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EVALUATION, VALIDATION AND APPLICATION OF THE H295R SYSTEM FOR THE ANALYSIS OF ENDOCRINE DISRUPTING EFFECTS

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Major Professor's Signature

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EVALUATION, VALIDATION AND APPLICATION OF THE H295R SYSTEM FOR THE ANALYSIS OF ENDOCRINE DISRUPTING EFFECTS

By

Tannia Rocío Gracia Bustos

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ABSTRACT

EVALUATION, VALIDATION AND APPLICATION OF THE H295R CELL SYSTEM FOR THE ANALYSIS OF ENDOCRINE DISRUPTING EFFECTS

By

Tannia Rocío Gracia Bustos

The continuously growing number of compounds synthetically derived or extracted from natural materials daily discovered, designed or produced in order to satisfy human's necessities has brought along unintended effects that have been of human concern for several decades. It has been clearly established that several of these synthetic and naturally occurring chemical substances in the environment are disrupting the normal functions of the endocrine system and its hormones in humans and wildlife. Despite the wide range of studies that have been conducted in this area, in many cases there is still insufficient data to demonstrate specific associations between environmental exposure and endocrine-mediated adverse effects. The need to evaluate endocrine disruption exerted by pathways other than receptor-mediated mechanisms has driven scientists to intensively work in the development of new, practical and more comprehensive bioassays in order to match up the growing speed of chemical design/production. In this dissertation the evaluation, validation and application of the new developed H295R cell bioassay for the evaluation of potential effects of environmental contaminants as endocrine disrupters, was conducted. Results from the executed studies corroborate the versatility of the H295R cell bioassay to identify the different effects of chemicals

on steroid production and to point through the different molecular and enzymatic mechanisms that may be responsible for such effects. This method is applicable to any environmental contaminants identified up to the present time, including complex mixtures of them. Moreover, the effectiveness shown in identifying the potential endocrine effects of realistic environmental samples of different matrices is without doubt one of its most valuables applications.

A mi madre, la mujer más valiente que he conocido.

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ABBREVIATIONS

ACETA	Acetaminophen
AhR	Aryl hydrocarbon Receptor
AMG	Aminoglutethimide
AMOXI	Amoxicillin
AMV	Avian Myeloblastosis Virus
ANOVA	Analysis of Variance
AR	Androgren Receptor
ATCC	American Type Culture Collection
ATP	Adenosine Triphosphate
CAFOS	Concentrated Animal Feeding Operations
cAMP	cyclic Adenosine Monophosphate
CYP	Cytochrome P450
CEPHA	Cephalexin
CLOFI	Clofibrate
CYPROT	Cyproterone
DEET	3-methyl-N,N-diethylbenzamide
DEPC	Diethylpyrocarbamate
DEXA	Dexamethasone
DHEA	Dehydroepiandrosterone
DMSO	Dimethylsulfoxide

DNA	Deoxyribonucleic acid
DNAse	Deoxyribonuclease
dNTP	Deoxyribonucleotide triphosphate
DOXYC	Doxycycline
EDCs	Endocrine Disrupting Compounds
EDSTAC	Endocrine Disruptor Screening and Testing Advisory Committee
EDTA	Ethylenediaminetetraacetic Acid
E2	Estradiol
EE2	Ethynilestradiol
EEC	European Economic Community
EIA	Enzyme immunoassay
ELISA	Enzyme-Linked ImmunoSorbent Assay
ER	Estrogen Receptor
ERAs	Environmental Risk Assessments
ERY	Erythromycin
FOR	Forskolin
FLUOX	Fluoxetine
GMP	Guanosine Monophosphate
HMGR	3-Hydroxy-3-Methyl-Glutaryl-CoA Reductase
HRC	Hormone Receptor Complex
HSP	Heat Shock Protein
IBUPR	Ibuprofen
ITS	Insulin, Transferin, Selenium

Jak/STAT	Janus Kinase/Signal Transducers and Activators of Transcription
KETO	Ketoconazole
LDLs	Low Density Lipoproteins
MeOH	Methanol
mRNA	messenger Ribonucleic Acid
MET	Metyrapone
NCI	National Cancer Institute
NHEERL	National Health and Environmental Effects Research Laboratory
NSAAID	Non-Steroidal Analgesic Antiinflamatory Drugs
ORD	Office of Research and Development
OXY	Oxytetracycline
P	Progesterone
PAHs	Polycyclic Aromatic Hydrocarbons
PBDEs	Polybrominated Diphenylethers
PCBs	Polychlorinated Biphenyls
PKA	Protein Kinase A
PKC	Protein Kinase C
Q-RT-PCR	Quantitative Real Time Polymerase Chain Reaction
SALBU	Salbutamol
SAR	Special Administrative Region
SF-1	Steroidogenic Factor 1
SSDS	Strategic Sewage Disposal Scheme
StAR	Steroidogenic Acute Regulatory Protein

STW	System Treatment Works
T	Testosterone
TBT	Tributylin
ThR	Thyroid Receptor
TRENB	Trenbolone
TRIME	Trimethoprim
TYL	Tylosin
USEPA	United States Environmental Protection Agency
USFDA	United States Food and Drug Administration
WWTP	Wastewater Treatment Plant
ZEARA	α-Zearalanol

INTRODUCTION

Daily, a large number of compounds synthetically derived or extracted from natural materials are being discovered, designed, produced and used in order to satisfy human's necessities. An unsurprising but unintended consequence of the increasing demand for the use of these compounds has been the introduction of them and/or their metabolites into the environment. For years it has been known that several of these synthetic and naturally occurring chemical substances in the environment are disrupting the normal functions of the endocrine system and its hormones in humans and wildlife. An endocrine disruptor compound is a chemical substance that interferes with, or has adverse effects on, the production, distribution, or function of these hormones. Clearly, interference with or damage of hormone could have major impacts on the health and reproductive system of humans and wildlife.

Endocrine Disrupting Compounds (EDCs) include a broad range of substances with different chemical structure and physico-chemical properties which define their potential. Human activities are the current major sources of concern although a wide range compounds are also produced naturally. EDCs can be found in air, water, soil, sediment, food and consumer products, and their potential effects of greatest concern are reproductive malfunction and developmental disorders in humans and wildlife. Despite the wide range of studies that have been conducted in this area, in many cases there is still insufficient data to demonstrate specific associations between environmental exposure and endocrine-mediated adverse effects.

It has been established that there are different ways in which synthetic or naturally occurring chemicals can affect the endocrine system. Direct interaction with

hormone receptors such as ER, AR, and ThR are the most studied mechanisms however, there are other, indirect mechanisms that do not involve contact with any of these receptors, and still the compounds may produce disruption of several endocrine activities, such as producing adverse effects on the enzymes involved in the hormone synthesis and their metabolism.

Because most of the bioassays available to evaluate endocrine disruption are based solely on direct interaction with hormone receptors, the main objective of this dissertation is to present research centered on the evaluation, validation and application of a new method that integrates the analysis of potential effects of EDCs on steroid production exerted by direct-acting hormone mechanisms as well as other indirect mechanisms or transduction pathways. The evaluated system is the H295R cell line which not only has the ability to express all the key enzymes required for steroid hormone synthesis, transport and metabolism, but is also capable of producing all the major hormones responsible for homeostatic and reproductive activities in humans and wildlife. This cell line therefore offers an integrative system to evaluate at the same time several important endpoints in endocrine-disruption including gene expression, enzyme activity and hormone production.

Chapter 1 of this dissertation presents a complete description of hormone production and EDC mechanisms of action, the H295R cell system, and the molecular techniques and criteria used in this EDC screening assay system. The biomolecular methods include Quantitative-Real Time-Polymerase Chain Reaction (Q-RT-PCR) for the evaluation of the expression of genes encoding for key enzymes driving

steroidogenesis, and the Enzyme-Linked Immunosorbent Assay (ELISA) used for quantification of hormone production.

Chapter 2 presents an evaluation of the H295R cell system using four model chemicals known to be inducers or inhibitors of hormone production. Exposures with individual model chemicals as well as with binary mixtures of inducers and inhibitors were conducted and the results evaluated. This chapter addresses the importance of the evaluation of mixture effects since EDCs do not generally occur in the environment individually but in complex mixtures, and they may interact synergistically increasing the potential to exert stronger adverse effects. Moreover, it corroborates the fact that every EDC reaching the environment varies in potency and in the level of exposure required to produce an adverse effect. This portion of the dissertation has been published in the journal Ecotoxicology and Evironmental Safety, 65 (3), 293-305.

Pharmaceuticals, one of latest emerging group of contaminants of environmental concern, were among the chemicals chosen for the validation of the H295R cell system whose results are described in Chapter 3. The potential endocrine disrupting effects of 15 of the most commonly used pharmaceuticals, including prescribed and over the counter drugs, and some natural estrogenic compounds were evaluated at high and environmentally relevant concentrations. Antibiotics of medium and broad spectrum, non steroidal analgesic anti-inflammatory drugs (NSAA), growth promoters, antidepressants, birth control compounds, β-blockers and hormone therapy drugs were part of the tested group. The steroidal side effects of these chemicals designed to execute specific biological functions in animals and humans were established, as well as the variation of endocrine responses with respect to the exposed

doses. Responses to mixtures of chemicals were also compared to individual compound behavior.

Chapter 4 describes the applicability of the H295R cell system to relevant environmental samples. H295R cells were exposed to extracts of water samples collected in several waste water treatment plants before and after treatment, fish culture zones, marine disposal areas and other aquatic environments in Hong Kong S.A.R, China. The potential of compounds present in these extracts to cause endocrine disruption was used to evaluate the relevance of EDCs in waters collected from one of the most polluted areas in Asia.

Studies presented in this dissertation confirm that the H295R assay system allows simultaneous analysis of the expression of key steroidogenic genes, enzyme activities and hormone production, and that this assay can be used as a cost-effective, sensitive and integrative screening system to evaluate the many potential effects of chemicals or mixtures of chemicals that can lead to endocrine disruption.

Chapter 1

STEROIDOGENESIS, ENDOCRINE DISRUPTION AND THE H295R CELL SYSTEM

INTRODUCTION

The Endocrine System

The endocrine system is the machinery in charge of the regulation coordination and control of several important biological functions in the organism such as growth and development, mood, tissue function, metabolism as well as sexual function and reproductive processes (Figure 1.1). This system is composed of a collection of glands that secrete chemical messengers called hormones that transfer information and instructions from one set of cells to another. The major glands of the endocrine system are the pineal body, hypothalamus, pituitary, thyroid, parathyroid, thymus, adrenals, pancreas (although it is also part of the digestive system because it also produces and secretes digestive enzymes), and the reproductive glands ovaries and testes (1). Combine together, more than fifty hormones are secreted by the endocrine system and released into the bloodstream. Each hormone is programmed to reach a target organ which has specific cells genetically programmed to receive and respond exclusively to its message. On their way to the target cells, many hormones bind to specific proteins, known as carrier proteins, which act as vehicles of transport and at the same time control the amount of hormone available to interact with the target cells (2). Usually, hormones linked to carrier proteins are more stable

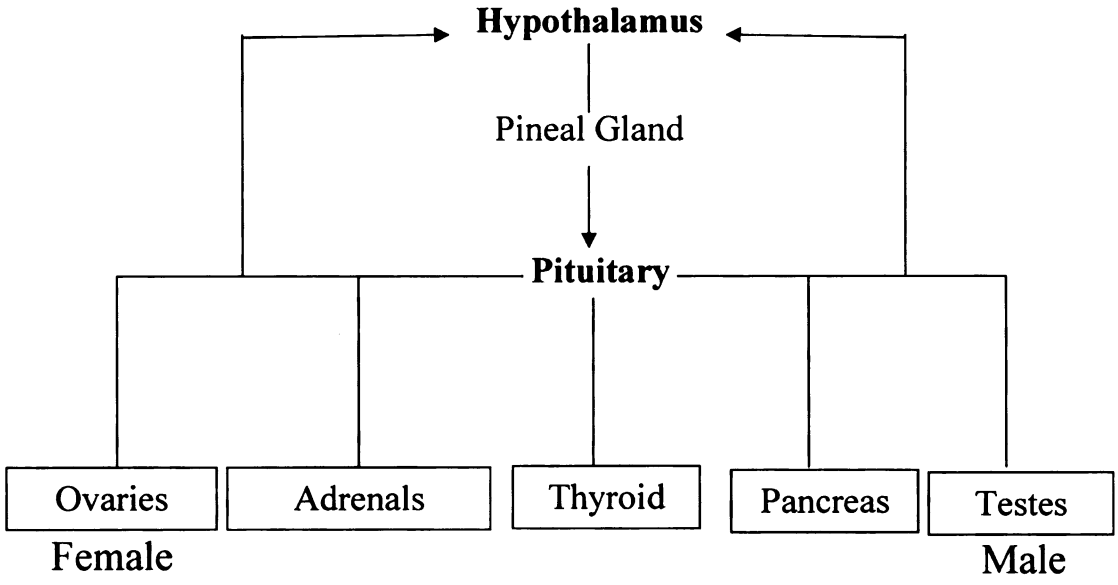


Figure 1.1 Simplified diagram depicting the major glands of the endocrine system in charge of the regulation of homeostasis and reproductive functions in humans and animals.

increasing their half-life in the circulation. It has been also suggested that some binding proteins can function in targeting and trafficking of hormones (3).

The endocrine system uses cycles and negative feedback to regulate physiological functions (4). Cycles of secretion maintain physiological and homeostatic control and they may last hours or months. Hibernation, mating behavior and body temperature are examples of activities controlled by biological cycles (5). Although the instances of positive feed-back certainly occur, negative feedback regulates the secretion of almost every hormone. When hormone levels reach a certain 'normal' concentrations, further secretion is controlled to maintain that necessary level of hormone in the blood. This down regulation of hormone production may involve the hormone itself or another substance in the bloodstream (6-7). Hormone levels can also be influenced by factors such as stress, infection, and changes in the balance of fluid and minerals in the organism.

The endocrine and nervous systems work in parallel to control growth and maturation along with homeostasis. The nervous system coordinates rapid and precise responses to stimuli using action potentials, while the endocrine system maintains homeostasis and long-term control using chemical signals (1, 8). Thus, nervous messages are immediate and of short duration while hormonal effects still prevail although hormonal levels return to basal values.

