuliia Savchuk

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by

Iuliia Savchuk, MD

Principal Supervisor:
Ass Professor Konstantin Svechnikov
Karolinska Institutet
Department of Women's and Children's Health

Co-supervisor(s):
Professor Olle Söder
Karolinska Institutet
Department of Women's and Children's Health

Opponent:
Professor Matts Olovsson
Uppsala University
Department of Women's and Children's Health

Examination Board:
Ass Professor Stefan Arver
(coordinator)
Karolinska Institutet
Department of Medicine/Huddinge
Division of Metabolism

Professor Eewa Nånberg
Karlstad University
Department of Health Sciences

Ass Professor Pauliina Damdimopoulou
Karolinska Institutet
Department of CLINTEC

Stockholm 2017

“Life is the art of drawing sufficient conclusions

from insufficient premises”

Samuel Butler

To my beloved family and my country Ukraine

ABSTRACT

We are living in a man-made world and often exposed to different chemicals known as endocrine disrupting chemicals (EDCs) that negatively influence our hormonal system. These compounds have the potential to interrupt a normal steroidogenesis and result in harmful effects on the reproductive health of humans and among other organisms. Several studies have demonstrated a considerable decrease in fertility biomarkers such as sperm counts, and increase in malformations and undermasculinization of the male reproductive tract of mammals including humans. Increasing incidence of cryptorchidism, hypospadias and micropenis in male babies may be a consequence of the detrimental effect of environmental chemicals on male fetuses due to disruption of the steroidogenic machinery in the adrenal glands and gonads. The general aim of this thesis was to examine the impact of selected EDCs on hormonal function of steroidogenic cells from mice and human.

In papers 1 and 2 we made use of primary cultures of immature mouse Leydig cells (LCs) to probe their response to bisphenol A and mono-phthalates. The cells were incubated with or without human chorionic gonadotropin (hCG) and a selection of chemicals ranging in concentrations over a certain period of time. It was uncovered that immature mouse LCs with varying abilities to produce testosterone were unaffected by estrogenic stimuli. Moreover, they exhibited a similar response to mono-phthalates. Our study showed that bisphenol A is able to stimulate testosterone production in the hCG pre-treated immature mouse LCs. However, among three mono-phthalates, only MEHP significantly increased basal androgen production via upregulation of StAR expression. In addition, mono-phthalates disturb a normal mitochondrial function, by attenuating ATP generation and increasing super oxide synthesis.

In papers 3 and 4 we had possibility to obtain human abortion material from terminated pregnancies at gestational week 9-12. In study 3, we used a primary culture of human adrenocortical cells, stimulated with or without adrenocorticotropic hormone (ACTH) with resveratrol treatment, whereas in paper 4, the whole tissue of the human fetal adrenal gland (HFA) was taken for analysis. We observed that resveratrol had a suppressive effect on the synthesis of androstenedione, dehydroepiandrosterone (DHEA) and cortisol by the primary culture of human fetal adrenocortical cells through inhibition of lyase activity of cytochrome Cyp17 and Cyp21 expression in these cells. With the help of GC-MS/MS expertize was detected that the HFA is limited to produce androgens to testosterone and androsterone at the first trimester of pregnancy. This finding correlates with an observation of fully activated

CYP17A1-POR-CYB5 complex and the concurrent upregulation of transcription factors (SF1, GATA-6) and downregulation of HSD3B2.
Altogether, our results indicated that environmental endocrine disruptive chemicals have diverse effect on androgen production in steroidogenic cells in rodents and humans. Bisphenol A and MEHP should be taken into consideration as possible causes of premature maturation in boys. In contrast, pregnant women at an early gestational stage should try to avoid taking resveratrol due its ability to suppress steroidogenesis in the human fetal adrenals.

Key words: steroidogenesis, Leydig cells, human fetal adrenals/cells, bishphenol A, MEHP, resveratrol

LIST OF SCIENTIFIC PAPERS

I. **Savchuk I**, Söder O, Svechnikov K (2013) Mouse Leydig cells with different androgen production potential are resistant to estrogenic stimuli but responsive to bisphenol A which attenuates testosterone metabolism. PLoS ONE 8(8): e71722. doi:10.1371/journal.pone.0071722

II. **Savchuk I**, Söder O, Svechnikov K (2015) Mono-2-ethylhexyl phthalate stimulates androgen production but suppresses mitochondrial function in mouse Leydig cells with different steroidogenic potential. Toxicol Sci. 145(1):149-56. doi: 10.1093/toxsci/kfv042.

III. **Savchuk I**, Morvan ML, Søeborg T, Antignac JP, Gemzell-Danielsson K, Le Bizec B, Söder O, Svechnikov K (2017) Resveratrol inhibits steroidogenesis in human fetal adrenocortical cells at the end of first trimester. Mol Nutr Food Res. 61(2) doi:10.1002/mnfr.201600522.

IV. **Savchuk I**, Morvan ML, Antignac JP, Gemzell-Danielsson K, Le Bizec B, Söder O, Svechnikov K (2017) Androgenic potential of human fetal adrenals at the end of the first trimester. Endocr Connect. 6 (6): 348-359 doi: 10.1530/EC-17-0085.

Additional Publications (Not included in the thesis)

1. Svechnikov K, Stukenborg JB, **Savchuck I**, Söder O. Similar causes of various reproductive disorders in early life. Asian J Androl 2014;16:50-9

2. Svechnikov K, **Savchuk I**, Morvan ML, Antignac JP, Le Bizec B, Söder O. Phthalates Exert Multiple Effects on Leydig Cell Steroidogenesis. Horm Res Paediatr. 2015 Nov 12.

3. Wagner IV, Klöting N, Atanassova N, **Savchuk I**, Spröte C, Kiess W, Söder O, Svechnikov K Prepubertal onset of obesity negatively impacts on testicular steroidogenesis in rats. Mol Cell Endocrinol. 2016 Dec 5;437:154162.doi:10.1016/j.mce.2016.08.027. Epub 2016 Aug 17

4. Pampanini V, Germani D, Puglianiello A, Stukenborg JB, Reda A, **Savchuk I**, Kjartansdóttir KR, Cianfarani S, Soder O Impact of uteroplacental insufficiency on postnatal rat male gonad. J Endocrinol. 2016 Nov 24. pii: JOE-16-0418

CONTENTS

LIST OF ABBREVIATIONS

17OH4P	17α-Hydroxyprogesterone
17OH5P	17α-Hydroxypregnenolone
17OH-Allo	17OH-allopregnanolone
17OH-DHP	17OH-dihydroprogesterone
21-OHD	21-hydroxylase deficiency
3α-Diol	3α-Androstanediol
3αHSD	3α-Hydroxysteroid dehydrogenase
A4	androstenedione
ACTH	adrenocorticotropic hormone
ALC	adult Leydig cells
ATP	adenosine triphosphate
BPA	Bisphenol A
CRH	corticotrophin releasing hormone
CYB5	cytochrome B5 Type A
DAX1	dosage-sensitive sex reversal, adrenal hypoplasia critical region, on chromosome X, gene 1
DBP	di-n-butyl phthalate
DDT	dichlorodiphenyltrichloroethane
DEHP	di-2-ethylhexyl phthalate
DES	diethylstilbestrol
DHEA	dehydroepiandrosterone
DHEAS	dehydroepiandrosterone sulfate
Dhh	Desert Hedgehog
DHT	dihydrotestosterone

DZ	definitive zone
EDCs	endocrine disrupting chemicals
EPA	Environmental Protection Agency
ERs	estrogen receptors
ERα	estrogen receptor α
Erβ	estrogen receptors β
FLC	fetal Leydig cells
FSH	follicle stimulating hormone
FZ	fetal zone
GATA6	GATA Binding Protein 6
GATA4	GATA Binding Protein 4
GnRH	gonadotropin releasing hormone
hCG	human chorionic gonadotropin
HFA	human fetal adrenal gland
HFAC	human fetal adrenocortical cells
HPG	hypothalamus-pituitary-testicular axes
HSD17B3	17β-hydroxysteroid dehydrogenase, type 3
HSD17B6	17β-hydroxysteroid dehydrogenase, type 6
HSD3B2	3beta-hydroxysteroid dehydrogenase/delta(5)-delta(4)isomerase type II
LCs	Leydig cells
LH	luteinizing hormone
MBeP	monobenzyl phthalate
MBP	mono-butyl phthalate
MC2R	melanocortin 2 receptor
MEHP	mono-(2-ethylhexyl) phthalate
P4	progesterone

P5	pregnenolone
PBBs	polybrominated biphenyls
PCBs	polychlorinated biphenyls
POR	cytochrome P450 Oxidoreductase
ROS	reactive oxygen species
S	11-Deoxycortisol
SF1	steroidogenic factor-1
SRD5A1	5α-reductase type I
SRD5A2	5α-reductase type II
StAR	steroidogenic acute regulatory protein
TDS	testicular dysgenesis syndrome
TZ	transitional zone

1 INTRODUCTION

Steroidogenesis is a dynamic process to produce steroid hormones from the cholesterol derivatives that occur in a variety of tissues, most prominently in the adrenal glands and gonads. The normal production of steroids plays a pivotal role to support natural body homeostasis and reproductive function. This process is very complex and needs an accurate orchestrated performance between single cells and all organs. Biosynthesis of steroids is regulated by the hypothalamus-pituitary endocrine axes through the classical negative feedback loop where steroid hormones produced by the target endocrine organs (e.g., testes, adrenals, ovaries) suppress production of releasing hormones by the hypothalamus, and adrenocorticotropins and gonadotropins by the pituitary (Fig.1).

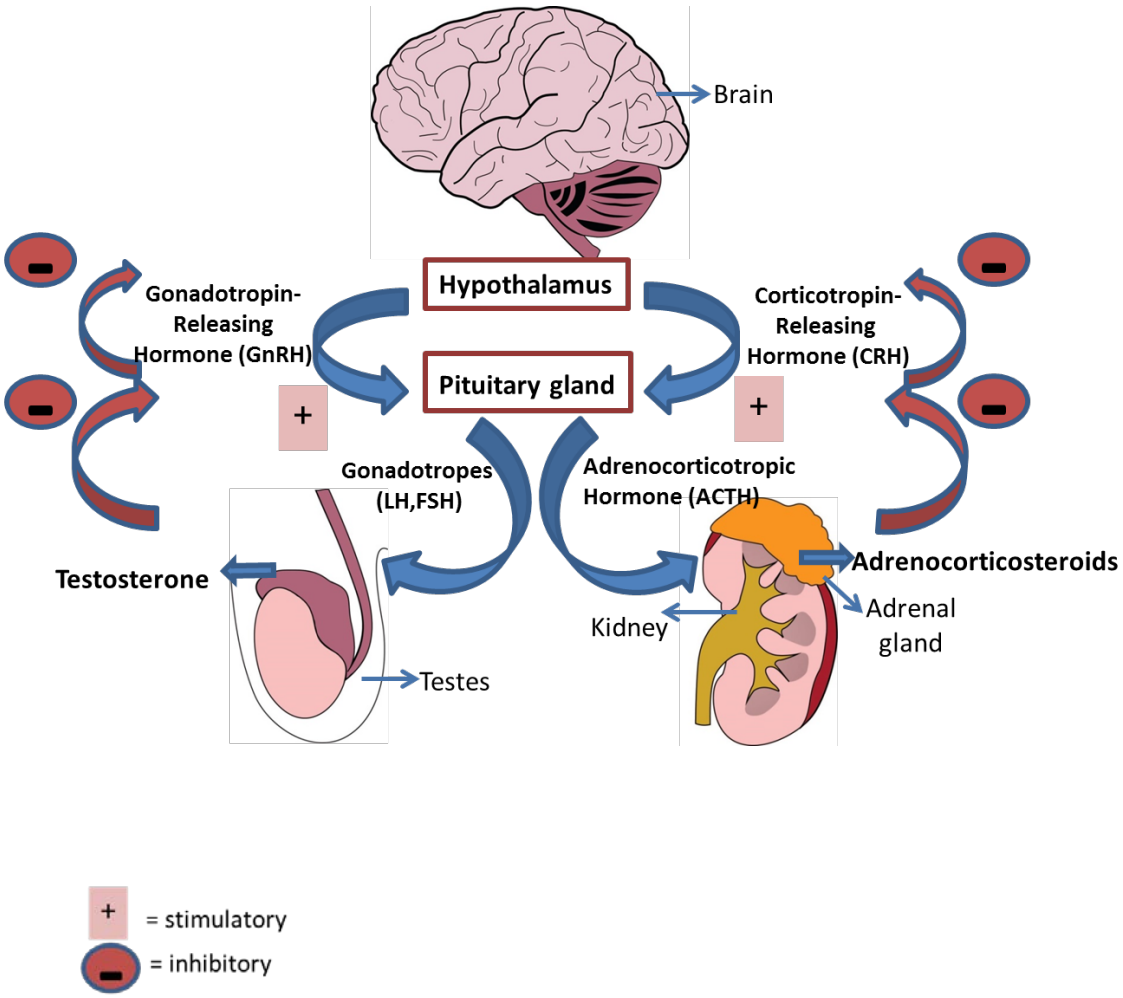

Figure 1. *Schematic illustration of the hypothalamus-pituitary-gonadal and adrenal axes. LH, luteinizing hormone; FSH, follicle stimulating hormone.*

1.1 SEX DIFFERENTIATION

Gonadal anlagen during the embryonic period can develop into either testes or ovaries. Sexual differentiation in the human follows four successive steps:

1. Formation of the genetic sex occurs when the sperm fertilizes the egg creating an XX (female) or XY (male) zygote, dependent on the chromosomal set-up of the sperm.

2. Sex determination occurs when the yet undifferentiated gonad turns into a testis in XY and an ovary in XX embryos. The gonad regulates further development.

3-4. the development of the internal and the external genitalia is regulated by the gonadal hormones. In males this process is dependent of androgens produced by LCs and anti Müllerian hormone secreted by Sertoli cells in the testis.

Every step in this process is important for normal sexual differentiation.

1.2 THE TESTIS

The testis is responsible for the sperm production and production and secretion of testosterone.

There are two different compartments in the testes, separated from each other by a basal lamina: a tubular and an interstitial one. The tubular compartment where the spermatogenesis takes place consists of tubuli seminiferi surrounded by the peritubular cells, and the Sertoli and germs cells. The interstitial compartment where the steroidogenesis occurs is composed byLCs, macrophages and other immune cells and blood and lymph vessels.

The function of testis is controlled by the hypothalamus-pituitary-testicular axes (HPG) to the same extent as by paracrine and autocrine factors. Gonadotropin releasing hormone (GnRH) stimulates the anterior pituitary to release luteinizing hormone (LH) and Follicle stimulating hormone (FSH), further these hormones activate androgen production by LCs and convert the signal to the germ cells via Sertoli cells, respectively (Morohashi, 1997).

1.3 LEYDIG CELLS

Franz Leydig in 1850 first described testicular cells that are situated in the interstitial compartment of the testis and their function was unclear until testosterone was first isolated in 1935. Leydig cells (LCs) are polygonal cells which contain lipid droplets and a large amount of smooth endoplasmic reticulum with the main function to produce testosterone which is needed for spermatogenesis and normal male development. There are two types of LCs: fetal

Leydig cells (FLC) and adult Leydig cells (ALC) with the same function to generate androgens, but with different active period and origin.

The fetal Leydig cells

Fetal Leydig cells (FLC) population start to arise prenatally (around 12.5days of conception (dpc) in mice and 14.5dpc in rats, whereas in humans, they are recognized from week 6-7 of pregnancy) and produce testosterone which is needed for the normal masculinization of the male fetus (Svechnikov *et al.*, 2010b).

The origin of FLC is still unknown but there are some studies that suggest their development from the neural crest (Middendorff *et al.*, 1993; Mayerhofer *et al.*, 1996), the mesonephros (Nishino *et al.*, 2001), adrenal-gonadal primordium (Hatano *et al.*, 1996) or the coelomic epithelium (Karl and Capel, 1998). There are many factors that influence the natural differentiation of the FLC: genetic background (a normal Y-chromosome containing an intact Sry region is essential), some factors produced by Sertoli cells (Dhh and PDGF-A) (Brennan *et al.*, 2003; O'Shaughnessy *et al.*, 2006) and paracrine components. Interestingly, this process is LH independent, since fetal testosterone production in a LHR-knock out mice was not affected (Pakarinen *et al.*, 2002).

The number of FLC is significantly higher compare to the ALC (Migrenne *et al.*, 2001), but they regress before the birth, although some cells could be found up till 3 month postpartum (Ariyaratne and Chamindrani Mendis-Handagama, 2000).

The adult Leydig cells

The precursors of ALC are still under discussion. Several studies showed evidence that peritubular mesenchymal-like cell types give rise to the progenitors of LCs (Benton *et al.*, 1995; Siril Ariyaratne *et al.*, 2000; Habert *et al.*, 2001), others suggested that vascular smooth muscle cells and pericytes undergo trans differentiation into steroidogenic cells (Ariyaratne and Chamindrani Mendis-Handagama, 2000).

One can divide postnatal differentiation of LCs into 3 stages: (1) progenitor stage (in the rat testis between day 11-28 and before 3 years of age in humans) LCs are elongated and have spindle shape as stem cells, but they have developed mitochondria and lipid droplets and are able to produce small amount of androgens; (2) immature stage (from day 28-56 in rats, between 3-8 years in humans) LCs are rounder and contain a lot of lipid droplets, express 5α reductase II and 3αHSD, which help to convert testosterone to 5α-androstane-3α,17β-diol; (3) mature stage (day 56 in rats, in humans at age 9-11 with an end of maturation around 15 years

of age) LCs are round and have less lipid droplets and mitochondria, cytoplasm contains large amount of smooth endoplasmic reticulum and a well-differentiated Golgi apparatus; they produce testosterone as the main steroid and have limited ability to proliferate.

1.4 THE ADRENALS

1.4.1 The fetal adrenals

The fetal adrenal gland possesses a remarkable transformation in size, morphology and functioning during the fetal and perinatal life. The human primitive adrenal gland can be recognized by 3 to 4 weeks of gestation. During the fetal life 3 distinct zones can be identified: the fetal zone (FZ) that consists of large eosinophilic cells, and the outer definitive zone (DZ), which is comprised of small, densely packed basophilic cells (Hanley *et al.*, 2001), furthermore with the help of ultrastructural studies the third zone was distinguished, named as the transitional zone (TZ).

The fetal zone

The fetal zone (FZ) consists of large (20–50 mm) cells that express steroid acute regulatory protein (StAR), CYP11A1, CYP17 and CYP21 and 3βHSD2 and therefore they are able to produce dehydroepiandrosterone (DHEA) and dehydroepiandrosterone sulfate (DHEAS), a substrate for placental estrogen synthesis, as well as pregnenolone sulphate and 17α-hydroxypregnenolone sulphate (Mesiano *et al.*, 1993; Goto *et al.*, 2006). The FZ works similar to the adult zone reticularis.

The transitional zone

Cells that lay between the fetal and definitive zone form the transitional zone, where CYP11A1, CYP17 and CYP21 and 3βHSD2 genes expression was found. The transitional zone may produce glucocorticoids and will give rise to the cortisol-producing zone fasciculata (Mesiano *et al.*, 1993; Goto *et al.*, 2006).

The definitive zone

The DZ is composed of a tight group of small (10–20 mm) cells that show structural characteristics typical of cells in a proliferative state with poor lipid droplets during the midgestation with ability to accumulate cytoplasmic lipid and resemble steroidogenic cells. The steroidogenic gene's profile of this region characterized with the weakly detected

expressed StAR, CYP11A1, CYP21, CYP11B1/CYP11B2 expression and mostly undetectable CYP17(Goto *et al.*, 2006). The definitive zone induces formation of the zona glomerulosa in the adult adrenal.

The adrenal cortex increases in weight almost 10-fold from gestation week 8 to 10, and by week 20 of gestation reaches the same size as fetal kidney and equal to the adult adrenal gland. Nevertheless after the birth, gland undergoes quick involution due a rapid dissipation of the FZ and formation of the zonae glomerulosa and fasciculate (Sucheston and Cannon, 1968).

1.4.2 The adult adrenals

The adult human adrenal located on top of the upper pole of kidney and weigh approximately 4gram. Each gland is divided into two compartments: the medulla (an inner part, responsible for the catecholamines, adrenalin and noradrenaline synthesis) and cortex (an outer region), which is involved in the steroidogenesis. The adrenal cortex itself is divided to 3 zones: zona glomerulosa (where aldosterone is secreted), zona fasciculata (responsible for the cortisol and corticosterone production) and zona reticularis (answerable for the androgens synthesis).

1.4.3 Function and regulation

The adrenal is under control of the hypothalamus-pituitary-gland axis, where corticotrophin releasing hormone (CRH) activates secretion of ACTH in the anterior pituitary, which stimulates cells in the adrenal to generate glucocorticoids (Morohashi, 1997).