Hormones

A hormone is a chemical substance synthesized by particular endocrine glands that later enters the bloodstream to be carried to a target tissue or cell type. The target

tissues have specific receptors that bind these messenger chemicals (9) (Figure 1.2). This series of events defines the so called endocrine and neuro-endocrine signaling mechanisms. Some hormones are not secreted into bloodstream; instead, they reach their nearby target cells by diffusion. The effects generated by binding to the specific receptors establish the different signaling mechanisms used by hormones. A simplified mechanism of selecting target hormonal receptors is shown in Figure 1.3. An intracrine mechanism involves the production and use of the signal/hormone within the same cell. In autocrine signaling the cell produces and releases its own signal/hormone. A paracrine mechanism is established between neighbor cells within a tissue or organ (10-11).

The effects of hormones are broad and can include induction or inhibition of cell death, stimulation or suppression of growth, regulation of metabolism, control of life stages such as puberty and menopause, and in several systems hormones are capable of regulating the production and release of other hormones (12). Although hormones are produced mostly by classical endocrine glands other 'non-endocrine' organs such as the brain, thymus, heart, lungs, liver, kidneys, placenta and skin are also capable of producing and releasing hormones (13-14).

Hormones are present at all times in the bloodstream, albeit at low concentrations, in order to maintain receptors in the target tissues and to keep the tissue 'prepared' to mount a response. They are normally found in plasma and interstitial tissues in trace amounts ranging between $10^{-9} - 10^{-6}$ g/l (15). The physiological effects of hormones depend mostly on their concentration. Hormonal over- or under-

production, as well as non-functional receptors that make the target cells un-sensitive to hormonal messages,

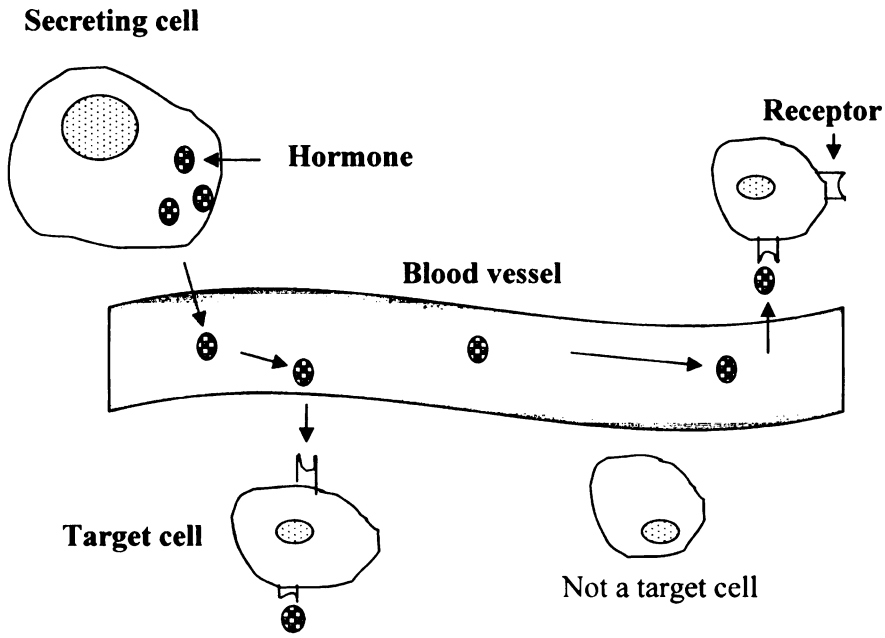


Figure 1.2 The role of hormones selecting targets and delivering the hormonal message. Once released to the blood stream hormones bind specific receptors on target cells only.

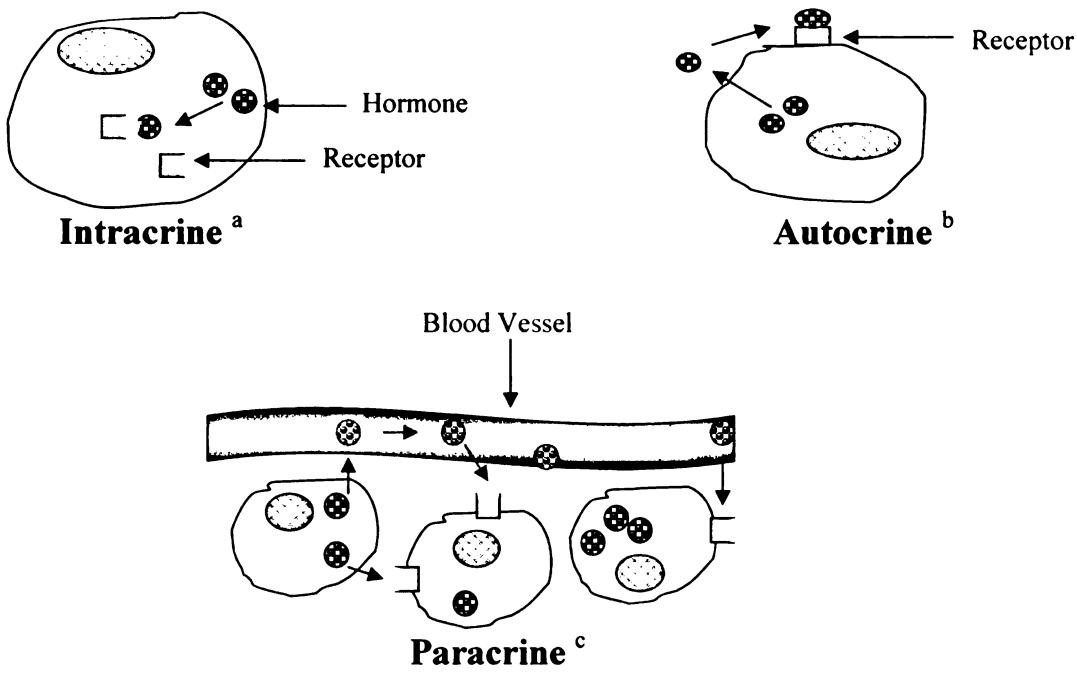


Figure 1.3 Different mechanisms of hormonal signaling. a) A chemical messenger acts inside the secreting cell. b) Cells secrete chemical messenger that signal the same cell. c) The target cell is close to the signal releasing cell.

are the most common causes of endocrine-related problems (16). Therefore, precise control of circulating hormonal levels is crucial. In general, the concentration of a hormone in the bloodstream is determined by three factors (17):

- Rate of production: Regulated by positive and negative feedback mechanisms.
- Rate of transport: Determined by blood flow.
- Rate of degradation and excretion: Established by metabolism and elimination processes, which determine hormone half-life.

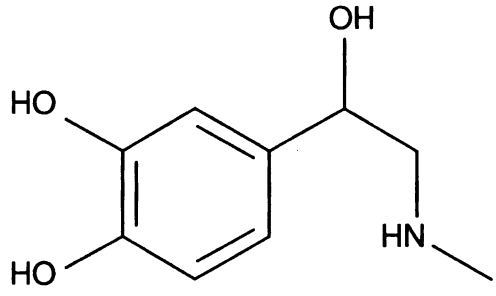
The function of hormones is to serve as a signal to target cells to maintain homeostasis and drive and coordinate changes in an organism. Hormone actions are determined by the pattern of hormone secretion and by the signal transduction capabilities of the receiving tissue.

Types of Hormones

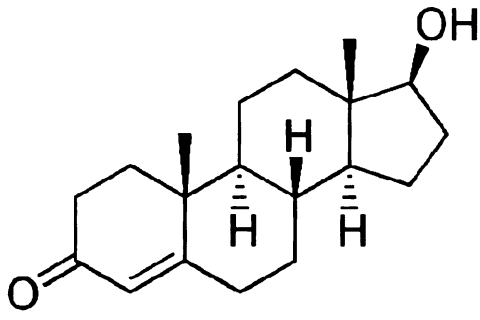
Endocrine hormones are grouped into four major structural groups (18):

- Polypeptides, proteins and glycoproteins
- Amino-acid derivatives
- Fatty acid derivatives - Eicosanoids
- Steroids

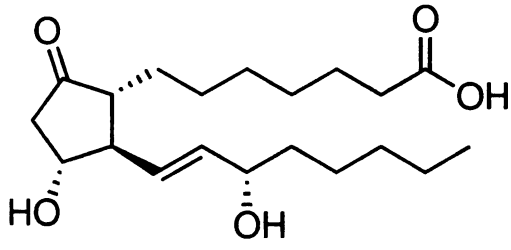
Figure 1.4 shows chemical structures of representative hormones.



Adrenaline [a]



Testosterone [b]



Prostaglandin E1[c]

Figure 1.4 Chemical structure of representative hormones. a) Amino-acid derivative hormone and neurotransmitter which drives the response to stress. b) Steroid hormone from the androgenic group. It is the principal male sex hormone and plays key roll in health and well being in male and female organisms. c) Fatty acid derivative hormone important in the constriction and dilatation of muscle and mediator in inflammatory process.

Peptide and amino-acid derivative hormones are secreted by the pituitary, parathyroid, thyroid, heart, stomach, liver, kidneys and adrenal medulla. Polypeptides and proteins are products of translation and vary considerably in size. The amino acid sequence information of a protein is contained in the coding region of the gene that codes for the protein in sequences of bases known as the genetic code; the code is copied from DNA into mRNA by transcription and the mRNA coordinates the protein synthesis by the process of translation (Figure 1.5). They can be as small as peptides formed by 3 amino acids or as large as glycoproteins formed by multisubunits of hundreds of amino acid residues. Several hormones belonging to the peptide group are synthesized in the endoplasmic reticulum as pro-hormones, to later go through proteolytic changes and convert them to the mature or acting form of the hormone; in other cases the hormone is part of the sequence of a larger precursor that is proteolytically cleaved several times to release the active hormone (19). Once synthesized, some peptide hormones are sent to the Golgi apparatus and packaged into secretory vesicles for export (20). Because most of the peptide hormones circulate un-attached to carrier proteins in the blood stream, their half-life is typically in the range of a few minutes.

Tyrosine, tryptophan and glutamic acid are the amino acids from which several hormones are synthesized. Thyroid hormones and catecholamine are derived directly from tyrosine. Catecholamines such as epinephrine and norepineprhine have a dual function; they can function as hormones and as neurotransmitters (21). While the half-life of thyroid hormones is in the order of a few days, catecholamines are rapidly degraded with half lives in the range of a few minutes (22-23). Tryptophan is the

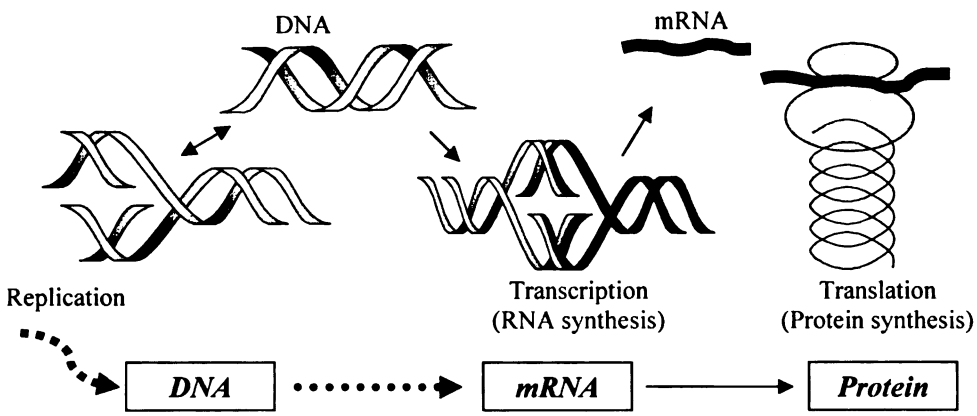


Figure 1.5 Protein synthesis. After DNA replication a transcription process translates DNA into mRNA to be translated to protein in the ribosome.

precursor of serotonin, which plays an important role in the regulation of mood and sleep; tryptophan is also a precursor of melatonin which communicates information about environmental lighting (24). Glutamic acid is converted to histamine, involved in immune responses as well as regulating physiological functions in the gut and acting as neurotransmitter (25).

Eicosanoid hormones are mostly derived from the arachidonic and other essential fatty acids and are found virtually in all tissues and organs. Among the eicosanoid hormones are prostaglandins, prostacyclines, thromboxanes and leukotrienes (26). They are important agents in allergic and inflammation processes and control blood flow and pressure. Leukotrienes are made by leukocytes and are extremely potent in vasoconstriction (27).

Steroid hormones are derivatives of the lipid cholesterol. They are synthesized and secreted mainly by gonads and the adrenal glands (28). Gonad tissues specifically produce the sex steroids estrogen and androgen that are responsible for female and male secondary sexual characteristics. Female gonads also synthesize progestins, responsible for the maintenance of pregnancy (29-31), while the adrenal cortex synthesizes mineralocorticoids such as aldosterone, which increases sodium, chloride and bicarbonate absorption to maintain blood flow and pressure. The glucocorticoids, such as cortisol, are also produced by the adrenal cortex and their function is to promote gluconeogenesis and fat and protein degradation (32). Once produced, steroid hormones are immediately released and depending on the species and the hormone may be bound by specific plasma protein carriers (33). In general steroid hormones are inactivated by metabolic transformations and excreted in urine or bile (34).

Hormone Release

Internal signals or external stimuli from the environment such as smell, light, temperature and sound trigger electrical responses in the nervous system causing the release of 'releasing hormone' in the hypothalamus which are transported to the pituitary through the portal system (10). This event induces the release of the trophic hormones that are responsible for the release of the ultimate hormone by the target glands (Figure 1.6). The critical characteristic of this domino effect is the intensity of the response in each event. Releasing hormone is produced in nanograms amounts, which induces production of the trophic hormone at the microgram level while the final hormone is produced in miligram quantities. This is a good example of 'amplified responses'. The release of hormones can also be stimulated by the levels of several metabolites (35).

In general, hormone secretion is a chain of events meticulously coordinated and regulated in a rhythmic manner.

Mechanism of Hormone Action

Hormones induce a trigger effect in order to modulate the activity of specific target cells. Most of the hormones act by binding to specific receptors, which are proteins that are normally present in small numbers in the target cells and may be of two kinds, plasma membrane receptors, also known as cell-surface receptors, and intracellular receptors. Therefore, there are two mechanisms by which hormones can activate/inactivate enzymes, alter membrane permeability and influence gene expression

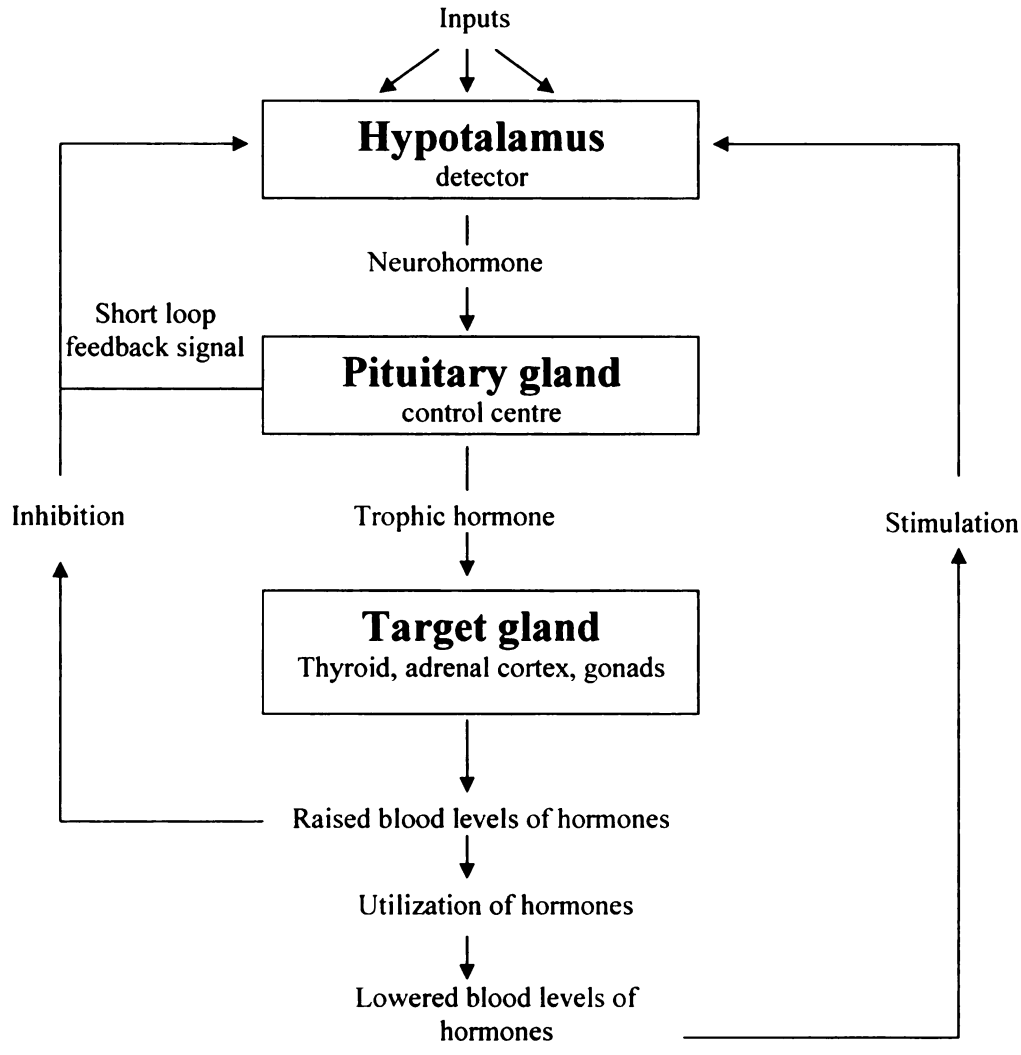


Figure 1.6 Mechanism and regulation of hormone release. External or internal inputs induce the hypothalamus to produce releasing hormones which stimulate the pituitary gland to release trophic hormones that consequently induce the activity of target endocrine glands.

producing significant changes in target cells which translate later into a variety of observed physiological effects (36).

Non-steroid or water soluble hormones, because of their lipophobicity, do not diffuse across the cell membranes; usually, they interact with cell-surface receptors.

Most of the receptors for peptide and protein hormones are G-protein linked receptors which use a second messenger to transmit the hormone signal from the outside to the inside of the cell. Hormone-receptor interactions provoke the dissociation of the intracellular trimeric G protein, which results in the opening of ion channels or the activation of membrane enzymes that may stimulate or inhibit the production of second messengers. As is shown in Figure 1.7, the activated molecules or second messengers then cause the phosphorylation of specific proteins that finally elicit the response. Calcium, cAMP, GMP, and inositol triphosphate are the most common second messengers (37).

Thyroid and steroid hormones, because of their lipophilicity, are believed to pass passively through the plasma membrane and once inside the cell bind to specific intracellular receptors forming an activated hormone-receptor complex, which binds to DNA activating or suppressing the expression of specific genes (38) (Figure 1.8). Type 1 receptors are linked to heat shock proteins (HSPs) and are located in the cytoplasm; this type of receptor is used for glucocorticoids, mineralocorticoids, androgens and progestins; the estrogen receptors usually travel between the cytoplasm and nucleus. Once the steroid hormone-receptor complex is formed the HSPs are released and the receptor undergoes dimerization with another receptor. The dimeric structure translocates to the nucleus where it binds a specific DNA sequence. Thyroid hormone

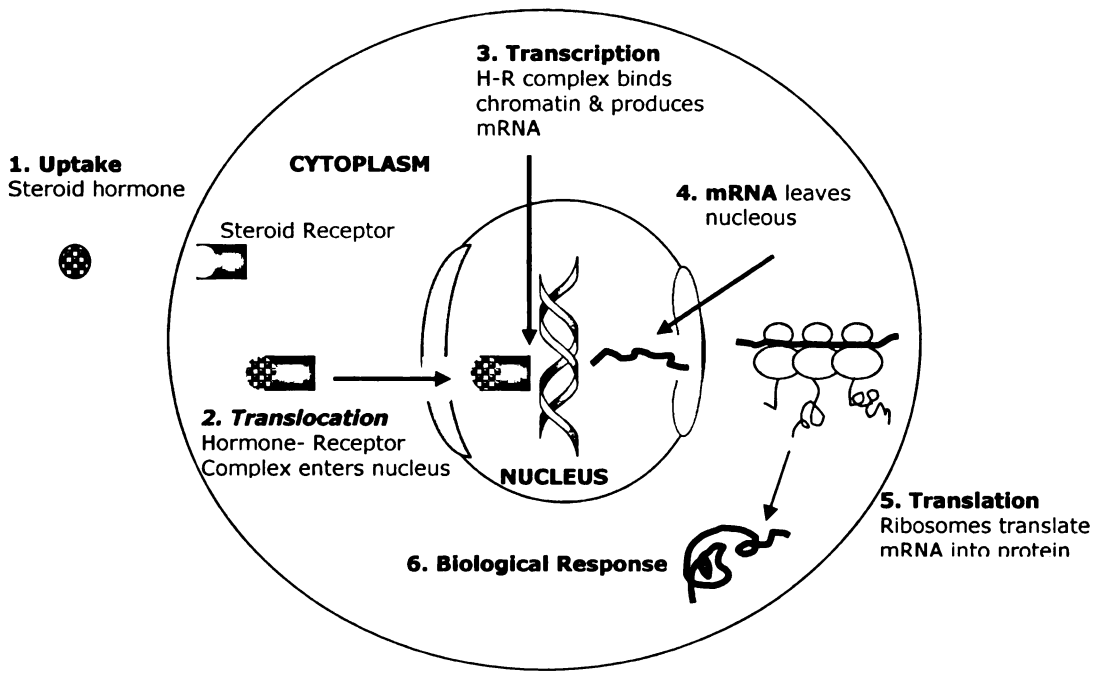


Figure 1.7 Mechanism of action of steroid hormones. After binding of the hormone to the receptor the formed Hormone-Receptor Complex (HRC) moves to the nucleus. A dimmer of HRC interacts with hormone-responsive elements on specific genes. As a consequence, template sites on DNA habilitate sites for RNA polymerase and increase transcription. Once produced, mRNA leaves the nucleus and undergoes translation in the ribosome.

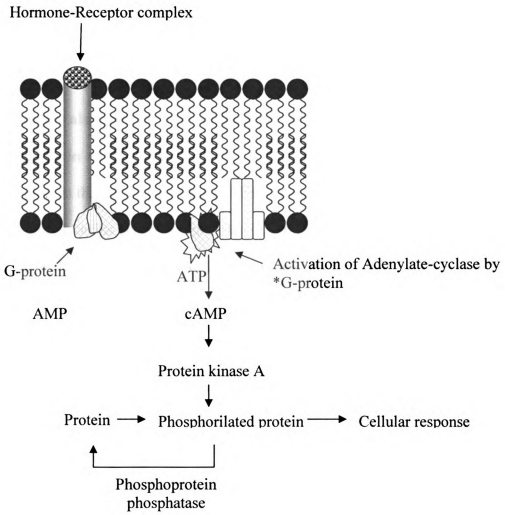


Figure 1.8 Cyclic AMP second messenger system. The enzyme adenylate cyclase catalyses the production of cAMP from ATP. The cAMP produced then activates protein kinases which phosphorylate intracellular proteins to alter their activity and produce a cellular response.

and retinoic *X* receptors are mostly found in the nucleus, bound to DNA and are known as type 2 receptors. In contrast to steroid receptors, type 2 receptors undergo dimerization with other non-identical receptors forming heterodimers; in some instances they may initiate transcription in the monomeric form (39). Despite the ability of lipophilic hormones pass through the cell membrane, some steroid or thyroid hormones also interact with cell-surface receptors to produce rapid non-genomic responses in target organs or cells. They may stimulate the production of protein receptors at the cell surface level or affect the intracellular production of protein kinases and other proteins needed in the action of peptide hormones (40).

After the interaction with the hormone-receptor occurs, both elements form a single condensed structure that induces vesicular formation followed by endocytosis. The hormones may be degraded by cell-surface enzymes and the receptors re-used or catabolised by lysosomic enzymes. Second messengers may be disintegrated or inactivated by phosphorylation/dephosphorylation processes (41).

Metabolism and excretion of hormones

Inactivation or degradation of hormones must occur in order to ensure steady-state levels of hormones in the bloodstream this is achieved using feedback mechanisms. Hormone inactivation and catabolism may occur in peripheral organs or tissues such as liver and kidney, but can also occur in target tissues immediately after the hormone trigges of the biological responses. The half-life of hormones in circulation is directly related to hormone action (42).

Peptide and protein hormones are degraded by peptidases at specific structural points, like carboxy or amino terminal groups (43). Usually, steroidal hormones are not re-used and they must be inactivated first, and then converted to a more soluble form in order to be excreted. P450 enzymes drive the inactivation of steroid hormones through hydroxylation processes and then transferase enzymes conjugate these inactive metabolites with glucoronic or sulfate groups to make them hydrophilic so they can be eliminated via urine and bile (44).

STEROIDOGENESIS

The biological synthesis of steroids is termed steroidogenesis. Steroid hormones are derivatives of cholesterol synthesized in the adrenal cortex and gonads, and in smaller quantities by a variety of other tissues such as brain and placenta (Figure 9). Synthesis occurs by two major types of enzymes located in both, mitochondria and endoplasmic reticulum (45-46). The identified sources of cholesterol include cholesterol synthesized *de novo* from acetate within the cell, cholesterol esters from lipid droplets and from cholesterol-containing low density lipoproteins (LDLs), are considered the main source of cholesterol in humans (47).

Initially, a chain of reactions activated by trophic hormones hydrolyze cholesterol complexes to form free cholesterol. This free cholesterol, by an assisted mechanism, is transported to the inner mitochondria where it is converted by the P450 side chain cleavage enzyme to pregnenolone, not a hormone itself, but a precursor for the synthesis of all steroid hormones (47-48). Subsequent biosynthetic steps involving

a movement of the various substrates thru the enzymatic batteries located in the endoplasmic reticulum

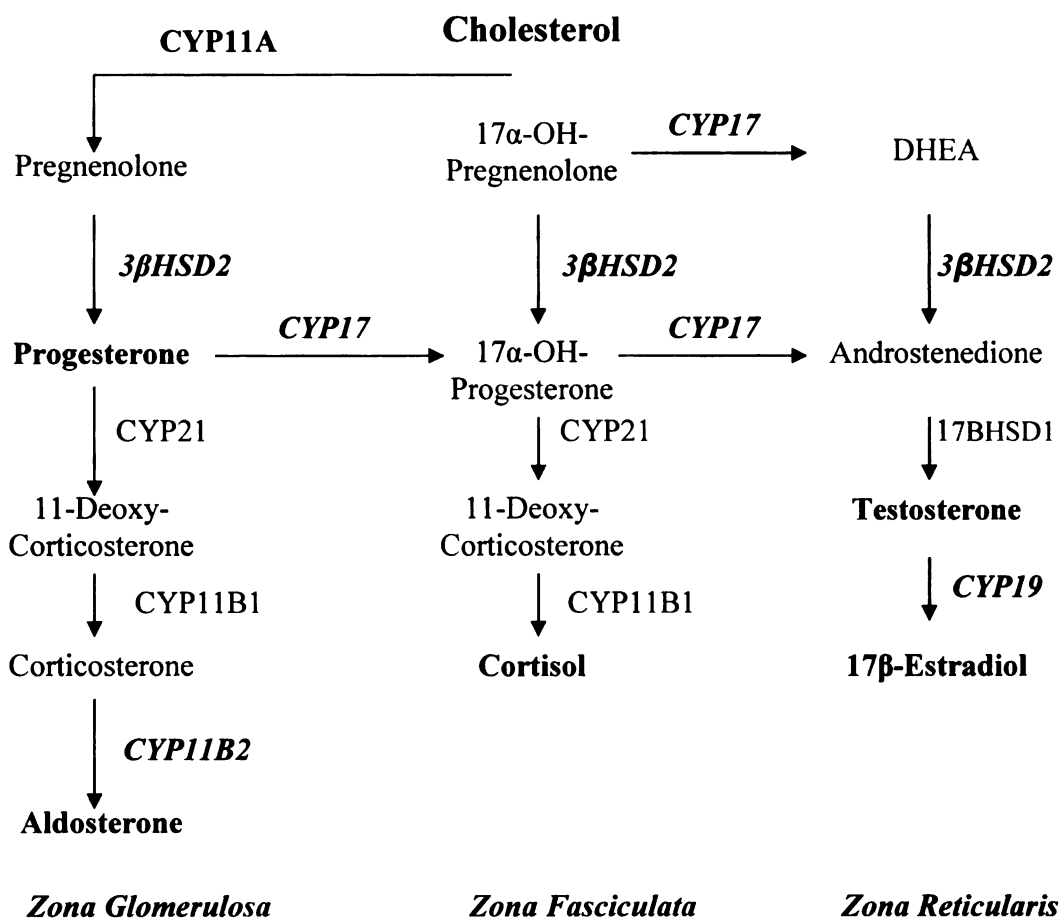


Figure 1.9 Synthesis of several adrenal steroid hormones from cholesterol. Enzymes leading the production of precursors and active hormones are shown. Chemical structures are presented only for terminal main hormones such as aldosterone and cortisol and for main precursors such as corticosterone an androstenedione.

and mitochondria, give origin to all steroid hormones (49).