The HFA cortex is an active endocrine organ that plays a pivotal role in intrauterine equilibrium and the maturation of certain fetal organs, in addition fetal cortisol is involved in the triggering parturition. During the first trimester of pregnancy, the HFA is the source of the steroid precursors that would be used by placenta to syntheses estrogens. The concept of feto-placental unit was described first by Diczfalusy (Diczfalusy, 1964) where the placenta, the fetal and maternal adrenal glands and the fetal liver work in collaboration in the steroids production and metabolism.

A dysfunction of the adrenal gland leads to the numerous of endocrine diseases. Overproduction of cortisol causes Cushing's syndrome, however deficiency of it is associated with Addison's disease. Genetic mutation in the genes that involved in the glucocorticoid production can cause congenital adrenal hyperplasia.

1.5 STEROIDOGENESIS

Steroidogenesis is a biochemical process to produce steroid hormones from cholesterol. There are several sources of cholesterol: synthesis of cholesterol from acetate within the cell; from cholesterol ester stores that are located in intracellular lipid droplets or from uptake of cholesterol-containing low density lipoproteins.

Synthesis of steroid hormones required a cascade of oxidative enzymes located in both mitochondria and endoplasmic reticulum. The only limitation in this process is the transport of free cholesterol from the cytoplasm into mitochondria that happens with the help of steroidogenic acute regulatory protein (StAR). Cholesterol undergoes conversion to pregnenolone by an enzyme in the inner membrane of mitochondria, called side-chain cleavage enzyme P450scc (CYP11A1). At present, two steroidogenic pathways (Δ5 and Δ 4) are described in LCs. The Δ 5 pathway is mostly operative in humans, while Δ4 pathway is prevalent in rodents. In these pathways pregnenolone is converted to testosterone via DHEA (Δ5 pathway) or progesterone (Δ4 pathway) (Fig.X)

In human LCs, pregnenolone is converted to17α-Hydroxypregnenolone (17OH5P) by CYP17A1 hydroxylase activity. Further, 17OH-Preg is converted to DHEA by the 17,20 lyase activity of CYP17A1. This reaction is also supported by oxidoreductase (POR) and cytochrome b5 (CYB5). DHEA is then converted to testosterone through androstenedione steroid product of 3β-hydroxysteroid dehydrogenase type II (HSD3B2/3βHSDII) activity. Testosterone may be converted to the most potent androgen dihydrotestosterone (DHT). This conversion is catalyzed by 5α-reductase type II (SRD5A2 /5αRed2) which is expressed in genital skin and the prostate. In rodents, the Δ4 pathway is prevalent in LCs and converts

pregnenolone to testosterone via progesterone (Fig.2) (Fluck *et al.*, 2003; Payne and Hales, 2004).

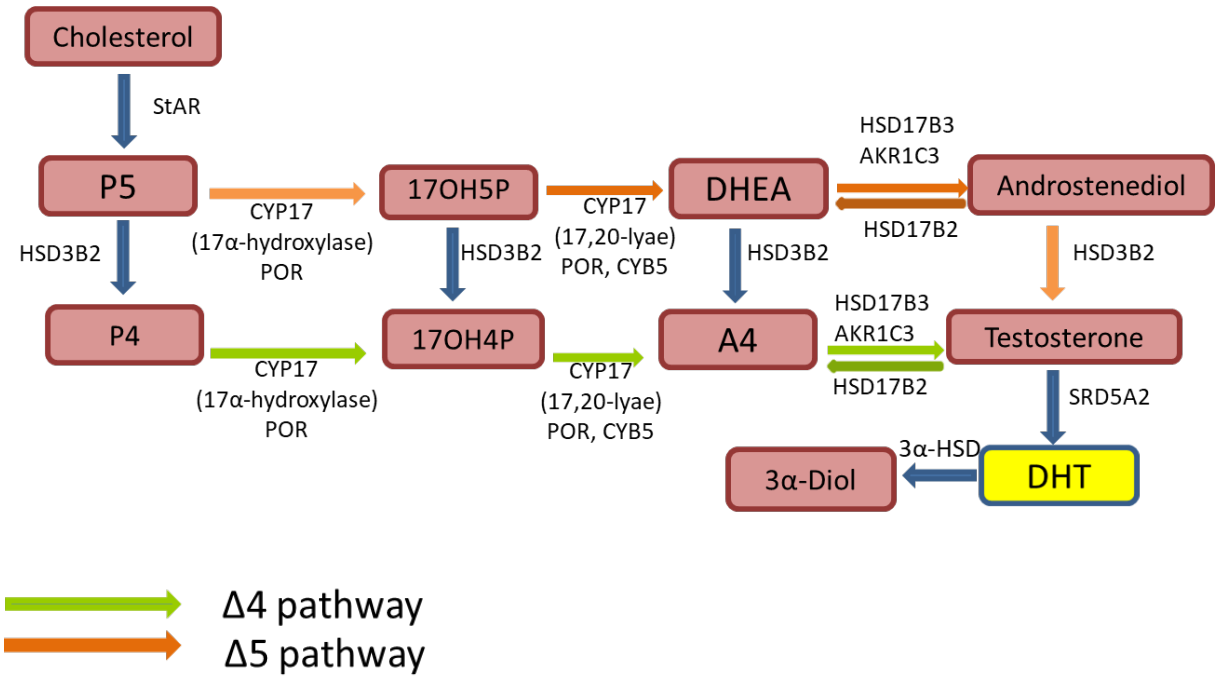

Figure 2. *Diagram showing the Δ4 and Δ5 pathways involved in biosynthesis of androgens.*

The human adrenal cortex synthesizes mineralocorticoids, glucocorticoids and large amount of a weak androgen DHEA. In contrast, rodent adrenals do not express CYP17A1 and have no capacity to synthesize DHEA. The key adrenocortical enzyme involved in the synthesis of mineralocorticoids and glucocorticoids is 21-hydroxylase (CYP21A2/P450c21), which hydroxylates progesterone (in rodents) and 17-hydroxyprogesterone (in humans) at the 21 position to deoxycorticosterone and 11-deoxycortisol, respectively. These steroids are further converted to corticosterone (in rodents) and cortisol (in humans) by 11β-hydroxylase (CYP11B1). Mineralocorticoid aldosterone is synthesized from corticosterone in rodents and human by action of aldosterone synthase (CYP11B2) (Fig.3)

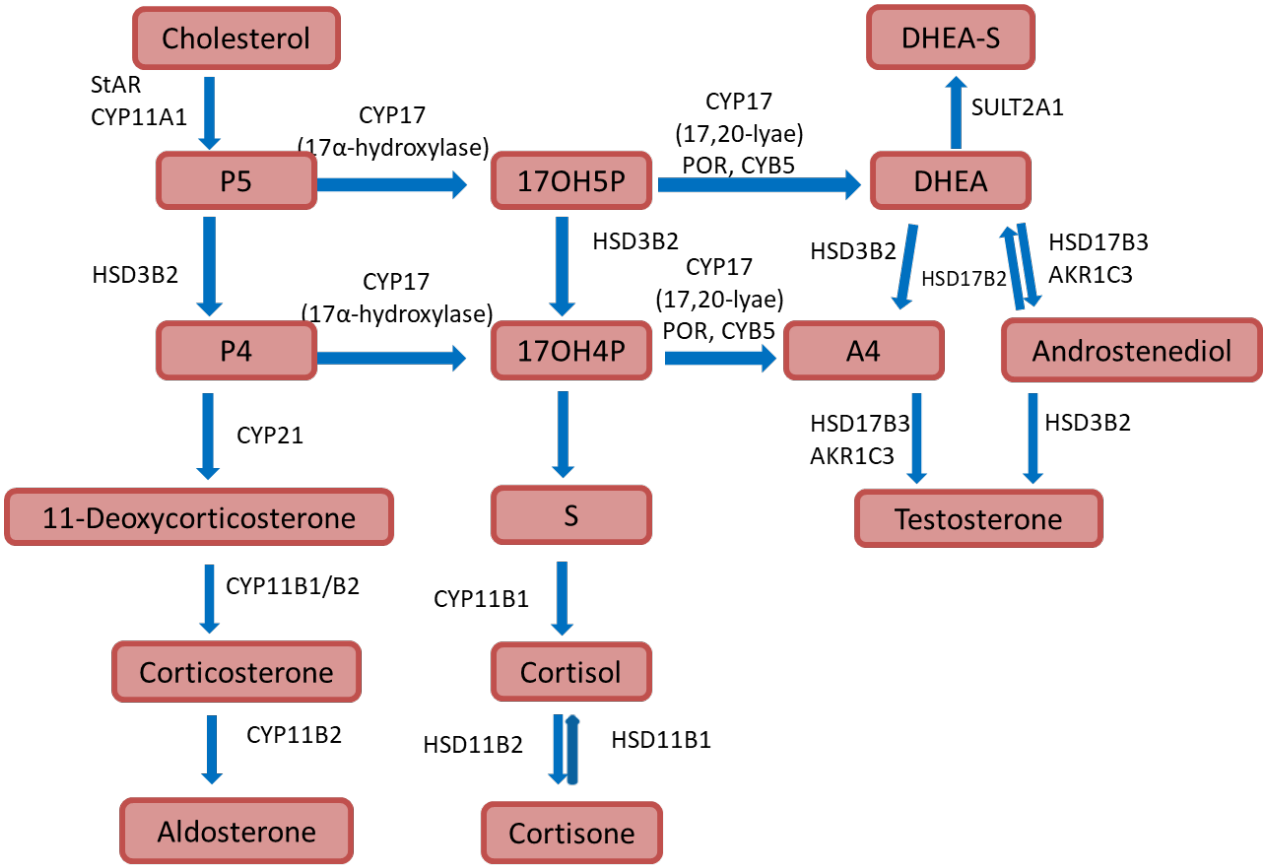

Figure 3. *Overview of steroidogenesis in the adrenal gland.*

HFA has their own pattern to synthesize steroid hormones that have features of the Δ 5, Δ 4

and the backdoor pathways to generate androgens with limitation to androsterone and testosterone (Savchuk *et al.*, 2017a). Cortisol production by HFA takes place on biphasic manner; first it occurs in the early gestational age when the HPA feedback is established and then shut down until late of gestation (Goto *et al.*, 2006).

Recently, it was shown existence of the backdoor pathway (Fig.4), where generation of DHT occurs via testosterone independent pathway. According to it, 17OH4P is 5α-reduced to 17OH-dihydroprogesterone (17OH-DHP) by 5α-reductase type I (SRD5A1/5αRed1) and further converted to 17OH-allopregnanolone (17OH-Allo) by the 3α-HSD1-4 (AKR1C1-4) enzymes. 17OH-Allo is then sequentially converted to androsterone, androstanediol and finally to DHT by actions of CYP17, 17β-hydroxysteroid dehydrogenase, type 3 (HSD17B3) and 17β-hydroxysteroid dehydrogenase, type 6 (HSD17B6). This alternative way to produce androgens was described in testes of pouch young marsupials (Wilson *et al.*, 2003), in

immature mice testes (Mahendroo *et al.*, 2004) and suggested in humans but not proved yet (Fluck *et al.*, 2011). Recent studies have reported the presence of a backdoor pathway of DHT synthesis in newborn untreated patients with 21-hydroxylase deficiency (21-OHD), suggesting that the HFA could contribute to virilization of the female fetuses with 21-OHD via production of this potent androgen (Kamrath *et al.*, 2012).