Classification of Steroid Hormones

Steroid hormones are classified according to their physiological behavior, a consequence of their actions on one or more specific steroid hormone receptors (50):

- Progestins: Essential for reproduction
- Mineralocorticoids: Maintain electrolyte balance
- Glucocorticoids: Mobilize carbohydrates
- Androgens: Induce male secondary sex characteristics
- Estrogens: Induce female secondary sex characteristics

Since they arise from a common series of pathways, all steroid hormones share the cyclopentanoperhydrophenanthrene chemical structure (Figure 10).

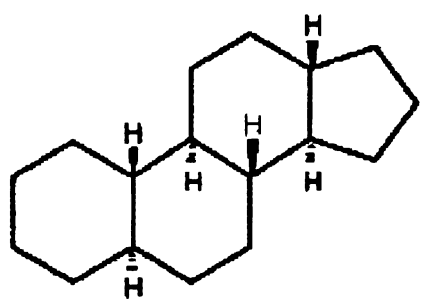


Figure 1.10 Chemical structure of the Cyclopentaneperydrophenantrene ring.

Enzymology of the Steroid Production

Steroid oxydoreductases and Cytochrome P450 (CYP) are the two major types of enzymes driving the pathways of steroid hormone biosynthesis. The capacity of a cell

to synthesize specific steroids is directly linked to its enzymatic expression; steroidogenic capacity then, is regulated mainly by tissue and cell specific expression of enzymes. Each enzyme involved in steroid production is encoded by one specific gene (Table 1.1), which controls and regulates its expression in different tissues and organs (49). The group of

Table 1.1 Enzymology of steroid production.

Functional name	Common name	Standard name/Gene
Desmolase; side chain cleavage enzyme	P450scc	CYP11A
3β- hydroxyseteroid-dehydrogenase	3β-HSD2	3BHSD2
17-α-hydroxylase/17,20 lyase	$P450_{C17}$	CYP17
21-hydroxylase	$P450_{C21}$	CYP21
11β-hydroxylase	$P450_{11\beta}$	CYP11B1
Aldosterone synthase	$P450_{11AS}$	CYP11B2
Aromatase	$P450_{arom}$	CYP19
17β-hydroxysteroid-dehydrogenase	17β-HSD(1-7)[a]	17β-HSD(1-7)

[a] Cytochrome P450s and oxydoreductases controlling the production of steroid hormones in the adrenal cortex. Each one of the steroidal P450 enzymes are encoded by a single gene, instead, the oxydoreductases are usually encoded by different genes, one for each isoform or isozyme. Human 17β-hydroxysteroid-dehydrogenase has 7 known isozymes.

enzymes in the adrenal cortex and testis does not change during adult life. In contrast, in the ovaries the amount of enzymes changes dramatically, especially during the final stages of follicle development (51). Steroidogenic factor 1 (SF1) has been identified as the transcription factor required for tissue-specific expression of steroid P450s enzymes as well as the gonadal and adrenal-specific isoforms of oxydoreductases. Despite the mandatory presence of SF1 for cell specific adrenal and gonadal expression, there are other factors necessary for determining the amount and specificity of steroidogenic

gene expression (52). One unique property of several of the steroidogenic enzymes is that they may catalyze more than one step in the pathway of steroid production.

The important role of enzyme expression in regulation of steroidogenesis is demonstrated by the fact that the hormonal output is mainly regulated by enzyme dependent events (51), such as:

- Substrate availability, which depends on enzyme-depended cholesterol mobilization.
- Steroidogenic enzyme level, determined by the mRNAs encoding the enzymes.
- Steroidogenic enzyme activity, determined by intracellular conditions.
- Tissue growth, determined by cell division and directly related with enzyme production.

Cytochrome P450 (CYP): These are oxidative enzymes, found mostly in the inner membrane of mitochondria or in the endoplasmic reticulum. Their main characteristic is wavelength of maximum absorption at around 450nm when the heme iron is reduced and complexed to carbon monoxide, this is the reason they have been termed "Pigment at 450nm" or P450. Hydroxylation is the most common reaction catalyzed by P450 enzymes but members of this superfamily are able to perform a wide range of reactions such as sulfoxidation, deamination, dehalogenation, N-oxydation or dealkylation (49-53).

Besides driving biosynthesis and catabolism of steroids, CYP enzymes are also involved in several other important physiological activities in mammals such as drug metabolism, cholesterol biosynthesis and blood homeostasis to name a few. Their

actions are also very important in metabolic processes of insects and in higher plants due to their involvement in the synthesis of numerous secondary metabolites like flavonoids, alkaloids and terpenoids (54-58).

The major steroidogenic CYP enzymes are:

- *P450scc*: This enzyme initiates the biosynthesis of all steroid hormones. In the inner mitochondrial membrane, cholesterol is converted to the precursor pregnenolone through monooxygenation processes that include hydroxylations at C-22 and C-20 ending with cleavage of the C-20, C-22 bond. This step is considered by many, the rate-limiting step of steroidogenesis. P450scc is encoded by the gene CYP11A and is expressed in the three zones of the adrenal cortex, ovaries, testis and brain. Genetic lesions affecting the activity of this enzyme are mostly rare, but if present, may result in an inability to synthesize any steroid hormone with consequent immediate death due to mineralocorticoid deficiency (49, 59-60).
- *P450c17*: Encoded by the gene CYP17, this 17-alpha-hydroxylase is required for androgen production and regulation of substrate supplies for aromatization, as well as for cortisol biosynthesis. Cleavage of C17-C20 bond in C21 steroids leads to androgenic production, while their 17-alpha-hydroxilation produces cortisol. This enzyme is well expressed in zona fasciculate and zona reticularis of the adrenal cortex and in smaller amounts in ovaries (49, 61). This enzyme is expressed in most steroidgoenic tissues. Some vertebrate species, like rats, do not express P450c17; thus, corticosterone rather than cortisol is the major

glucocorticoid. The activity of this particular enzyme is said to be strongly affected by the intracellular concentrations of substrates and products (62).

- *P450c21*: At the endoplasmic reticulum this enzyme executes hydroxylation on C21 in progesterone to the formation of cortisone and cortisol precursors, 11-deoxycorticosterone and 11-deoxycortisol, respectively; P450c21 is key in the production not only of mineralocorticoids but also glucocorticoids and is exclusively expressed in all three zones of the adrenal cortex. CYP21A and CYP21B are the genes encoding this enzyme, but only CYP21B encodes the active form of the enzyme (49, 63). Its deficiency, the most commonly observed genetic lesion, conduces to congenital adrenal hyperplasia, an illness characterized by deficient gluco and mineralocorticoid production and to excessive androgenic biosynthesis showed morphologically through enhancement of male characteristics, also known as virilization (64-65).
- *P450c11*: Is the final enzyme in the synthesis of adrenal mineralocorticoids and glucocorticoids. P450c11 exerts not only 11β-hydroxylase, but also 18-hydroxylase, and 18 aldehyde synthetase activities to mediate the conversion of 11-deoxycortisol to cortisol and the three final steps in the biosynthesis of aldosterone from deoxycorticosterone (66). The known two forms of this enzyme are localized mainly in the adrenal cortex, specifically in the inner mitochondrial membrane. Type I works directly on cortisol production, while type II focuses on aldosterone synthesis. Although the two forms of P450c11 are different in only 32 out of the 503 aminoacids of their primary sequence, their catalytic properties are clearly different. It has been proved that CYP11B2

does not use efficiently cortisone as substrate for the aldosterone biosynthesis, therefore the main mineralocorticoid is produced from 11-deoxycorticosterone as precursor. The deficiency in P450c11 hydroxylase and aldosterone synthase activity is the second most common cause of congenital adrenal hyperplasia, the first one is the lack of P45021c activity (68).

- *P450aro*: It mediates the aromatization of C18 estrogenic steroids from C19 androgens in the endoplasmic reticulum. The aromatization process involves 2 hydroxylations at C19 methyl group and a third one at C2. These hydroxylations result in the loss of C19 and the consequent aromatization of the A ring of the steroid (49). CYP 19 is the gene encoding for P450aro, commonly known as aromatase. This gene is the longest of all the steroidogenic genes and its main characteristic is the presence of alternative promoters that are used in tissue-specific manner (69). Aromatase is widely expressed in the ovary, mostly in the granulose cells where the major estrogen production occurs. This enzyme is also present in many other tissues besides gonads, such as breast, skin, placenta and adipocytes (70-71).

Oxidoreductases: These enzymes that catalyze the transfer of electrons from one molecule, the oxidant or hydrogen donor, to another, the reductant or hydrogen acceptor. Oxidoreductases are more commonly known as dehydrogenases. 3β-HSDs and 17β-HSDs are the main steroidogenic dehydrogenases. They belong to the same protein family as the short-chain alcohol dehydrogenase reductase superfamily. While P450 enzymes are encoded by single genes, the steroid dehydrogenases are usually encoded by at least 2-3 homologous genes since they

present several isoforms in the case of 3β-HSDs, or isozymes as is the case for 17β-HSDs. The number of isoforms or isozymes varies between species (49, 51, 62).

- 3β-HSD/isomerases: Are the enzymes catalyzing the production of the first biologically important steroid in the pathway, progesterone. Currently two isoforms, I and II, of 3β-HSD have been identified in humans; each isoform is the product of a distinct gene. Only type II is expressed in the 3 zones of the human adrenal cortex and in gonads, but not in placenta. Instead, type I is found in skin, placenta and breast tissue. Their functions are to convert 3β-hydroxy-5-ene steroids into 3-keto-4-ene, pregnenolone into progesterone, 17α-hydroxypregnenolone into 17α- hydroxyprogesterone and DHEA into androstenedione (49, 53, 57, 72-73).
- 17β-HSDs: Their main function is controlling the last step in the biosynthesis of all gonadal androgens and estrogens. These isozymes convert inactive 17-ketosteroids into their active 17β-hydroxy forms; 17β-HSDs are responsible for the interconversion of 17-ketosteroids, such as dehydroepiandrosterone, androstenedione and estrone with the respective 17β- hydroxysteroids, like androst-5-ene-3β, 17β-diol and 17β-estradiol (E2) (74). Usually they are found membrane bound or as soluble enzymes. Currently, 11 different 17β-HSDs have been identified. Each isoenzyme presents individual cell-specific expression, subtrate specificity and regulatory mechanisms. Among the 11 known 17β-HSDs only 3 forms actively participate in the production of gonadal steroidal hormones, types I, III and VII (75). 17β-HSD1, which is found in ovary, placenta and mammary glands, was the first of these ketosteroid-

dehydrogenases to be characterized. In humans, the substrate of preference for 17β-HSD1 are estrogens and its main function is to catalyze the inter-conversion between estrone and estradiol, 17β-HSD7 only from estrone to estrone and 17β-HSD3 is responsible of the conversion of weak androgens into potent androgens, that is, from androstenedione to testosterone. The androgenic 17β-HSD3 is exclusively expressed in testes (76).

Other Enzymes

In the early steps of steroid production, before the formation of pregnenolone, two critical events, enzymatically controlled by other type of enzymes, occur. One event is the production of cholesterol, since it is the basic building block for all steroids, and consequently, its transport to the inner mitochondrial membrane where all the initial enzymatic machinery for steroidogenesis is present. Cholesterol biosynthesis is controlled by hydroxymethylglutarylCoA reductase (HMGR) and its transport to the inner mitochondria is directed by the steroidogenic acute regulatory protein, StAR.

- *HMGR:* Cholesterol production is controlled by 3-hydroxy-3-methyl-glutaryl-CoA reductase or HMGR, which is the first enzyme in the metabolic pathway that produces cholesterol and other physiologically important biomolecules such as non-sterol isoprenoids and coenzyme Q (77). HMGR is a polytopic transmembrane protein driving the key step in the mevalonate pathway and its activity is regulated by transcription, translation, degradation and phosphorylation (78-79). Since in humans the

mevalonate pathway is the main mechanism of cholesterol production, this enzyme is the main target in the design of hypercholesterolemia and atherosclerosis drugs (80). The enzyme activity is mostly regulated by a negative feedback mediated by its own products, thus in mammalian cells this enzyme is suppressed by cholesterol derived from the internalization and degradation of LDL (77). This enzyme can also be found in prokaryotes and plants.

- *StAR*: The steroidogenic acute regulatory protein is a mitochondrial phosphoprotein that defines the well considered rate-limited step in steroid production (81). This enzyme/transport protein regulates the delivery of cholesterol from the outer to the inner mitochondrial membrane where is cleaved by the P450scc also known as CYP11A enzyme. The lipid properties of cholesterol make it difficult for it to cross the aqueous phase between the two membranes requiring the assistance of several proteins (82). StAR is found in the adrenal cortex and gonads where its presence increases the steroid production. The expression of this enzyme is stimulated by the cAMP signaling pathway and also by trophic hormones stimulation. Patients with mutations in the StAR gene suffer from congenital lipoid adrenal hyperplasia, which drastically impairs adrenal and gonadal biosynthesis (83).