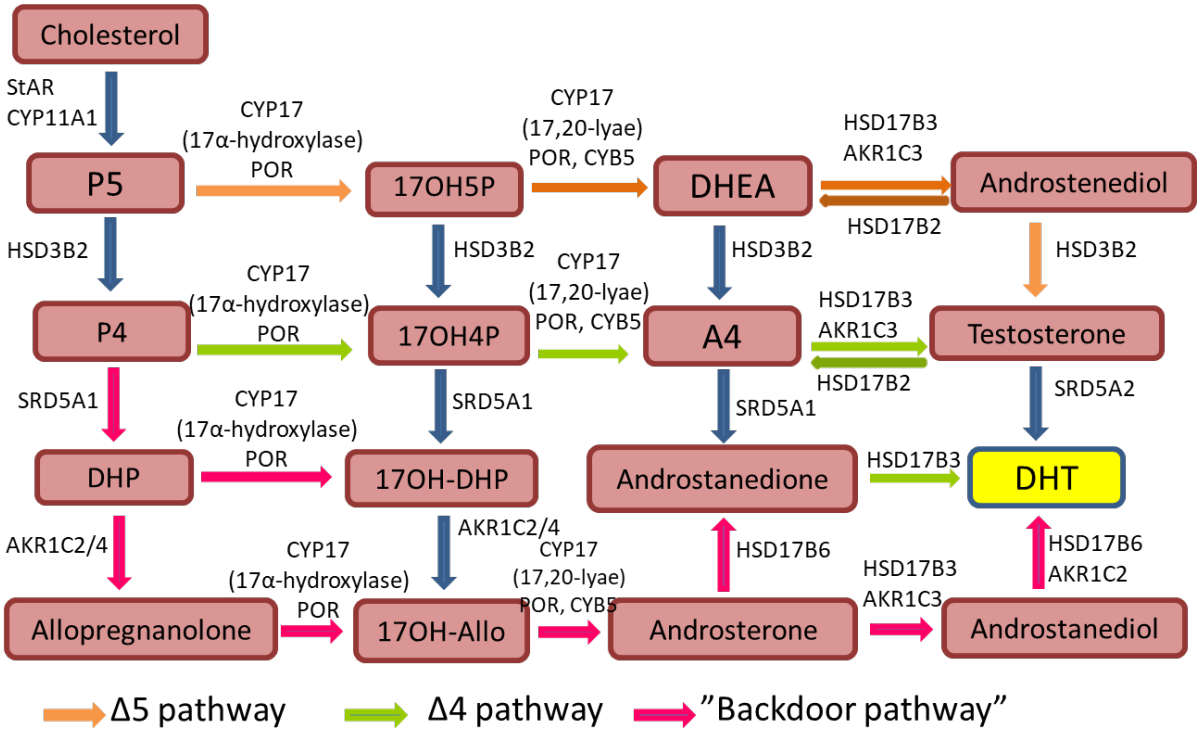

Figure 4. *The "Backdoor pathway" of synthesis of DHT in humans.*

1.6 ENDOCRINE DISRUPTING CHEMICALS (EDCS)

The U.S. Environmental Protection Agency (EPA) specified an endocrine-disrupting compound as "an exogenous agent that interferes with synthesis, secretion, transport, metabolism, binding action, or elimination of natural blood-borne hormones that are present in the body and are responsible for homeostasis, reproduction, and developmental process."

Over the past 20 years observations about the harmful impact of environmental chemicals on human health and wildlife dramatically increase. Numerous of studies highlighted negative influence of endocrine disruptors that can cause adverse effect on the development, malformation of the sex organs, cryptorchidism, reproduction, alterations of the puberty, the same as harm the neurological and immune systems, thyroid dysfunction, obesity and metabolic disorders, diabetes in both humans and animals (Bergman *et al.*, 2013). First, it

was detected the low sperm concentration in men from industrialized countries in 1990 (Swan *et al.*, 1997) . Several other studies reported about the increase incidence of testicular cancer, declining of sperm quality, impaired development of testes and urogenital abnormalities. These features are characteristics of a testicular dysgenesis syndrome (TDS) possibly caused by exogenous factors such as EDC exposure (Nordkap *et al.*, 2012).

Several epidemiological studies showed a correlation of negative effect of EDCs on male reproductive health in boys (Thankamony *et al.*, 2009; Bornehag *et al.*, 2012). Elevated risk of cryptorchidism among baby-boys was linked to their mothers, who worked as gardeners with pesticides (Weidner *et al.*, 1998). Sons, whose fathers were exposed to dioxins (Seveso accident in 1997), had increased episodes of hypospadias (Baskin *et al.*, 2001).

Endocrine disruptors is highly heterogeneous group of molecules that combines synthetic chemicals used as industrial solvents/lubricants and their byproducts (polychlorinated biphenyls (PCBs), polybrominated biphenyls (PBBs), dioxins), plastics (bisphenol A (BPA)), plasticizers (phthalates), pesticides (methoxychlor, chlorpyrifos, dichlorodiphenyltrichloroethane (DDT)), fungicides (vinclozolin), and pharmaceutical agents (diethylstilbestrol (DES)), comparable with EDCs that has natural origin, such as phytoestrogens, including resveratrol, genistein and coumestrol etc. (Diamanti-Kandarakis *et al.*, 2009).

Exposure to the EDCs happens through different routes: food consumption, water, dust, inhalation of gases and particles in the air. These chemicals can be delivered via placenta or breast milk from mother to child. EDCs can act via nuclear receptors, non-nuclear steroid hormone receptors (membrane ERs), orphan receptors and may affect different steroidogenic enzymatic pathways as well as disturb numerous other mechanisms that are important in normal functioning of endocrine and reproductive systems. These chemicals often have a phenolic compound that can imitate natural steroid hormones and interact with steroid hormone receptors as analogs or antagonists. It was reported that 48% of EDCs have estrogenic effect, 19% anti-androgenic, 15% anti-estrogenic, 4% androgenic and approximately 42% possess thyroid action (Choi and Lee, 2004).

There are a lot of evidences that prove negative effect of environmental chemicals on the reproductive system (Hardell *et al.*, 2006; Parent *et al.*, 2015; Skakkebaek, 2016). Not only industrial xenoestrogens even natural examples have a negative impact on the steroidogenesis in both adrenocortical and testicular cells (Singleton and Khan, 2003).

The compounds examined here

1.6.1.1. Bisphenol A (BPA)

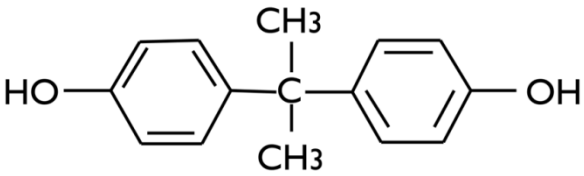

Figure 5. *The chemical structure of bisphenol A (BPA).*

Bisphenol A (BPA) is an organic synthetic compound from the group of diphenylmethane derivatives and bisphenols, with two hydroxyphenyl groups(Fig.5), first was discovered by Russian chemist Aleksandr Dianin and started to be in commercial use since 1957.

BPA has been used to harden polycarbonate plastics, and make epoxy resin which is contained in the lining of food and beverage containers, water and infant bottles, compact discs, impact-resistant safety equipment, and medical devices, dental sealants and composites.

People could be exposed to BPA mostly through the diet; other possible sources are air, water and dust. It was discovered that 90% of US population has BPA in their urine samples (Calafat *et al.*, 2008; Lakind and Naiman, 2008).It was shown that the levels of BPA in maternal plasma could reach 0.3-18.9 ng/ml, while in the fetal plasma the elvel of the compound were around 0.2-9.2 and 0.28-0.97 in the breast milk (Schonfelder *et al.*, 2002; Sun *et al.*, 2004).

BPA is a xenoestrogen, exhibiting estrogen-mimicking, hormone-like properties that raise concern about its suitability in some consumer products and food containers. It was shown that high doses of BPA during pregnancy reduce plasma testosterone at birth in the rat (Tanaka *et al.*, 2006) and acting via estrogen receptors (ERs)-dependent mechanism(Akingbemi et al., 2004) suppressed protein expression of the 17beta-hydroxysteroid dehydrogenase enzyme (HSD17B3), thereby decreasing androgen secretion by rat LCs (Nanjappa et al., 201*2).*

1.6.1.2. Phthalates

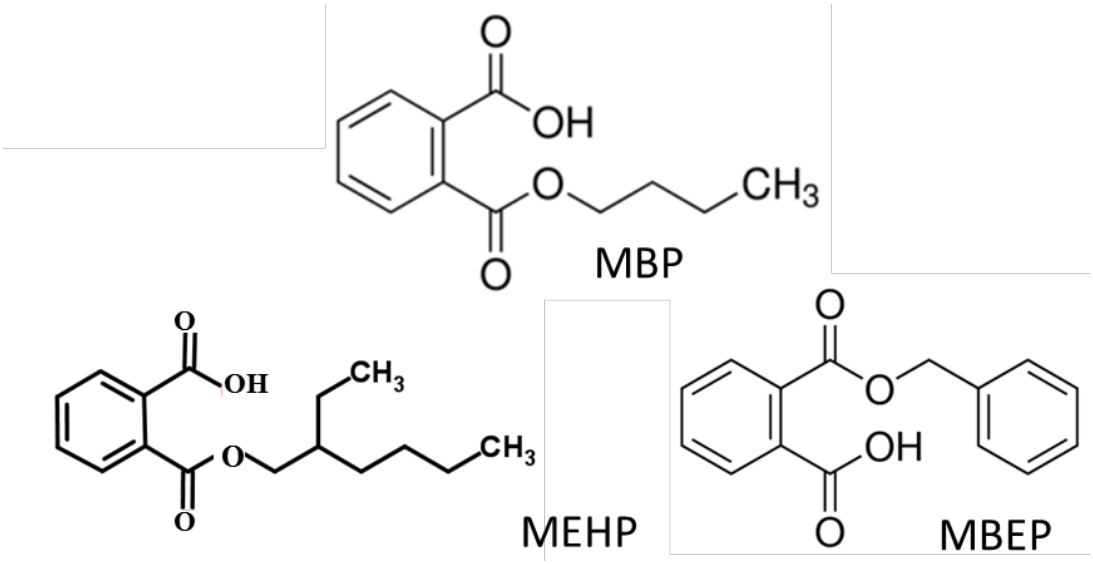

Figure 6. *The chemical formulas of mono-phthalates tested in this thesis.*

Phthalates or phthalate esters are esters of phthalic acid and are mainly used to soften and increase the flexibility of plastic and vinyl. Phthalates are a group of chemicals that widely used in hundreds of consumer products.

One can find phthalates in cosmetics and personal care products, including perfume, hair spray, soap, shampoo, nail polish, and skin moisturizers. They are used in products such as flexible plastic and vinyl toys, shower curtains, wallpaper, food packaging, and plastic wrap.

Phthalates are also used in wood finishes, detergents, adhesives, plastic plumbing pipes, lubricants, medical tubing and fluid bags, solvents, insecticides, medical devices, building materials, and vinyl flooring.

High exposure to phthalates, 25mg/kg/day, were detected in group of children who went through hemodialysis and blood transfusion (Koch *et al.*, 2006).

Several studies reported that European population mostly exposed to the 2 commonly used phthalates, di-n-butyl phthalate (DBP) and di-2-ethylhexyl phthalate (DEHP) that are further metabolized to mono-butyl phthalate (MBP) and mono-2-ethylhexyl phthalate (MEHP) (Fig.6), accordingly, and have a negative effect on male reproductive system (Wormuth *et al.*, 2006; Clewell *et al.*, 2010; Albert and Jegou, 2014). Phthalates have anti-androgenic effect by attenuation of testosterone production by LCs (Svechnikov *et al.*, 2010a; Svechnikov *et al.*, 2015).

High level of phthalate metabolites in urine samples from pregnant women was found to correlate to relatively short ano-genital distance in their male babies (Swan *et al.*, 2005). Further, different types of phthalates have ability to inhibit steroidogenesis by LCs in primates and rodents (Koch *et al.*, 2003; Sharpe, 2005; Hallmark *et al.*, 2007; Svechnikov *et al.*, 2008).

1.6.1.3. Resveratrol

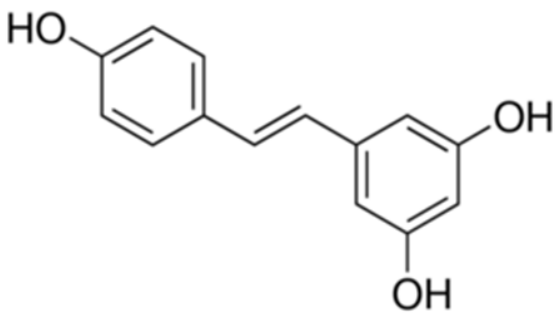

Figure 7. *The chemical structure of resveratrol.*

Resveratrol (3, 5, 4'-trihydroxy-*trans* stilbene) is a stilbenoid, a type of natural phenol, and aphytoalexin naturally produced by several plants in response to injury (Fig.7).

First, it was isolated by Michio Takaoka in 1939 from the plant Veratrum album. In mid-1990 the"French Paradox" was described, an observation that French people despite having

high fat diet had low rates of cardiovascular diseases. This phenomenon was linked to favorable anti-inflammatory and antioxidant effects of resveratrol consumed from red wine (Renaud and de Lorgeril, 1992; Artaud-Wild *et al.*, 1993).

Resveratrol was found in various plants, including berries and peanuts. This compound exists as cis- and trance- isomers but only trance-form has a biological effect: anti-oxidant, anti-inflammatory, cardioprotective and anti-tumor (Bhat *et al.*, 2001; Baur *et al.*, 2006; Park and Pezzuto, 2015).

Resveratrol was reported to have an estrogenic effect, resulting in suppression of expression of some steroidogenic enzymes (Supornsilchai *et al.*, 2005; Oskarsson *et al.*, 2014). It was shown that resveratrol and its analogs decrease DHEA, testosterone and cortisol levels through inhibition of CYP17A1 enzyme activity in human adrenocortical carcinoma cells, H295R (Oskarsson *et al.*, 2014), moreover it can attenuate steroidogenesis in human fetal cells at the end of first trimester (Savchuk *et al.*, 2017b). We found (Svechnikov *et al.*, 2009) that resveratrol inhibited hCG-activated steroidogenesis in LCs through suppression of the expression of StAR protein and cytochrome P450c17.

2 PROJECT AIMS

The general aim of this thesis was to explore mechanisms of action of selected environmental endocrine-disrupting chemicals (EDCs) on hormonal function of steroidogenic cells from mice and human.

The specific aims of the projects were:

1. To explore the relationship between steroidogenic potential of mouse Leydig cells, their expression profile of estrogen receptors and responsiveness to estrogenic agonists and the xenoestrogen BPA.
2. To investigate effects of different mono-phthalates on mouse Leydig cell steroidogenesis and mitochondrial function and link these effects to steroidogenic potential of these cells.
3. To characterize the effect of resveratrol on steroid production in human fetal adrenocortical cells (HFAC) at the end of the first trimester of pregnancy.
4. To explore the early onset of steroidogenesis in the human fetal adrenals (HFA) at the end of the first trimester of pregnancy.

3 METHODOLOGY

3.1 ETHICS

All studies were performed with permission from an Ethical Board. Animal studies were approved by the local animal ethics committee (Stockholm North Animal Ethics Committee permits number Dnr N311/12). Human fetal adrenals are collected following elective termination of pregnancy. Women give written consent to participate in the project. The project is approved by Etikprövningsnämnden (EPN dnr 2014/1022-32).

3.2 CELL CULTURE

In papers 1 and 2 Leydig cells were prepared from the testes of immature mice as described previously (Svechnikov *et al.*, 2001). Briefly, to obtain purified LCs, a suspension of testicular cells was loaded on the top of a discontinuous Percoll gradient and centrifuged. Isolated LCs were cultured in 96-well plates with /without stimulation by hCG and treated with selected EDCs (BPA and mono-phthalates) in several concentrations for different time points.

In paper 3 HFA (GW9-12) were used to prepare individual primary cultures of HFAC. Gestational age was validated by measuring fetal limb length as described previously (Evtouchenko *et al.*, 1996). Adrenocortical cells were plated into each well of 96-well plates and incubated in the presence or absence of ACTH with or without resveratrol for 24h.

3.3 HORMONES MEASURMENT

In papers 1 and 2 culture medium samples were stored at -20ºC prior to analysis of the concentrations of testosterone, 17b-estradiol, 5α-androstane-3a, 17b-diol. The concentrations of the sex steroids were quantified employing the Coat-a-Count RIA kit (Diagnostic Products Corp., Los Angeles, CA, USA). Concentration of 5a-androstane-3α,17b-diol was also determined by RIA using specific antisera (Cosmo Bio Co. LTD.,Tokyo, Japan). 5a-[9, 11-3H (N)] Androstane-3a, 17b-diol (specificity activity, 40 Ci/mmol) was obtained from NEN Life Science Products (Boston, MA, USA). Serum levels of mouse LH were measured by specific ELISA (ABIN415551) from Antibodies online (Antibodies-online, Inc, GA, USA) in accordance with manufacturer's instructions.

In papers 3 and 4 the media from HFAC cultures and HFA tissue were stored frozen until preparation for analysis. Detection and quantification of androgens and estrogens were performed on a Scion 436 gas chromatograph coupled to a Scion TQ triple quadrupole mass spectrometer (Bruker, Fremont, CA, USA) as described previously [15, 17]. The LC–MS/MS analyses of glucocorticoids were carried out using a Dionex Ultimate 3000 UHPLC with integrated Transcend TLX TurboFlow sample preparation system coupled to a TSQ Quantiva triple quadruple mass spectrometer.

3.4 PROTEIN EXPRESSION

3.4.1 Wes automated Western blotting and analysis (Papers 2- 4)

All reagents were prepared and used according to manufacturer's recommendations for use on Wes (ProteinSimple, San Jose, California, www.proteinsimple.com/simon.html). Reagents included: biotinylated molecular weight ladder, streptavidin-HRP, fluorescent standards, luminol-S, hydrogen peroxide, sample buffer, DTT, stacking matrix, separation matrix, running buffer, wash buffer, matrix removal buffer, capillaries, containing a proprietary UVactivated chemical linked reagent, and antibody diluent and antibodies (goat-anti rabbit secondary antibody). Samples were diluted to adjust protein concentration to 0.8µg in 4µl with sample buffer and further diluted 4:1 by adding 1 µl of the 5x master mix. The final samples of 5 µl each were boiled 5 min, placed on ice for 5 min, briefly centrifuged and applied to proper wells. After plate loading, the separation of proteins by electrophoresis and immunodetection took place in the capillary system and were fully automated. Simple Western analysis was carried out at room temperature, and instrument default settings were used. The digital images were analyzed with Compass software (ProteinSimple) on Wes. Band density differences were expressed as percent of control values.

3.4.2 Immunohistochemistry (Paper 4)

Paraffin-embedded fetal adrenal tissue was cut to a thickness of 5 µm and mounted on microscope slides (P/N10143352, Superfrost Plus, Thermo Scientific, MA, USA) and placed at 60°C for 40 min in the oven. Tissue sections were dewaxed with xylene (P/N 02080, HistoLab, Gothenburg, Sweden) for 20 minutes and then rehydrated in graded ethanol (99.6, 96, and 70%). Antigen retrival in 0.01M citrate buffer (pH2 6.0) for 20 minutes, in a water bath was used for all slides. Samples were incubated with 3% H2O2 in 96% methanol for 10 min at RT for non-specific endogenous peroxidase blocking. After washing two times for five min each with phosphate-buffered saline (PBS) and one time with phosphate-

buffered saline (PBS) plus 0.01% Tween20 (P/N P1379, Sigma Aldrich, St.6 Louis, MO), the sections were treated with normal 3% goat serum plus 1% bovine serum albumin (BSA) in phosphate-buffered saline (PBS) for 60 minutes at RT to avoid non-specific biding. Slides were subsequently incubated with primary or unspecific IgGs (for negative control) dissolved in 3% goat serum × PBS, overnight at 4°C. After washing step, slides were incubated with biotinylated secondary antibody (P/N ab64256, Abcam, Cambridge, MA), and then with avidin-biotin-peroxidase complex prepared using Vectastain ABC kit (P/N PK-6100, Vector Laboratories, CA, USA) for 60 min at RT. After washing slides were stained with DAB (P/N SK-4105, Vector Laboratories, CA, USA) for 20-40 sec at RT, washed twice for five min in H2O, counter-stained with Hämalaun solution (1.09249.1000, Merck, NJ, USA), rinsed for five min with running tap water, dehydrated with gradually increasing concentrations of ethanol, cleared with xylene and mounted with cover glass. IgG negative sections exposed to non-immune rabbit and mouse serum in the absence of primary antibody were included in all immunohistochemistry runs and showed no positive immunostaining.