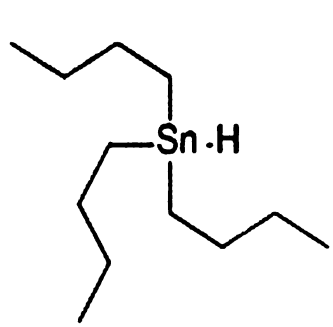
ENDOCRINE DISRUPTION

Among all the compounds released to the environment there are a range of chemicals that have the ability to adversely affect the normal functioning of the endocrine system. Specifically, a variety of chemicals with a diversity of physicochemical properties (Figure 1.11) are able to interfere with endogenous hormone activities so having major impacts on the health and reproductive functions in humans and wildlife. The ability of these chemicals to interfere with endocrine regulatory systems has lead to their being described as endocrine disrupting compounds (EDCs) (84-85). EDCs have been officially defined by the U.S. EPA as "exogenous agents who interfere with the synthesis, secretion, transport, binding, action, or elimination of natural hormones in the body that are responsible for the maintenance of homeostasis, reproduction, development and/or behavior" (86). Among the most frequent adverse effects of EDCs postulated to occur in animals and humans are: reproductive effects, birth defects, cancer, low sperm count, sexual dysfunction, heart disease, cognitive disorders, sex reversal and premature puberty (87-97). The complexity of EDC effects results to a great extent from the inherent complexity of the endocrine system and the fact that via a variety of signaling mechanisms the endocrine system is connected to the immune and nervous systems. As a

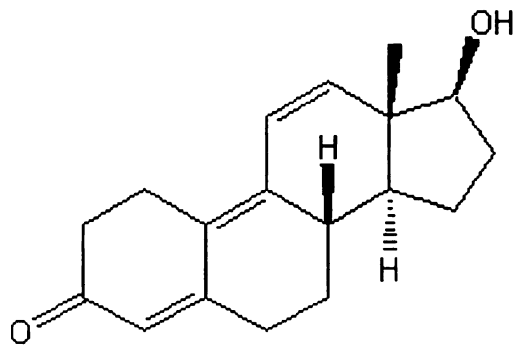
result some compounds classed as EDCs can also reduce the effectiveness of the immune system increasing the incidence of infections or produce neurotoxicity (98).

Natural sources of EDCs include plants and animals as well as human activities. A variety of naturally produced phytochemicals in foods, most of them with 'estrogenic' activities, may act in a similar way to estrogen due to their ability to bind to the estrogen receptor. However, phytoestrogens are much weaker than actual

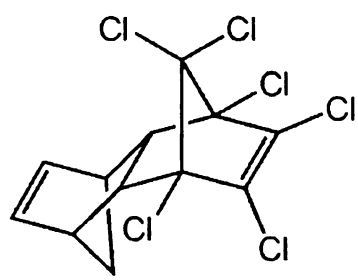
estrogen, so their effects are different from those of the natural hormone (99). Moreover, the consumption of a phytoestrogen-rich diet has been linked to either reduced or increased rates of heart disease and cancer (100-101). Good examples of natural phytoestrogen sources include soybeans, legumes, clover, yams and flax.



Tributyltin [a]



Trenbolone [b]



Aldrin [c]

Figure 1.11 Chemical structure of several known Endocrine Disrupter Compounds (EDCs). a) Tributyltin, biocide used as antifoulant paint additive. b) Trenbolone, anabolic steroid used by veterinarians on livestock to increase muscle growth and appetite. c) Aldrin, a pesticide commonly used in crop fields.

Humans and animals also produce hormones naturally; they also consume hormonal products by taking pharmaceuticals such as birth control pills and by using hormone replacement therapy containing mixtures of estrogens, progestagens and androgens; once they are excreted from humans and animals, these hormonally active chemicals can easily flow into sewage treatment and drinking water systems. An area of current high concern is the waste produced by concentrated animal feed operations (CAFOs) since they discharge environmentally relevant doses of natural and pharmaceutically produced hormones. Pregnant animals on CAFOs naturally produce high levels of estrogens and several pharmaceuticals such as growth promoters and antibiotics are administrated to maximize animal growth and health (102-106). Residues of these chemicals can be excreted in manure and by run-off they may easily reach aquatic environments (107-109). Since these compounds were designed to produce a biological effect, once they reach the environment, unwanted effects on non-target organisms may be exerted (110). Besides pharmaceuticals, some compounds used in the cosmetic industry have also raised concern. Chemicals used in dental sealants, skin lotions, and makeup are suspected of causing endocrine disruptor effects (111-113).

Other human activities also release synthetic compounds capable of disrupting the normal functioning of the endocrine system. These ubiquitous EDCs are used in plastics, food production and packaging, paints, wetting agents, carpets and furniture, detergents and pesticides. Phthalates, for example, are chemicals used as plasticizers to manipulate the physical properties of plastics. Although it has been established that human exposure does not currently appear to be toxicologically relevant, several bans haven been introduced in Europe for these compounds in children's toys as a

precautionary measure (114). Bisphenol A is widely used as an inner lining on food and beverage cans. Despite several studies that have shown Bisphenol A to produce hormone related effects at high doses, there is still an ongoing debate about whether or not these results are toxicologically relevant (115-116).

Tributyltin (TBT) is a compound primarily used as an antifoulant paint additive on ship and boat hulls, docks, fishnets, and buoys to discourage the growth of marine organisms such as barnacles, bacteria, tubeworms, mussels and algae (117). TBT concentrations in harbors and bays in Britain, France and the United States were historically high enough to significantly affect oyster and mussel production (118-120). At low levels, TBT can cause structural changes, growth retardation, and inhibit reproduction Imposex, the development of male characteristics in females, has been initiated by TBT exposure in several snail species (121). Other compounds, such as brominated flame retardants used in furniture, carpet and electronics manufacture have also been reported to occur in human breast milk, fatty tissue and blood. These compounds have been banned by the European Union for suggestive risks associated with accumulation processes (122-123).

Alkylphenols and their ethoxylates are compounds commonly used in liquid detergents and as wetting agents in different industrial applications. They have been shown to have both acute and chronic effects in aquatic organisms, including alterations in reproduction, feminization, hermaphroditism, and lower survival rates in fish and other aquatic organisms living in alkylphenol-contaminated waters (124-126). These effects have been found even at low doses.

There are several mechanisms whereby EDCs could modulate endocrine systems and potentially cause adverse effects. The most direct mechanism of action for EDCs is the direct binding of the compounds to hormone receptors at the cell surface, cytoplasm, or nucleus, followed by a complex series of events that can lead to changes in gene expression (127). Therefore, the most studied mechanisms of EDC action are those involving the best known receptors: estrogen (ER), androgen (AR), aryl hydrocarbon (AhR) and thyroid (ThR). However, there is evidence demonstrating that other receptor systems may also be of importance when evaluating EDC effects. These include the cytokine systems, the retinoic acid receptor and some of the 'orphan receptors', receptors without known ligands and/or function (128-131). Some of the EDCs can imitate and/or amplify the effects of the endogenous hormones, they may function by direct binding the hormone receptors and triggering the same response as the natural hormone would or in some cases producing even a stronger response. Others EDCs may instead, stimulate the production of more hormone receptors resulting in increased sensitivity or responses to natural hormones. In contrast, there are compounds that bind to a receptor and activate a weaker response than the natural hormone would or produce no biochemical effect but prevent hormonal action simply by taking the hormone's specific site on the receptor (132). All mechanisms involving direct interaction with any of the mentioned receptors are known as Direct Mechanisms of Endocrine Disruption.

The modes of action of EDCs are not limited to direct interactions with hormone receptors. There are other mechanisms that include inhibition of enzyme activity for hormonal synthesis, or binding to carrier proteins reducing their availability

to transport hormones or altering the endogenous hormone levels by accelerating their breakdown and elimination, or by deactivating the enzymes leading to hormone breakdown (133-135). Some compounds even react directly with hormones to alter their structure or modify their synthesis. Others less known mechanisms involve indirect receptor activation by phosphorylation or cellular complexes (136). Together, these mechanisms are known as Indirect Mechanisms of Endocrine Disruption.

There are numerous experimental assays available for the evaluation of specific interactions of xenobiotics with most of the endocrine receptors, ER, AR, ThR and AhR, However, there are very few methods currently available for the evaluation of integrated endocrine disrupting effects. Therefore, new practical experimental systems are needed for the identification of effects caused by pathways different to hormone-receptor interaction.

THE H295R CELL SYSTEM

The adrenal glands, also known as suprarenal glands, are the small, orange and triangular shaped endocrine glands located on the top of both kidneys. Each gland is divided into an inner medulla and an outer cortex, and both tissues receive input from the nervous system but through different mechanisms; the adrenal cortex is regulated mostly by negative feedback through the hypothalamus and ACTH, while the medulla is regulated by nerve impulses. The adrenal cortex is essential to life but the medulla can be removed withouth life-threatening effects (137).

Each part of the adrenal glands, medulla and cortex, performs separate specific functions (Table 1.2). The adrenal medulla is the main source of the catecholamines

adrenaline and noradrenaline. Adrenaline increases heart rate and relaxation of smooth muscles, while noradrenaline posessess strong vasoconstrictive effects, increasing blood pressure; these two hormones are secreted by stimulation via sympathetic nerves, particularly in response to stressful situations (138). The outer portion of the adrenal gland or adrenal cortex consists of three different regions: zona glomerulosa, zona fasciculata and zona reticularis, with each region producing different types of hormones, although chemically all of them are steroids. Aldosterone is the major mineralocorticoid secreted by the outermost portion, the glomerulosa; this hormone controls sodium and water absorption in the body. Glucorticoids such as cortisol are produced for the thicker zona fasciculate. Cortisol increases glucose levels in the bloodstream. Sex hormones are produced in the innermost region. Androgens and estrogens are secreted in minimum levels by both, male and female; their effects are usually masked by the hormones produced in testes and ovaries (139).

Table 1.2 The adrenal glands [a].

Adrenal Cortex	Zona Glomerulosa	**Mineralocorticoids** Aldosterone
	Zona Fasciculata	**Glucocorticoids** Cortisol
	Zona Reticularis	**Sex Steroids** Androgens & Estrogens
Medulla	**Catecholamines**: Epinephrine & Norepinephrine	

[a] Major hormones and neurotransmitters produced by the different zones of adrenal cortex and medulla of the adrenal glands.

In 1990, Adi Gazdar and his colleagues at the NCI-Navy Medical Oncology Branch established and characterized a continuous human adrenocortical carcinoma cell

line that expresses multiple pathways of steroid biosynthesis. The NCI-H295 adrenocarcinoma cell line was initiated from a portion of the adrenal tumor removed in 1980 from a 48-years old, black female patient born in the Bahamas. Pieces of the adrenal tumor were finely minced and placed in culture in microwells with one of four growth media. Initially, the cells grew slowly in all four media, some of them attached and some of them floating. Only HITES media, which lacks attachment factors, provided floating aggregated cells with fewer numbers of fibroblasts. Following culture of the NCI-H295 cells, mice were inoculated to evaluate its tumorogenic properties. Mice developed tumors at the inoculation site 6 to 9 weeks later. The cell line and developed tumors demonstrated their steroidogenic character after ultrastructural studies showed the presence of exaggerated numbers of mitochondria, smooth endoplasmic reticulum, prominent Golgi apparatus and nucleoli, meanwhile cytogenetic studies showed the presence of 65 marker chromosomes. Steroid secretion of the NCI-H295 was evaluated after 7-10 years of the cells being in culture and high concentrations of the precursor pregnenolone, 17-hydroxy pregnenolone and dehydroepiandrosterone were present. Aldosterone, deoxycortisol, progesterone and androstenedione were found in lower amounts. With the production of the quantified steroids, the presence of all major adrenocortical enzyme system including, 11β-hydroxylase, desmolase, 21α-hydroxylase, 17α-hydroxylase, lyase and sulfokinase was proved. The estrogens measured suggested aromatase presence. NCI-H295 cells were proven to be capable of synthesizing the cholesterol required for steroid production, since steroid synthesis occurred in serum-free, cholesterol-free medium. Androgens

were the main product in cells fed with serum free media. No steroids were to be found in control medium samples (140).

Some years after the establishment of this cell line it was demonstrated to possess all of the major pathways of adrenal steroidogenesis. In addition these pathways were based on expression of the same enzymes found in normal adrenal glands. It was also determined that the genes encoding the required enzymes responded to intracellular second messengers in a manner similar to that of normal human adrenals. Although this cell line is capable of producing most of the steroids synthesized in the three different zones of the adrenal cortex, there is supportive evidence that the NCI-H295 cell line is zonally undifferentiated (141).

It was subsequently demonstrated that this cell line also retains hormonal responsiveness and that because of its steroidogenic properties it could provide an *in vitro* model to help ascertain factors controlling adrenal androgen and glucocorticoid production. This would permit the examination of pharmacological, biochemical and molecular mechanisms that control adrenal steroid biosynthesis (142-143). Following studies corroborated the utility of the NCI-H295 cell line to expand the evaluation of endocrine disruption since prior to its use most of the assays available were restricted to chemical-receptor interactions (144-146). The potential for environmental contaminants to affect endocrine systems at levels other than receptor binding was now available. This cell line has also been found useful in the evaluation of adrenostatic compounds used in the treatment of patients with adrenal malfunctioning. Drugs as aminoglutethimide, metyrapone and etomidate, frequently used in patients with

Cushing's syndrome, have proven to not only suppress steroid enzyme activity but also influence both ACTH-receptor expression and cell proliferation in adrenal cells (147).

The NCI-H295 cell line is available through the American Type Culture Collection (ATCC) under the code CRL-10296. Because the population doubling time of the cells growing as free aggregates was higher then 96 hours, it was necessary to modify the growth conditions. A monolayer population of more actively growing H295 cells is now available for use and it is named H295R.

In previously reported studies from our laboratory we have established a standardized system to culture and expose H295R cells to evaluate the potential effects of chemicals on the expression of 10 genes involved in steroid production. The results of exposures with known inducers and inhibitors of steroidogenesis have proven the ability of the H295R cell line to identify similar expression patterns for chemicals acting through common mechanisms of action. Time-dependent expression profiles were also established through time-course studies (148). The reproducibility of this method was corroborated with later studies using a different method of gene expression evaluation and a different method of quantification, such as molecular beacon and the standard curve methods (149). Moreover, the H295R standard methods of exposure established by our group were proven to be effective in the evaluation of effects of environmental samples, such as fresh water sediments, on steroid production (150).