3.5 GENE EXPRESSION (PAPERS 1- 4)

3.5.1 RNA extraction and cDNA producing

Total RNA was extracted from control and treatment groups by RNeasy Mini Kit (74104,Qiagen, Hilden, Germany), according to the protocol provided by the manufacturer. The RNA was pretreated with DNAse (RNase-free DNase Set, Qiagen) according to the manufacturer's instructions. The amount of total RNA was measured by photometry (BioPhotometer, Hamburg, Germany). The RNA was kept at -80ºC until analysis. Total RNA was further processed using iScript cDNA Synthesis Kit (Bio-Rad Laboratories, Hercules, CA, USA) as proposed in the manufacturer's protocol.

3.5.2 Analysis by qPCR

The samples for qPCR were prepared using iQ SYBR Green Supermix (170-8882, Bio-Rad Laboratories, Hercules, California), and the PCR cycles were run at 95ºC for 10 s, 60ºC–62ºC for 45 s, 95ºC for 60 s, and 55ºC for 60 s followed by a melting curve from 55ºC–95ºC in steps of 0.5ºC and then held at 4ºC (iCycler iQ, Bio-Rad Laboratories, Hercules, California) after having estimated the best reaction conditions by running a temperature gradient. All values were normalized to b-actin (papers 1 and 2) or GAPDH (paper 3-4) as a housekeeping gene to balance possible irregularities in RNA concentration. To control the efficacy of the process, negative control (RT-) was always added to each qPCR assay.

Before use all primers were run on a gel to check their validity. The 2ΔDDCt method was used to calculate the fold changes in gene expression.

3.6 ATP ASSAY (PAPERS 2- 3)

Cellular ATP levels in the cells were assessed using the Promega luminescent cell viability assay kit (Promega, Fisher Scientific) according to the manufacturer´s protocol. Briefly, mouse LCs (Paper 2) or human fetal adrenocortical cells (Paper 3) were incubated with or without selected EDC for 24 or 48h and then lysed with Promega Cell Titer-Glo substrate at room temperature on an orbital shaker for 2 min, followed by a 10-min standing incubation at room temperature enabling cell lysis. Samples were analyzed for overall luminescence using a Wallac1420 microplate spectrofluorometer (Perkin Elmer).

3.7 WST-1 TOXICITY ASSAY AND MEASUREMENT OF EXTRACELLULAR SUPEROXIDE PRODUCTION (PAPERS 2- 3)

Tetrazolium salt WST-1 (Roche, Mannheim, Germany) was used to assay possible toxic effects of EDC on steroidogenic cells function. WST-1 is a cell-impermeable compound which is reduced to formazan by superoxide generated by specific trans-plasma membrane oxidoreductases. Primary cultures of LCs (Paper 2) and HFAC (Paper 3) were incubated with or without tested EDC and the media were collected for steroids measurement, and the cells underwent incubation in fresh DMEM/F12 phenol-free media with WST-1 for 3 h. The level of formazan produced was measured with a filter for 450/10 nm using a Wallac1420 microplate spectrofluorimeter (Perkin Elmer).

In paper 2 to prove that WST-1 was reduced into formazan by superoxide, the cells were preincubated with superoxide dismutase (SOD) (20 mg/ml), an enzyme that converts superoxide into hydrogen peroxide.

3.8 CELL PROLIFERATION ASSAY (PAPER 2)

The influence of MEHP on proliferation of immature LCs was assayed by CyQUANT Cell Proliferation Assay Kit (C7026, Invitrogen, Ltd, UK) according to the manufacturer’s protocol.

4 RESULTS AND DISCUSSION

4.1 EFFECTS OF BISPHENOL A AND MONO-PHTHALATES ON IMMATURE MOUSE LEYDIG CELLS ARE NOT CORRELATED TO THEIR ANDROGEN PRODUCTIONS POTENTIAL (PAPERS 1-2)

Increased incidences of developmental disorders in the male reproductive tract (e.g., hypospadias, cryptorchidism, micropenis) as well as testicular cancer and abnormal spermatogenesis are thought to be associated with exposure to estrogenic-like endocrine disruptors (EDCs) (Skakkebaek *et al.*, 2001). Two of the most widely used xenoestrogens are bisphenol A (BPA), exposure to which was found to suppress testicular function via an estrogen receptors (ERs)-dependent mechanism in rats (Akingbemi *et al.*, 2004) and phthalic acid esters, for with known toxic effects have been described to take place in the reproductive system and LCs (Skakkebaek *et al.*, 2001),and this have been proposed to be associated with their anti-androgenic potential. However, there are no reports concerning the responsiveness to estrogenic and/or anti-androgenic stimuli from LCs from mice with different genetic background.

In our 1 paper, we used 4 different strains of mice (C57BL/6j, CBA/Lac, 129S2, BALB/c) that are commonly used in laboratory investigations. We found that all of them have their own pattern for steroidogenesis. There were significant variations in the serum levels of T and E2 in different genotypes of mice. CBA/Lac mice had 20-times higher serum level of T, but 3-times lower level of E2 compared to C57BL/6j mice (Fig.8). This has been associated with lower levels of LH in the serum, suggesting marked differences in LC steroidogenesis in these mouse genotypes. The capacity of LCs in C57BL/6j mice to produce the androgen in the unstimulated condition and in response to hCG was impaired, while LCs from CBA/Ca mice had the highest capacity to produce T. This is in line with the hypothesis that reduced responsiveness to hCG- stimuli by LCs from C57BL/6j mice correlates with the higher levels of LH, secreted by the pituitary. The expression of the main steroidogenic genes, such as LHR, StAR, 3βHSD1 and Cyp17a1 in LCs from C57BL/6j mice was downregulated compared to CBA/Lac mice; whereas Cyp 19 gene expression was upregulated in C57BL/6j mice. There is some evidence that proves that the overexpression of Cyp 19 has a negative effect on androgen production and fertility in male mice (Li *et al.*, 2001).

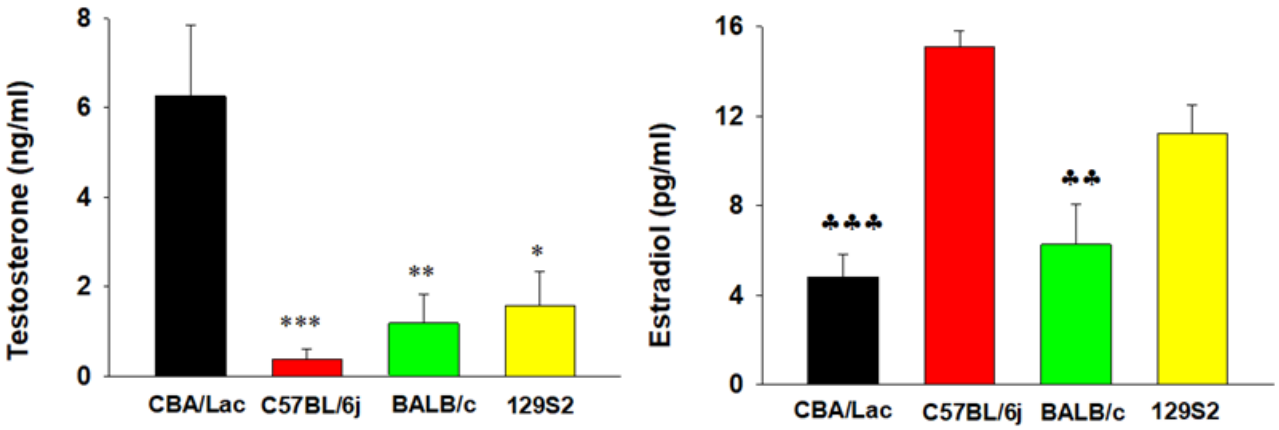

Figure 8. *Strain-related differences in in serum levels of sex hormones in mouse Leydig cells*

The data are expressed as means ± S.E.M (n = 7–15). *P<0.05, **P<0.01, ***P<0.001compared to CBA/Lac; ♣♣P<0.01, ♣♣♣P<0.001 compared to C57BL/6j

No differences in the expression of estrogen receptors ERα and Gpr30 were found, however ERβ expression was much more prominent in LCs from CBA/Lac mice. Interestingly, no response to the treatment with estrogenic ERα and ERβ agonist was detected, except after treatment with bisphenol A, which significantly increased hCG-activated androgen production by LCs from all strains (Fig.9), by suppressing the conversion of testosterone into its metabolite 5α-androstane-3α,17β-diol (Fig.10). This finding allowed us to hypothesize that LCs with different potential to produce testosterone are resistant to estrogenic stimuli, while bisphenol A can influence the steroidogenic function of immature LCs and potentiate the induction of early puberty and affect the paracrine environment required for the normal development of germ cells.

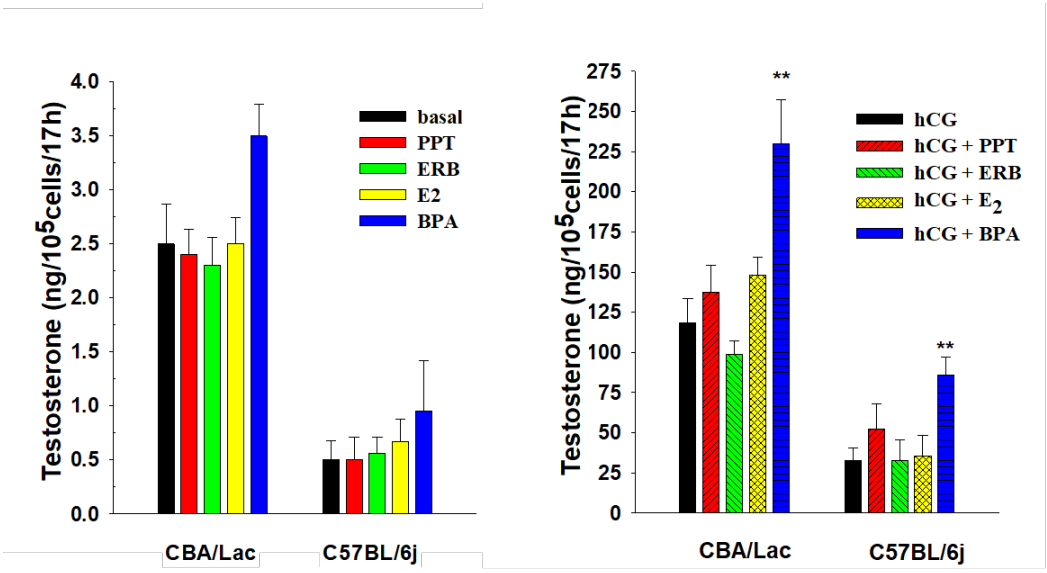

Figure 9. *The effect of agonists of estrogen receptors and BPA on basal and hCG-stimulated testosterone production by LCs from C57BL/6j and CBA/Lac mice* **P<0.01 compared to hCG treatment.