The continuous development of molecular biology techniques lends them to the study and understanding of the multiple mechanisms that drive life. The creative molecular methods available today, not only have made it possible to find explanations for normal and abnormal processes, but also have provided the basic tools for

diagnostics and research. For the purposes of this dissertation, the standardized methods for the use of the H295R cell in the evaluation of gene expression and hormone production chosen were the Polymerase Chain Reaction (*PCR*), which is an *in vitro* molecular biology technique used to replicate enzymatically specific regions of DNA and the Enzyme Linked Immunosorbent Assay (*ELISA)*, a molecular method that combines the specificity of antibodies or antigens with the sensitivity of simple enzyme assays.

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Chapter 2

THE H295R SYSTEM FOR THE EVALUATION OF ENDOCRINE DISRUPTING EFFECTS

ABSTRACT

The present studies were undertaken to evaluate the utility of the H295R system as an *in vitro* assay to assess the potential of chemicals to modulate steroidogenesis. The effects of four model chemicals on the expression of ten steroidogenic genes and on the production of three steroid hormones were examined. Exposures with individual model chemicals as well as binary mixtures were conducted. Although the responses reflect the known mode of action of the various compounds, the results show that designating a chemical as "specific inducer or inhibitor" is unwise. Not all changes in the mixtures exposures could be predicted based on results from individual exposures. Hormone production was not always directly related to gene expression. The H295R system integrates the effects of direct-acting hormone agonists and antagonists as well as chemicals affecting signal transduction pathways for steroid production and provides data on both gene expression and hormone secretion which makes this cell line a valuable tool to examine effects of chemicals on steroidogenesis.

Keywords: Bioassay, Steroidogenesis, Screening, Endocrine Disruptors, Mixtures

INTRODUCTION

Concern about the potential effects of chemicals on the endocrine systems of wildlife (Ankley et al 1998) and humans (Kavlock et al 1996) has increased over the past few years. On October 26th, 2000 the European Parliament adopted a resolution on endocrine disrupters, emphasizing the application of the precautionary principle and calling on the Commission to identify substances for immediate action. In 2004, the Commission presented an update on the implementation of the strategy which among other recommendations includes an adaptation/amendment of current legislation to consider potential effects of Endocrine Disrupters. In particular, Regulation No 793/93 of the European Economic Community (EEC) on risk assessment and Directive 67/548/EEC on the classification of dangerous substances have been promulgated. In the United States, legislation such as the Safe Drinking Water Act Amendments of 1995 and the Food Quality Protection Act of 1996 have been promulgated. These legislative mandates require screening for endocrine disrupting properties of chemicals used in commerce or resulting from process that might occur in drinking water or food.

It has been difficult to develop the necessary screening tools because there are so many potential effects that could lead to endocrine disruption. In fact, any stressor, chemical or otherwise that forces an organisms out of its' normal homeostatic range could be defined as an endocrine disruptor. The federal Endocrine Disruptor Screening and Testing Advisory Committee (EDSTAC) recommended that chemicals be screened as agonists or antagonists of estrogen (ER), androgen (AR), aryl hydrocarbon (AhR) and thyroid (ThR) hormone receptors (EDSTAC 1998). Specifically, much of the early

research focused on the effects of direct-acting effects such as chemicals that act as hormone mimics by acting as agonists for hormone receptors, in particular interest focused on the ER. In 1938, Dodds and Lawson conducted perhaps the first published study to show the estrogenicity of Bisphenol A and Alkylphenols using ovariectomized rats. Although more evidence for the endocrine disruption effects of these compounds was found in the 70's (Muller and Kim 1978) health concerns were only raised when effects of bisphenol A and nonylphenol on cultured human breast cells were observed (Kirshnan et al 1993; Soto et al 1991). A yeast screen containing a human androgen receptor showed that bisphenol A can also act as an antiandrogen (Sohoni and Sumpter 1998). In the late 90's the list of endocrine disrupters grew when some PCBs metabolites were found to mimic oestradiol (McKinney and Waller 1994) and dioxins showed not only to alter hormone production but also alter the immune system (Grassman et al 1998). The pesticide *o,p*-DDT was also found to be a weak estrogen agonist (Colborn et al 1997). The relevance of this finding is questionable since the primary form of the DDT metabolites found in the environment and animal tissues is actually *o,p*-DDE. Nevertheless, much of the initial research, public interest and legislation focused on hormone mimics. Furthermore, most of the initial work was on developing methods of predicting the ability of chemicals to serve as ER agonists. This included both structure activity models to predict ER binding (Kanno et al 2001) as well as ER binding assays (Legler et al 1999). More recently several eukaryotic cell based expression assays have been developed where an endogenous or exogenous reporter gene is expressed under control of the estrogen receptor (Pons et al 1990; Legler et al 1999) or androgen receptor (Sonneveld et al 2004; Wilson et al. 2002). In

addition, there have also been some in vitro systems based on prokaryotic cells (Routledge and Sumpter 1996). Together these systems have made possible the identification of many environmental contaminants which may act by binding directly to hormone receptors. The utility of in vitro assay systems for the identification of novel mechanisms of endocrine disruption was demonstrated by the observation that some chemicals are able to alter the production of enzymes involved in steroid production (Sanderson et al 2000). While some chemicals have been shown to modulate the endocrine system as direct receptor agonists or antagonists (Villenueve et al 1998) other chemicals can cause effects by non-receptor-mediated mechanisms (Sanderson et al 2000). In particular, chemicals that alter the expression of steroidogenic enzymes have the potential to alter rates, as well as absolute and relative concentrations of hormones in blood and tissues (Hilscherova et al 2004).

The H295R assay system is now being developed and validated for use in a tiered screening approach by the US EPA (EDSTAC Final Report 1998) and the results of preliminary work conducted in our laboratory have been presented to the OECD at their annual meeting in Paris (2005). At this time the US EPA is considering using the H295R system to replace two currently used assays, the Hershberger uterotropic assay for estrogenicity (Kanno et al 2001) and the rat minced testis assay for determining effects on aromatase (CYP19). If the H295R assay is adopted, it is anticipated that it will result in more rapid, accurate and less expensive assays as well as obviating the need for the use of live animals, which is required in the in vivo or ex vivo assays currently being utilized. Because of the great potential utility of the H295R assay as a screening tool and to discern the mechanisms of action of specific endocrine

modulating compounds, we are presenting this demonstration of the utility of the assay as a "frontiers" article, indicative of a publication with novel contributions in sciences.

The H295R cell line was derived from a human adrenal carcinoma and has all the enzymes necessary to produce steroid hormones (Gazdar et al 1990; Rainey et al 1993; Staels et al 1993). H295R cells have physiological characteristics of zonally undifferentiated human fetal adrenal cells and as a result have the ability to produce the steroid hormones of each of the three phenotypically distinct zones found in the adult adrenal cortex (Gazdar et al 1990; Staels et al 1993). Since the cells maintain the ability to express these genes and produce these enzymes, they are a useful model system for the study of potential effects on steroidogenesis. The genes measured in the current studies include *CYP11A* (cholesterol side-chain cleavage), *CYP11B2* (aldosterone synthetase), *CYP17* (steroid 17α-hydroxylase and/or 17,20 lyase), *CYP19* (aromatase), *17β-HSD1* and *17β-HSD4* (17β-hydroxysteroid dehydrogenase, type 1 and 4), *CYP21B2* (steroid 21-hydroxylase), *3β-HSD2* (3β-hydroxysteroid dehydrogenase), *HMGR* (Hydroxymethylgutaryl CoA reductase) and the cholesterol transfer protein *StAR* (steroid acute regulatory protein). Treatment with a variety of agents has been shown to alter steroid production in H295R cells (Ohno et al 2002). Previous studies have demonstrated that measurement of gene expression in the H295R system not only permit the evaluation of the potential of chemicals to interfere with the expression of steroidogenic enzymes, but also provides a means of profiling the modes of action of chemicals (Hilscherova et al 2004; Zhang et al 2005). Furthermore, the H295R cell line has also shown to be useful for measuring the activity of the enzymes as it has been observed in the studies conducted by Sanderson et al (Sanderson et al 2000 and 2001)

where it was demonstrated that commonly used 2-chloro-s-triazine herbicides dose-dependently induced aromatase (CYP19) activity in this cell line.

The H295R system therefore represents a unique bioassay system in that it allows for the measurement of alterations in gene expression and at the same time permits determination of alterations in steroid hormone production by the same cell cultures. In this paper we report further on the development of the H295R assay system and for the first time present data demonstrating the relationship between gene expression and steroid production.

MATERIALS AND METHODS

Test Chemicals

Forskolin, ketoconazole and aminoglutethimide, were obtained from Sigma (St. Louis, MO, USA), metyrapone was obtained from Aldrich (St. Louis, MO, USA); purity of all test chemicals exceeded 98%. The chemicals used in this study were chosen based on the known effects on steroid metabolism as well as their effects on steroidogenic gene expression (Hilscherova et al, 2004).

Experimental design

The H295R human adrenocortical carcinoma cell line was obtained from the American Type Culture Collection (ATCC # CRL-2128, ATCC, Manassas, VA, USA) and cells were grown in 75 cm^2 flasks with 12.5 ml of supplemented medium at 37°C with a 5% CO_2 atmosphere. Supplemented medium was a 1:1 mixture of Dulbecco's modified Eagle's medium with Ham's F-12 Nutrient mixture with 15 mM HEPES buffer. The medium was supplemented with 1.2 g/l Na_2CO_3, ITS+ Premix (BD Bioscience, 1 ml

Premix/100 ml medium), and 12.5 ml/500ml NuSerum (BD Bioscience, San Jose, CA, USA). Final component concentrations in the medium were: 15 mM HEPES; 6.25 μg/ml insulin; 6.25 μg/ml transferrin; 6.25 ng/ml selenium; 1.25 mg/ml bovine serum albumin; 5.35 μg/ml linoleic acid; and 2.5 % NuSerum. The medium was changed 2-3 times a week and cells were detached from flasks for sub-culturing using trypsin/EDTA (Sterile 1x Trypsin- EDTA (Life Technologies Inc.). Cells were exposed to test chemicals dissolved in DMSO using 6-well Tissue Culture Plates (Nalgene Nunc Inc., Rochester, NY, USA). Cells were detached from flasks with trypsin/EDTA (Sterile 1x Trypsin- EDTA (Life Technologies Inc.) and were harvested into a final volume of 11mL of medium. Cell density was determined using a hemocytometer. For dosing, 3 ml of cell suspension containing 1 x 10^6 cells/ml were placed in each well.

In the dose-response experiment, H295R cells were exposed to 0.03, 0.1, 1.0, 3.0, 10, or 50 μM forskolin for 24 h while only the 10 and 50 μM concentrations were measured at 48 h. The solvent used in these experiments was DMSO at a final concentration of 0.1%. Matching solvent controls were run concurrently and used to evaluate gene expression at each time interval.

To ascertain the effects of chemical mixtures on H295R cells, cells were treated with forskolin in combination with other chemicals previously shown to alter gene expression (Hilscherova et al, 2004) (Table 2.1). The chemicals used in this study were chosen based on their variety of known effects on steroid metabolism. Among other effects aminoglutethimide is an aromatase inhibitor (Bastisda et al, 2001); forskolin increases cellular cAMP concentrations (Thomson et al, 2001); ketoconazole works principally by inhibition of cytochrome P450 14 α-demethylase *(P45014DM)*; and

Table 2.1 Chemical mixtures exposure. [a]

Chemical 1	**Chemical 2**
Solvent control	na
10 μM Forskolin	300 μM Metyrapone[b]
10 μM Forskolin	300 μM AMG[b]
10 μM Forskolin	20 μM Ketoconazole[b]

[a] H295R cells were exposed to individual or chemical mixtures for 24 h.

[b] Gene expression data for individual chemicals were previously reported in Hilscherova et al 2004.

na not applicable

metyrapone is a specific inhibitor of 11 β-hydroxylase (Parthasarathy et al, 2002). The data used in these analyses are a compilation of several different exposure studies where some data for individual compounds were run separately from those that evaluated chemical mixtures. All chemical mixtures contained 10 μM forskolin in combination with 300 μM metyrapone, 300 μM aminoglutethimide or 20 μM ketoconazole.

Cell viability/Cytotoxicity

Before nucleic acid isolation and hormone analysis, cell viability was determined. Cells were visually inspected under a microscope to evaluate viability and cell number. In addition, cell viability was determined with the Live/Dead cell viability kit (Molecular Probes, Eugene, OR, USA). While ketoconazole inhibited cell growth at concentrations greater than 30 μM, no adverse effects on cell growth or viability were observed for any of the tested chemicals at concentrations up to 300 μM. In instances where exposure to model compounds resulted in cell death or decreased viability, the data were not used to evaluate gene expression or hormone production.

RNA Isolation

For nucleic acid extraction, after removal of the medium, cells were lysed in the culture plate, by the addition of 580 μL/well of Lysis Buffer-β-ME mixture (Stratagene, La Jolla, CA, USA) and RNA was isolated as described in (Hilscherova et al. 2004). Briefly, lysed cells were mixed and then centrifuged in a pre-filter spin cup and the mixture centrifuged. The filtrate was diluted with 70% ethanol and vortexed. The

mixture was transferred to an RNA spin cup and centrifuged for 1 min. The filtrate was discarded and the spin cup was washed with a low-salt buffer and then centrifuged for 1 min. RNase-Free DNase I solution (Strategene, La Jolla CA, USA) was added to the fiber matrix inside the spin cup and the sample was incubated at 37°C for 15 min. The sample was then washed with high-salt followed by a low salt buffer. After each wash cycle, the filtrate was discarded. After the final wash, the sample was centrifuged and nuclease-free water was added directly to the fiber matrix inside the spin cup. The tube was incubated for 2 min at room temperature and centrifuged. This elution step was repeated to maximize the yield of RNA. The purified RNA was used immediately or stored at –80°C until needed. An appropriate dilution of the RNA sample (1:50) was prepared for RNA quantification. The absorbance of the RNA solution was measured at 260 nm and 280 nm and the 260/280 ratio was calculated. The concentration of total RNA was estimated using the A_{260} value and a standard with an A_{260} of 1 that was equivalent to 40 μg RNA/ml.

cDNA preparation

Total RNA (1-5 μg) was combined with 50 μM oligo-$(dT)_{20}$, 10 mM dNTPs, and diethylpyrocarbamate (DEPC)-treated water to a final volume of 12 μL. RNA and primers were denatured at 65°C for 5 min and then incubated on ice for 5 min. Reverse transcription was performed using 8 μL of a master mix containing 5*x* cDNA synthesis buffer (Carlsbad CA, USA) and DEPC-treated water. Reactions were incubated at 50°C for 45 min and were terminated by incubation at 85°C for 5 min. Samples were

either used directly for PCR or were stored at –20°C until analyzed.