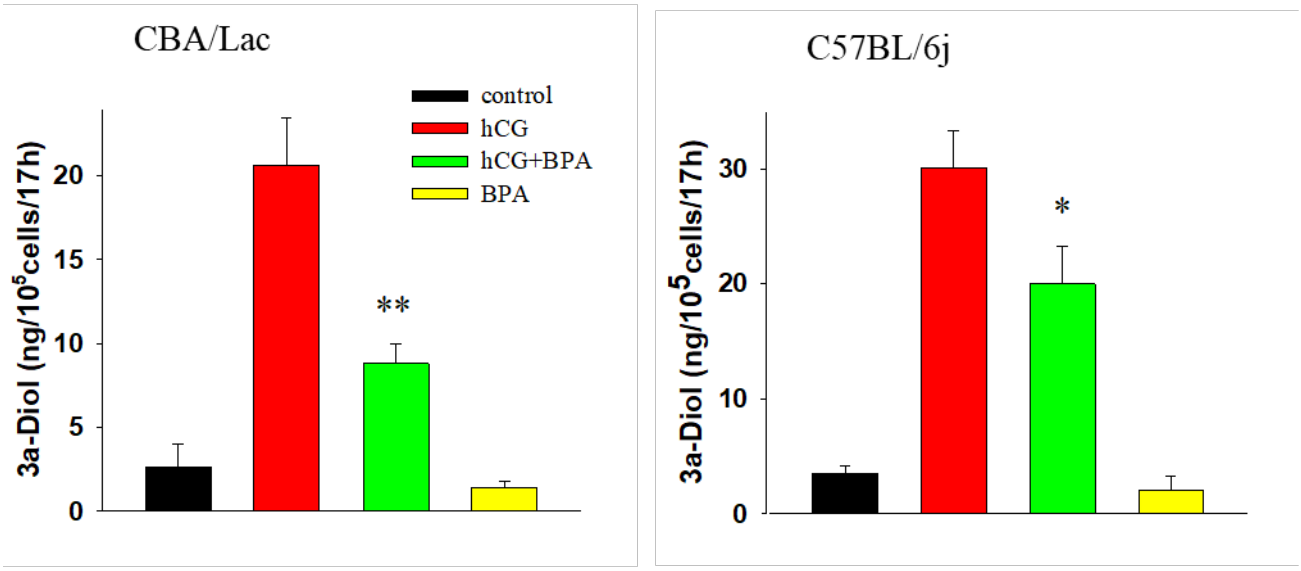

Figure 10. *BPA suppressed testosterone metabolism by mouse Leydig cells.* The data are expressed as means ± S.E.M (n = 7–15). *P<0.05, **P<0.01, compared to hCG

In contrast, no effects of MEHP, MBP and MBeP on hCG-stimulated T production by LCs from both mouse genotypes were found. Among three mono-phthalates tested, MEHP at the highest concentration (90μM) had the most prominent potential to stimulate basal T production by immature LCs from both CBA and C57BL mice and that was coupled with a significant upregulation of StAR protein expression in these steroidogenic cells. The observation that co-treatment of hCG with mono-phthalates did not lead to higher production of testosterone in comparison to stimulation by hCG or MEHP underlines the mechanism that preserves the LCs from testosterone overproduction via downregulation of StAR (Houk *et al.*, 2004).

The stimulatory effect of MEHP on basal androgen production was associated with an increased reduction of WST-1 to formazan by LCs from both mouse genotypes, suggesting an elevated production of extracellular reactive oxygen species (ROS) by the cells that could be one of the reasons for StAR upregulation (Zhao *et al.*, 2012). Moreover, our study showed that MEHP can suppress mitochondrial function by decreasing ATP production albeit having no effect on the proliferative status of these cells. Our study hypothesized that MEHP can disturb mitochondrial function and elevate the production of reactive superoxide in LCs that may cause oxidative stress in neighborhood cells such as Sertoli and germ cells and induce apoptosis.

Altogether, our data show that C57BL/6j mice have attenuated steroidogenesis. This should be taken into consideration when performing experiments that focus on the steroidogenic potential of these mice. The main finding of this study is that immature mouse LCs with different androgenic potential are resistant to the estrogenic stimuli and this is not associated with their responsiveness to mono-phthalates. Xenoestrogen BPA induces hCG-activated steroidogenesis via testosterone metabolism suppression. In addition, we showed that among the 3 mono-phthalates (MBP, MBeP, MEHP), only MEHP had the potential to stimulate basal testosterone production via upregulation of StAR expression and affect normal function of mitochondria. Consequently, this can disturb the normal development of germ cells and both bisphenol A and MEHP should be taken into consideration when evaluating the different reasons of premature maturation of LCs.

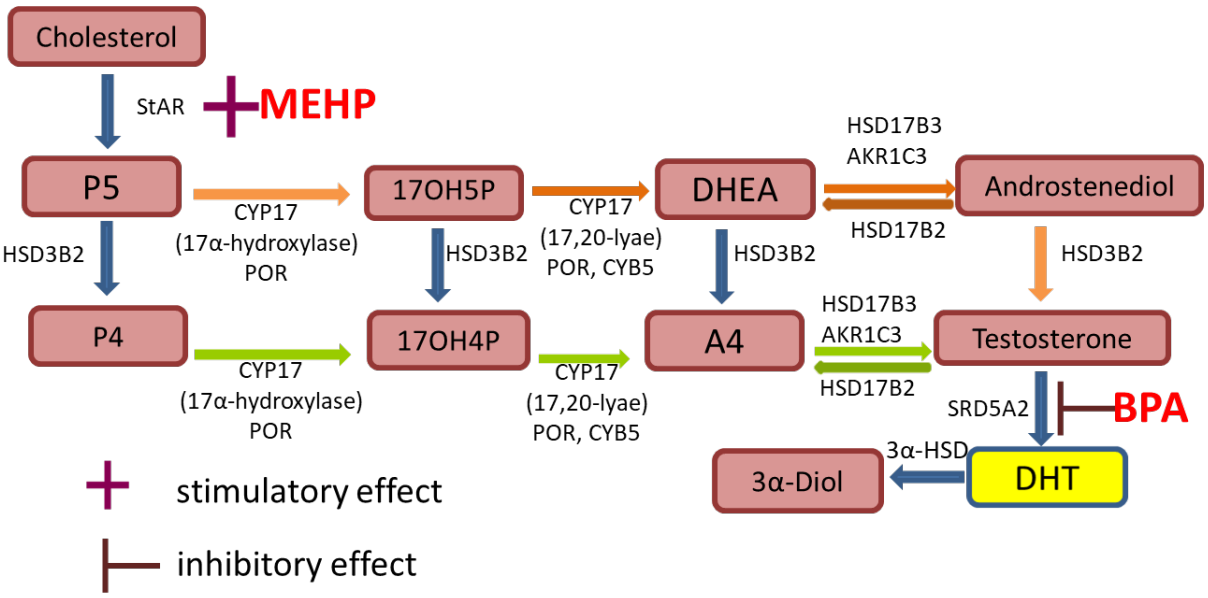

Figure 11. *MEHP activated StAR expression and steroidogenesis in mouse LCs, while BPA potentiated hCG-stimulated testosterone production by inhibition its metabolism.*

4.2 ANDROGENIC POTENTIAL OF HUMAN FETAL ADRENALS AND THE RESPONSE OF HUMAN FETAL ADARENOCORTICAL CELLS TO RESVERATROL (PAPERS 3-4)

The fetal adrenal glands play a decisive role in maintaining the normal homeostasis in the uterus during the pregnancy and in the maturation of hormone-dependent organs of the developing fetus. Since the capacity of the human adrenal glands (HFA) to produce DHT, the most potent androgen, in the first trimester, is poorly investigated, we focused our research on the steroidogenic machinery of the HFA and responsiveness of their cells to resveratrol stimuli, which is known to suppress steroidogenesis by the adrenals (Supornsilchai *et al.*, 2005).

In paper 4, we performed a comprehensive analysis of androgen production by the HFA and their cells *in vitro* and linked them to the expression of steroidogenic enzymes together with transcription factors. Our data showed that during the first trimester HFA are able to produce steroids of different pathways: Δ5, Δ4, including the backdoor of the DHT synthesis with restriction to the production of 17α- OH- dihydroprogesterone, androsterone and androstenedione (Fig.12). DHT was not detectable and only its precursors were found such as testosterone and androsterone. It is worth mentioning that less than 1% of androstenedione was converted to testosterone corroborating a study that demonstrated low levels of

testosterone in organ cultures with or without stimulated conditions (Goto *et al.*, 2006). In contrast, our experiments demonstrate that the primary cell culture of HFA was not capable of producing testosterone. There are two possible explanations for this: the HFA cells lose their ability to produce testosterone in culture or the level of testosterone in the media was under the threshold of detectability by GC-MS/MS. Nevertheless, we found two forms of testosterone in the tissue of HFA, 17α- and 17β- testosterone. This suggests that androstenedione could be converted to both forms with the help of 17αHSD, 17βHSD3 and 17βHSD5 as was reported previously (Goto *et al.*, 2006; Fluck *et al.*, 2011).