Real-time PCR

Real-time PCR (quantitative PCR) was performed by using a Smart Cycler System (Cepheid, Sunnyvale, CA, USA) in 25 μL sterile tubes using a master mix containing 25 mM $MgCl_2$, 1U/μL AmpErase (Applied Biosystems, Foster City, CA, USA), 5 U/μL *Taq* DNA polymerase AmpliTaq Gold, 10*x* SYBR Green (PE Biosystems, Warrington, UK), nuclease free water and between 10 pg and 1 μg of cDNA. The thermal cycling program included an initial denaturation step at 94°C for 10 min, followed by 25-35 cycles of denaturation (95°C for 15s), primer annealing (at 60-64°C for 40-60s), and cDNA extension (72°C for 30s); a final extension step at 72°C for 5-10 min was also included. Melting curve analyses were performed immediately following the final PCR cycle to differentiate between the desired amplicons and any primer-dimers or DNA contaminants. Specifics of the assay parameters such as primers used and annealing temperatures have been published previously (Hilscherova et al, 2004).

For quantification of PCR results C_t (the cycle at which the fluorescence signal is first significantly different from background) was determined for each reaction. C_t values for each gene of interest were normalized to the endogenous control gene, β-actin. Normalized values were used to calculate the degree of induction or inhibition expressed as a "fold difference" compared to normalized control values. Therefore, all data were statistically analyzed as "fold induction" between exposed and control cultures. Gene expression was measured in triplicate for each control or exposed cell culture and each exposure was repeated at least three times.

Hormone Quantification

Hormone extraction and quantification by ELISA were conducted as previously (Hecker et al 2005). Briefly, frozen media samples were thawed on ice, and the hormones were extracted twice with diethyl ether (5 ml) in glass tubes. To determine extraction recoveries a trace amount of ^{3}H-testosterone was added to each sample prior to extraction. The solvent extract was separated from the water phase by centrifugation at 2,000 x g for 10 min and transferred into small glass vials. The solvent was evaporated under a stream of nitrogen, and the residue was dissolved in EIA buffer from Cayman Chemical Company and either immediately measured or frozen at –80°C for later hormone determination. Concentrations of hormones in media were measured by competitive ELISA using Cayman Chemical® hormone EIA kits (Cayman Chemical Company, Ann Arbor, MI, USA; progesterone [Cat # 582601], testosterone [Cat # 582701], estradiol [Cat # 582251]). Because the antibody to progesterone exhibits cross-reactivity with pregnenolone of 61% and the method does not allow for the separation of these two hormones, progesterone concentrations are expressed as progesterone/ pregnenolone. The working ranges of these assays for the determination of steroid hormones in H295R media were determined to be: Progesterone (P): 7.8 - 1000 pg/well; testosterone (T): 3.9 - 500 pg/well; 17ß-estradiol (E2): 7.8 - 1000 pg/well. Media extracts were diluted 1:25 and 1:100 for T while for P and E2 dilutions were 1:50 to 1:100 and 1:2 to 1:10, respectively.

Statistical Analysis

Statistical analyses of gene expression profiles were conducted using SYSTAT (SYSTAT Software Inc., Point Richmond, CA, USA). Differences in gene expression were evaluated by ANOVA followed by Tukey's Test. Differences with $p<0.05$ were considered significant.

RESULTS

Chemical Dose and Time Courses

Results from the time course study indicated that gene expression profiles could be grouped into three general categories. These categories were based on gene expression levels measured in H295R cells exposed to a range of forskolin concentrations for either 24 or 48 h (Figure 1). The genes were grouped as follows:

Group I Genes (CYP21, CYP19, 3β-HSD2, and CYP11β2). At 24 h, the dose-response curve for Group I genes was characterized by a relatively great increase in gene expression from the solvent control levels to 10 μM that was followed by a 'plateau' in expression from 10 to 50 μM. At 48h, the pattern in gene expression was similar to that observed at 24 h except that gene expression was approximately 2 to 3-fold greater than that observed at similar concentrations at 24 h. Overall, the genes in this group showed the greatest levels of induction with gene expression levels commonly being 10-fold in excess of that observed in solvent controls.

Group II Genes (CYP17 and CYP11A). At 24 h, gene expression was characterized by rapid increase that reached a maximal level between 5 to 10 μM forskolin. At

concentrations greater than 10 μM forskolin, gene expression increased but to a lesser degree indicating that maximal expression levels may have not yet been reached. In the 48 h exposure, genes were characterized by an increase in expression up to approximately 10 μM forskolin followed by a 'plateau' up to 50 μM. However, unlike that observed at 24h, gene expression did not significantly differ between 10 and 50 μM indicating that these genes may have reached a maximal expression level. Finally, while the shape of the gene expression profile for these genes was similar to that observed with Group I genes, the induction of these genes was not as pronounced and was generally in excess of 3 fold but no greater than 10-fold.

Group III genes (StAR, 17β -HSD1 and 17β -HSD4). Unlike the profiles observed for Group I and II genes, the expression profiles at 24 and 48 h in the Group III genes differed considerably. At 24 h, the dose-response curve was characterized by relatively great increase in gene expression in cells exposed up to 3 μM forskolin. This was followed by a large decrease in expression at 10 μM. Levels of gene expression at 10 μM were similar to that observed in the solvent controls. However, this decrease was followed by a slight increase in activity at concentrations up to 50 μM. In contrast, in cells exposed for 48 h, gene expression increased sharply from control levels up to 10 μM forskolin that was followed by less than a 1.5-fold increase in activity in the 50 μM exposure. Overall, the level of gene expression observed at 10 and 50 μM at 48 h was similar to that observed at the 3 μM forskolin dose in cells exposed to 24 h.

Alterations in expression of HMGR did not appear to be similar to any of the above categories. The HMGR gene expression profile at 24 h was characterized by a 2-

fold increase in gene expression up to 3 μM that was followed by approximately a 4-fold decrease in expression at 10 μM and 50 μM. The gene expression profile at 48 h differed from that observed at 24 h in that gene expression was suppressed from control levels at all doses with the greatest suppression being observed in cells exposed to 50 μM. These steroidogenic genes were also categorized by the time of induction with Group I genes being capable of further induction relatively 'late' (48 h) in the exposure. In contrast, HMGR along with the Group II and III genes appeared to be induced by low concentrations of forskolin to a greater degree at 24 h than higher doses at 48 h, unlike the response observed for the Group I genes. The observed results were in good agreement with those published previously (Hilscherova et al, 2004) demonstrating the general robustness of the assay procedure. While there were slight differences in gene expression alterations between the two studies for Group II and Group III these differences were generally less than 2-fold between the two experiments. The most profound differences between the current study and Hilscherova's study were observed for Group I genes. In the current study, the expression levels of CYP11β2, CYP19, and CYP21 ranged from 3-fold less to 1.5-fold greater than the results observed by Hilscherova et al. for cells exposed under similar conditions. These results most likely represent differences in culture and exposure conditions.

The dose-response curves for forskolin at 24 h were tri-modal for all of the genes studied (Figure 2.1). This is particularly evident for HMGR, STAR, 17β-HSD1 and 17β-HSD4. For these genes, induction of expression was greatest at 1 and 3 μM forskolin and

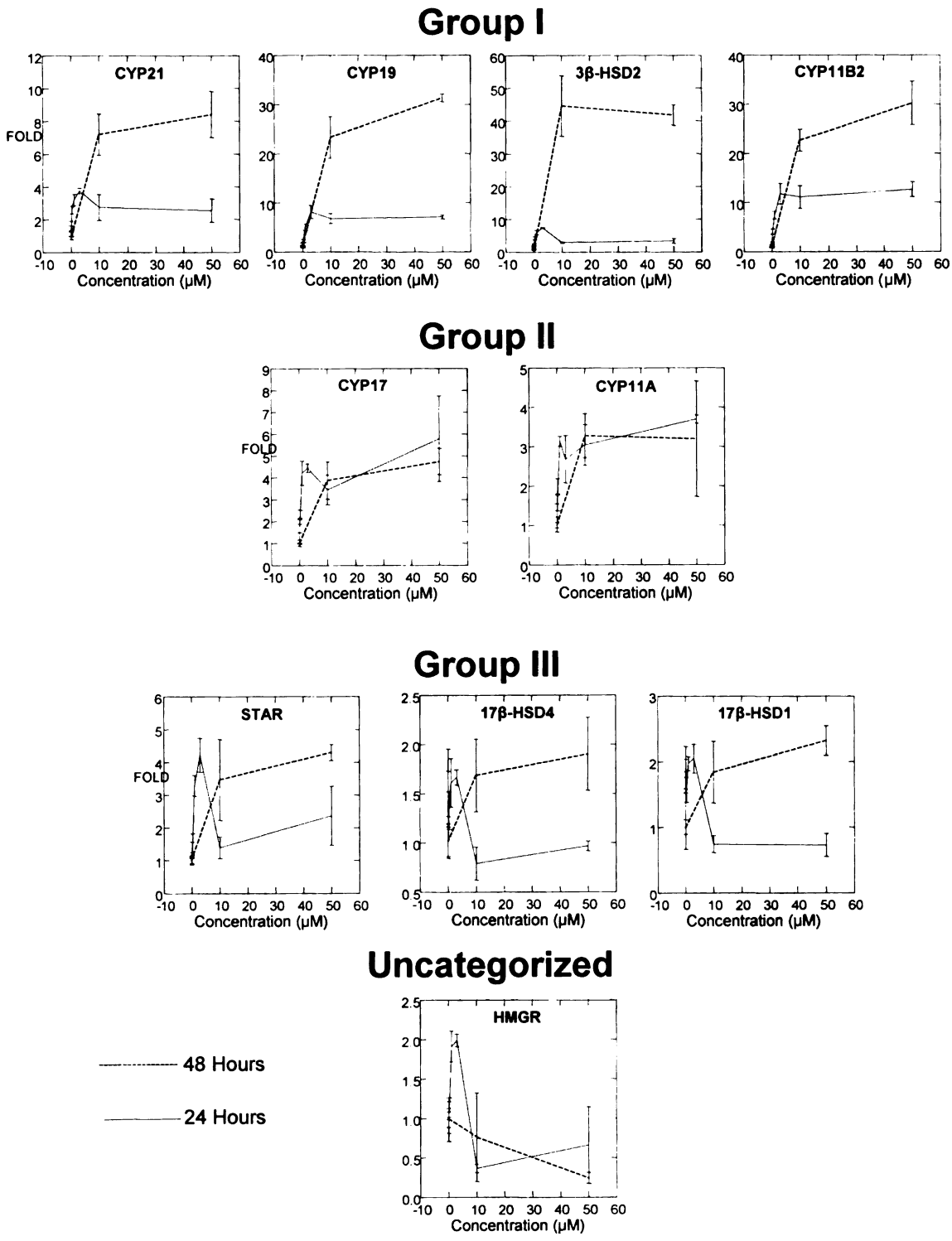

Figure 2.1 Alterations in expression of steroidogenic genes in H295R cells exposed to a range of concentrations of forskolin for 24 or 48 h.

was decreased markedly at 10 μM. While there was a moderate increase in expression between 10 and 50 μM, the expression at these greater concentrations never reached the levels attained at 3 μM. In contrast, nearly all the gene expression measures at 48h were unimodal and consistent, either increases or decreases, over the exposure range. The complex nature of the dose-response curve clearly demonstrates the complex, multiply regulated nature of the steroidogenesis pathway.

Patterns of Gene Response to Chemical Mixtures

Exposure of H295R cells to 10 μM forskolin for 24 h resulted in statistically significant (2-fold or greater) increases in the expression of the CYP17, CYP19, 17β-HSD4, CYP11A, StAR and CYP11β2 genes as compared to control levels (Table 2). No significant changes were observed in the expression of CYP21, 17β-HSD1, 3β-HSD2 or HMGR genes when compared to control gene expression levels. Furthermore exposure to forskolin was not associated with any decrease in the expression of the targeted genes indicating that this chemical was a general inducer of steroidogenic genes in H295R cells. As a result, all comparisons in the binary mixture studies were made relative to forskolin while changes in gene expression with single chemicals were evaluated relative to solvent controls.

Metyrapone did not alter the forskolin-induced expression of CYP19, 17β-HSD4, StAR or CYP11β2 in the mixture (Table 2.2). However, there was approximately a 2-fold decrease in forskolin-induced gene expression of CYP17 and CYP11A that was accompanied by an 11-fold decrease in 3β-HSD2 gene expression. The reduction of 3β-

Table 2.2 Gene expression in H295R cells exposed to single chemicals and to binary mixtures. [a]

Treatment [b]	CYP17	CYP19	CYP21	17βHSD1	17βHSD4	CYP11A	StAR	3βHSD2	HMGR	CYP11B2
Solvent Control	1.03	1.12	1.01	1.34	1.05	1.00	1.01	1.01	1.00	1.09
	(0.29)	(0.69)	(0.15)	(0.19)	(0.42)	(0.11)	(0.14)	(0.20)	(0.06)	(0.48)
Forskolin	3.10*	4.64*	1.36	1.00	4.03*	3.53*	1.98*	1.48	1.34	6.60*
	(0.64)	(0.80)	(0.25)	(0.06)	(1.50)	(1.45)	(0.45)	(0.20)	(0.19)	(1.87)
Forskolin + metyrapone	1.59*	4.56	1.13	0.77*	4.96	1.77*	1.63	0.13*	1.32	6.45
	(0.21)	(0.89)	(0.10)	(0.22)	(0.64)	(0.46)	(0.47)	(0.04)	(0.23)	(2.12)
Forskolin + aminoglutethimide	2.00	0.35*	1.39	0.45*	6.81*	1.80*	1.77	1.33	1.23	5.38
	(0.51)	(0.02)	(0.59)	(0.16)	(0.52)	(0.25)	(0.36)	(0.30)	(0.21)	(1.16)
Forskolin + ketoconazole	1.24*	4.32	0.61*	1.24	6.53*	1.62	2.59	0.38*	0.41*	37.0*
	(0.23)	(0.86)	(0.09)	(0.09)	(0.72)	(0.38)	(0.21)	(0.07)	(0.11)	(4.03)
Metyrapone	0.81	1.62	1.11	1.70*	1.15	0.83	1.05	0.48*	1.01	1.65
	(0.01)	(0.35)	(0.35)	(0.12)	(0.100	(0.03)	(0.42)	(0.18)	(0.06)	(0.42)
Aminoglutethimide	0.78*	1.21	0.89	1.41	0.41*	0.84	0.29*	0.92	0.97	0.87
	(0.08)	(0.12)	(0.19)	(0.23)	(0.44)	(0.10)	(0.07)	(0.30)	(0.26)	(0.19)
Ketoconzole	0.33*	1.00	0.75*	1.04	5.62*	0.81	1.18	0.56*	0.45*	4.79*
	(0.06)	(0.19)	(0.19)	(0.30)	(0.68)	(0.16)	(0.34)	(0.14)	(0.16)	(0.80)

All exposures were conducted for 24 h under standard conditions. All gene expression values for fold change relative to control given as means and standard deviations.
[b] Concentrations of single chemicals and mixtures exposures were: Forskolin (10 μM), Metyrapone (300 μM), aminoglutethimide (300 μM), ketoconazole (20 μM).
* Indicates statistically significant differences at $p < 0.05$. For individual treatments, comparisons made to solvent control. For mixtures, comparisons made to forskolin.