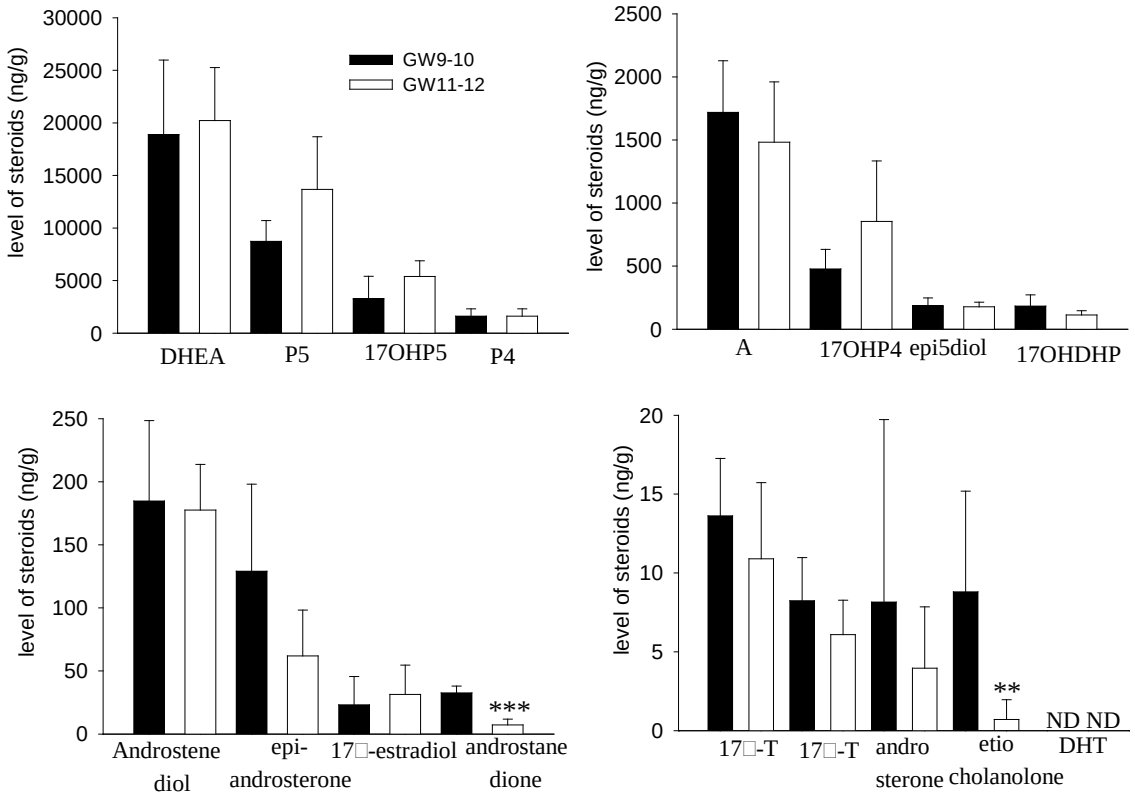

Figure 12. *The tissue levels (ng/g) of steroids in the HFA at the end of the first trimester. The values shown are means ± SE for five-six HFA tissue samples.* **P<0.01, ***P<0.001 compared to control. ND – not detectable.

We also detected high levels of DHEA and its metabolites that were linked to the low expression of 3βHSD2. Interestingly, 3βHSD2 peaked at GW10, but significantly decreased at GW11. However the major steroid produced by the HFA was DHEA indicating high activity of CYP17A1 and its assistance proteins P450 oxidoreductase (POR) and microsomal cytochrome *b*5 (CYB5A). This was correlated with an elevated expression of SF-1 and

GATA-6. The abundance of DHEA and its sulfated derivative serve as precursors for placental-derived estradiol, which is critical for sustaining pregnancy (Mesiano and Jaffe, 1997). Our study indicated that the CYP17A1-POR-CYB5A complex is fully functional in the first trimester of pregnancy and, together with transcriptional factors such as SF-1, GATA-6 and DAX1, plays a pivotal role in the normal development of the fetal human adrenals while maintaining a normal onset of steroidogenesis (Hammer *et al.*, 2005).

In paper 3, we investigated the influence of the natural polyphenol on steroidogenesis in the primary culture of HFAC during the first trimester and demonstrated that this natural stilbenoid significantly suppressed the biosynthesis of DHEA, androstenedione and 11-deoxcortisol by HFAC in stimulated and unstimulated conditions. Furthermore, we found that a downregulation of cytochromes 17α-hydroxylase/17, 20 lyase (CYP17) activities and expression of 21- hydroxylase (CYP21) in these cells (Fig.12). Hence, we concluded that the human fetal adrenals are very sensitive to resveratrol at gestational week 9-12, which is a critical period for normal development of the reproductive organ (e.g. gonads, external and internal genitalia). The risk of detrimental effects on differentiation should be taken into consideration and it is recommended that women who are at early stages of pregnancy avoid the intake of resveratrol.

Using the primary culture of the HFAC in paper 3, we also showed that ACTH upregulated steroidogenic enzyme expression and stimulated steroidogenesis in HFACs at GW9-12, indicating that ACTHR-coupled signaling pathways involved in the activation of steroidogenesis are functional in these steroid-producing cells at the end of the first trimester. Along the same line, we found that the expression of MC2R (the ACTHR) is significantly increased in the HFA during the last two weeks of the first trimester, suggesting active differentiation and maturation of the fetal adrenocortical cells.

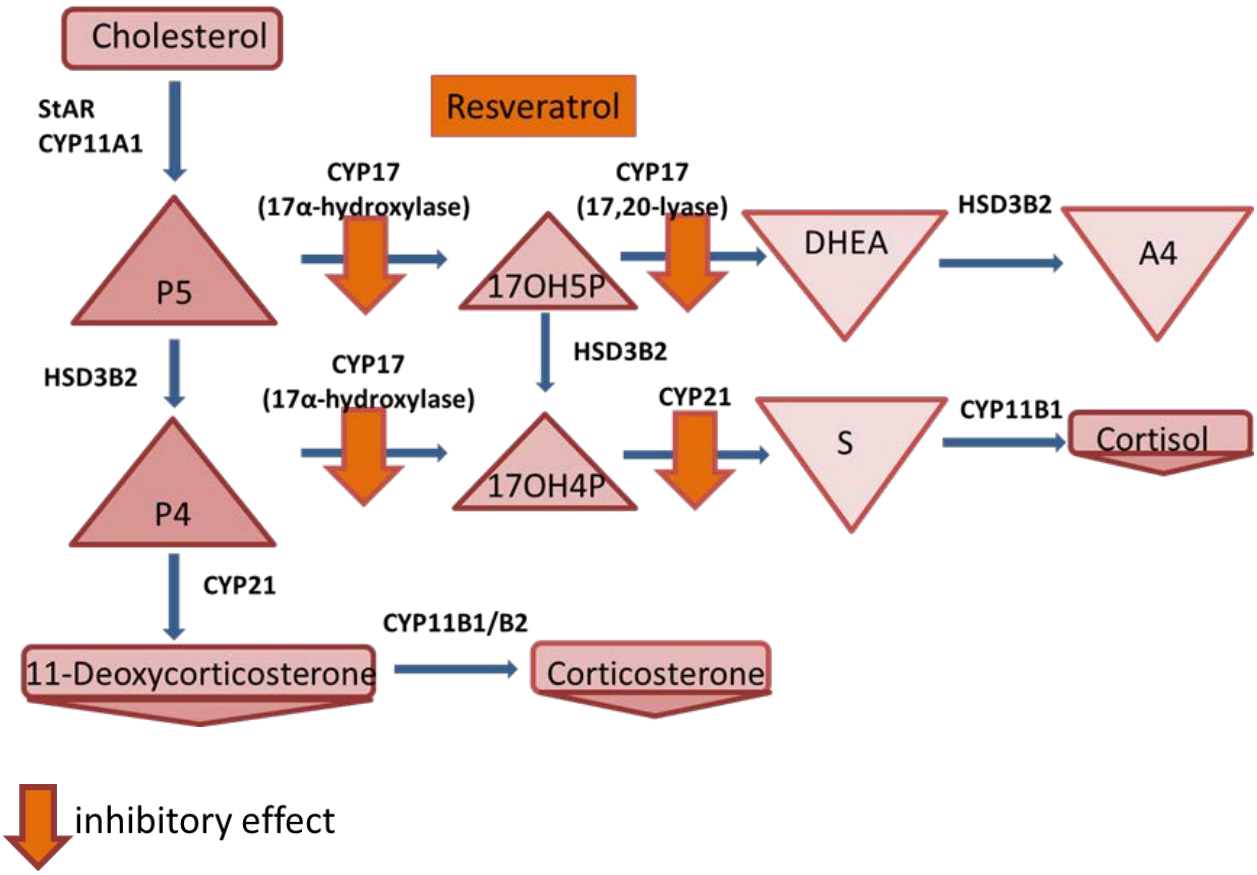

Figure 12. *Resveratrol inhibited expression and activity of Cyp 17 and Cyp21.*

5 A CRITICAL EVALUATION AND FUTURE PERSPECTIVES

The adverse effects of EDCs on reproductive health should not be neglected. This being said, the response to such compounds could depend on the species and the associated pharmacokinetics. Rats are known to be more sensitive to the harmful effects of EDCs compared to other rodents (Svechnikov *et al.*, 2016). The data about human steroidogenic cells are less clear.

In our first two studies we used a mouse animal model and found a significant effect of bisphenol A and MEHP on their steroidogenesis. Future plans would be to define their effect *in vivo*, since all chemicals could be metabolized and their metabolites may induce a detrimental impact on fetal and developing gonads and impair their ability to produce androgens, since it is known that those chemical compounds could go through the placental barrier and reach the fetus. Ultimately, an *in vitro* model would help in investigating of pathways that could be involved in this process and their action on mitochondrial function.

In studies 3-4, we afforded the chance to work with human abortion material from the first trimester of pregnancy and this helped us define the steroidogenic potential of HFA *ex vivo* and investigated androgen production of human fetal adrenocortical cells and their response to resveratrol. From a clinical perspective, we suggest that resveratrol, via suppression of the activity of certain steroidogenic enzymes (e.g., CYP21), may give rise to a hormonal pattern similar to that associated with certain types of autosomal recessive congenital adrenal hyperplasia.

Altogether, one can conclude that the action of EDCs on Leydig and adrenocortical cells producing androgens and glucocorticoids is a complex process that depends on the exposure route, dose, the developmental stage of the exposed target organism and many other factors. Together, these factors determine the potential risk for adverse consequences with long-lasting effects on male reproductive function, metabolism and stress response.

6 MAIN FINDINGS

- Immature mouse Leydig cells are resistant to estrogenic stimuli independently of their capacity to produce testosterone.
- The xenoestrogen BPA facilitates hCG-induced androgen production by immature mouse Leydig cells from both mouse genotypes by attenuating conversion of testosterone into its metabolite 3a-Diol.
- MEHP upregulates StAR expression and stimulates testosterone production by developing mouse LCs independently of their steroidogenic potential.
- MEHP attenuated ATP production and increased superoxide generation by both types of mouse LCs, indicating that mitochondrial dysfunction is affected by the mono-phthalate.
- Resveratrol inhibits synthesis of DHEA, androstenedione and 11-deoxicortisol by ACTH-activated and unstimulated HFAC, which was associated with attenuation of the activities and expression of CYP17 and CYP21 in these fetal adrenocortical cells.
- The androgenic potential of the HFA at GW9-12 is limited by the production of testosterone and androsterone.
- DHT production by the human fetal adrenocortical cells is limited to its steroid precursors such as 17α- OH- dihydroprogesterone, androsterone and androstanedion that correlate with expression of steroidogenic genes and their transcription factors.

7 ACKNOWLEDGMENTS

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