HSD2 is interesting in that forskolin alone induced 3β-HSD2 gene expression by about 1.5 fold whereas metyrapone decreased expression of this gene by approximately 2-fold when compared to control levels. However, the mixture resulted in 7.7-fold decrease in gene expression when compared to solvent control levels. All other metyrapone- forskolin mixture related changes in forskolin altered gene expression were less than 1.5-fold.

Aminoglutethimide did not significantly alter the forskolin-induced gene expression of StAR, CYP17, or CYP11B2 in the mixture exposure (Table 2). However, there was approximately a 2-fold reduction in the forskolin-induced expression of the CYP11A and 17β-HSD1 genes in cells exposed to the mixture. The greatest effect in the mixture experiment was observed for CYP19. The expression of this gene was reduced approximately 13- fold less than that observed in cells treated with forskolin alone. Ketoconazole did not alter the forskolin induced gene expression of CYP19, 17β- HSD1 or StAR while it reduced by approximately 2-fold the expression of CYP17 and CYP11A (Table 2). In addition, while forskolin itself did not alter the expression of HMGR, CYP21 and 3β-HSD2, the forskolin-ketoconazole mixture significantly decreased the expression levels of these genes when compared to controls. The reductions in the expression of these genes were similar to those observed in cells exposed to ketoconazole indicating there was no interaction but that the effects were being moderated only by ketoconazole. Forskolin and ketoconazole caused 6.6-fold and 4.8- fold increases in CYP11β2 expression respectively. However, a binary mixture of these two chemicals resulted in a much greater increase in expression (37-fold) than would have been predicted from

exposures to the individual chemicals. This super-induction was not observed for any of the other genes in that most other changes in gene expression were typically less than 3-fold. We hypothesize that this super-induction was due to the combined effects of increased CYP11A activity induced by forskolin and inhibition of CYP17 and CYP21 (Figure 2.2). Increases in CYP11A activity caused by forskolin would increase the flux towards and production of pregnenolone (Cauet et al 2001). At the same time the inhibition of CYP17 and CYP21 would prevent the conversion of pregnenolone to products other than progesterone (Hu et al 2001; Hu et al 2002). This should lead to an increase in the flux of metabolites to progesterone. The super-induced enzyme, CYP11β2 is responsible for the subsequent metabolism of progesterone such that under these experimental conditions, the large increase in expression of this enzyme could be reasonably expected. Several other enzymes also metabolize progesterone but their expression was not measured in this study. In addition at least one of these enzymes, 17- hydroxylase (EC 1.14.99.9) has previously been reported to be inhibited by ketoconazole (DiMattina et al, 1988). The interactive effects of the chemicals in this situation are also understandable since increased metabolism due to CYP11A induction by forskolin alone would not result in a great increase in CYP11β2 metabolism of progesterone. This is because the increased flux would be dispersed to other parts of the synthetic pathway by CYP17 and CYP21 activities. Also in the absence of increased CYP11A activity the inhibition of CYP17 and CYP21 would not necessarily lead to accumulation of progesterone and subsequent induction of CYP11B2.

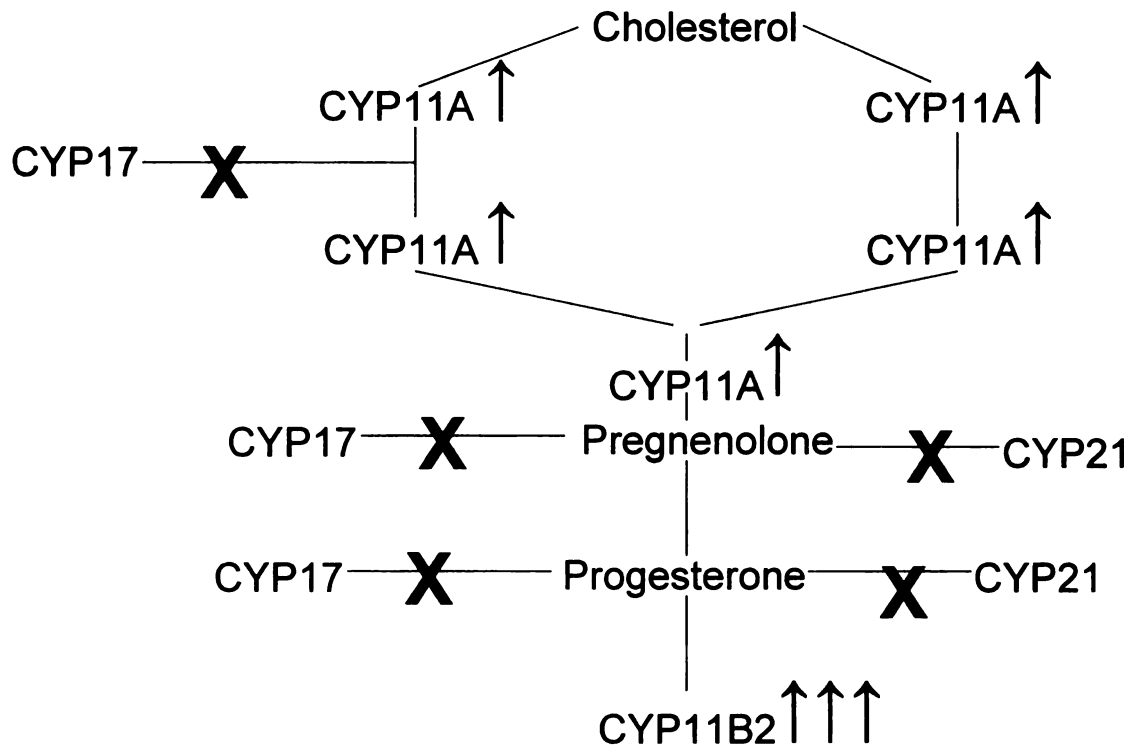

Figure 2.2 Potential mechanism for super-induction of CYP11B2 by a mixture of forskolin and ketoconazole. Arrows indicate increased gene expression crosses represent decreased gene expression.

Hormone production

Medium from the solvent controls and most of the chemical treatments contained measurable concentrations of P, T and E2 (Table 2.3). The average concentrations of E2, T and P in the solvent controls were 14.2 pg/ml, 3,845 pg/ml, and 13,948 pg/ml, respectively. Coefficients of variation for E2, T and P were 0.8, 3.4 and 49% respectively.

In H295R cells treated with forskolin, the production of P, T and E2 was increased from control levels by approximately 2.5-, 1.7- and 21-fold, respectively (Table 2.3). Thus, while forskolin increased all three hormone concentrations, the production of E2 preferentially increased when compared to the other two hormones. In contrast, treatment of H295R cells with aminoglutethimide and ketoconazole resulted in decreased production of all three hormones compared to solvent controls. In cells treated with aminoglutethimide, the production of E2 and P were decreased to below their assay detection limits (7.8 and 440 pg/ml respectively) while T was reduced 3-fold when compared to the solvent control. Treatment with ketoconazole resulted in approximately a 7-fold reduction in P, a 10-fold reduction in T and a 1.1-fold reduction in E2 compared to control levels.

In the forskolin-aminoglutethimide binary mixture, the concentration of P and E2 in the medium was reduced by approximately 50-fold and greater than 40-fold, respectively, from that measured in cells exposed to forskolin alone. In contrast, T concentrations were only reduced approximately 6-fold from forskolin-induced levels. However, when compared to solvent control, there were 20-fold and 3.5-fold reductions in P and T concentrations while E2 concentrations were only 2-fold less

than control. In the forskolin-ketoconazole mixture, there was approximately a 40-fold reduction of T concentrations from that observed in the forskolin alone experiment. However the reductions in P and E2 concentrations were only 5-fold and 1.5-fold, respectively, that observed with forskolin alone. A comparison of the mixture hormone data to the solvent control had a slightly different pattern in that P and T concentrations were reduced from control levels by 2-fold and 25-fold respectively.

Table 2.3 Hormone concentrations in media from H295R cells treated with single chemicals or in binary mixtures.[a]

	Progesterone	Testosterone	Estradiol
Treatment [b]	(pg/ml)	(pg/ml)	(pg/ml)
Solvent control	13948 ± 6907	3845 ± 129	14.2 ± 0.109
Forskolin (FOR)	35542 ± 6006	6513 ± 151*	303 ± 4.55*
Aminoglutethimide (AMG)	<440 [c]	440 ± 165*	< 7.3[c]
Ketoconazole (KETO)	1949 ± 116*	374 ± 3.81*	12.6 ± 1.88*
Metyrapone (MET)	na	na	na
Forskolin+AMG	693 ± 129*	1112 ± 190*	< 7.3 [c]
Forskolin+KETO	7429 ± 105*	149 ± 48.5*	207 ± 62.3
Forskolin+ MET	5902 ± 892*	508 ± 105*	< 7.3 [c]

[a] H295R cells exposed to either forskolin (10 μM); aminoglutethimide (300 μM); metyrapone (300 μM); ketoconazole (20 μM). Binary mixtures had the same chemical concentrations. All exposures were 24h.

[b] Statistical comparisons for individual chemicals were to the solvent control. Binary mixtures were compared to forskolin alone.

[c] Indicates that hormone concentrations were less than the assay detection limit

na is not analyzed

* Indicates a Statistically significant difference ($p < 0.05$ in a two-tailed test).

These reductions represent approximately a 50% change from that observed in the mixture. In contrast, E2 concentrations were more than 15-fold greater than observed in the solvent control confirming the observation that ketoconazole did not greatly affect E2 concentrations. In the forskolin-metyrapone exposure, concentrations of P and T were reduced approximately 6- and 13-fold from the forskolin alone while E2 was reduced by approximately 80-fold.

DISCUSSION

Previous studies have demonstrated the utility of the H295R assay system as a rapid, sensitive and predictive in vitro system to assess the potential effects of chemicals on steroidogenesis (Hilscherova et al 2004; Zhang et al 2005). However, to more fully interpret the results obtained from this system it was necessary to develop a more detailed understanding of the effects of exposure concentration and time on the results for model compounds. Additionally, to be of use in real world scenarios the response of the system to chemical mixtures needs to be understood. Finally, the results of alterations on gene expression can now be related to alterations in actual steroidogenic function as determined by rates of synthesis and release of hormones to the culture medium.

Dose and time dependent patterns

The results of the time course experiments for the forskolin exposure demonstrated the coordinated expression of distinct groups of genes based on the shape and time dependence of the dose-response curve. The ability to group genes based on

chemical- induced alterations in expression suggests a mechanistic linkage in the regulation of these genes. When a group of chemicals alter the same set of genes it is possible to establish the general mechanism by which they disrupt steroid production; furthermore, based on their chemical structures, response profiles may be established and used to predict the effects of other chemicals with similar chemical structure and unknown mechanism of action. Thus, the genes that exhibited the greatest change in transcription, those classified in Group I, are the genes coding for the enzymes involved directly in the production of the final steroid products such as aldosterone (CYP11β2) and E2 (CYP19). These downstream genes exhibited the greatest increase in expression when compared to the other genes studied in the 24 and 48 h exposure to forskolin. Group I also included genes involved in the production of key steroidogenic substrates such as 11-deoxycortisol, an important precursor in the production of glucocorticoids that is regulated by CYP21; and androstenedione, an indispensable substrate for the formation of sex steroids by 3β - HSD2; this gene, 3β-HSD2, is also involved in the production of progesterone, a key steroid end-product as well as an important substrate in the formation of glucocorticoids. As observed in earlier studies, forskolin has relatively little impact on the expression of 17β-HSD1 and 17β-HSD4. These two genes code for two of the ten types of mammalian 17β-hydroxysteroid dehydrogenases (17β-HSDs) (Baker, 2001). These enzymes have the crucial role of controlling the last step in the formation of the essential active estrogens and androgens as well as the role of inactivating these potent sex steroids to produce compounds with little or no biological activity. Although the ten 17β-HSDs belong to the same protein super-family, their amino acid sequences and structures

demonstrate relatively low degrees of identity. In addition, each protein demonstrates distinct tissue-specific expression, substrate specificity, regulatory mechanisms and catalytic activity. In particular, human 17β -HSD type 1 and 4 have distinctly opposite functions. While 17β-HSD1 catalyzes the formation of 17β-estradiol from estrone, 17β-HSD4 functions mainly in the conversion of 17β-estradiol to estrone and androst-5-ene-3β, 17β diol (Labrie et al, 1997), in the current study the alteration of expression of these two genes by forskolin was minimal. The greatest induction observed relative to control was approximately 2-fold at 48 h for both, 17β–HSD1 and 17β-HSD4.

The relationship observed between HMGR and the other genes evaluated in this study was also of interest. At 24 h, the HMGR expression in cells exposed to up to 3 μM forskolin increased rapidly to approximately 2-fold control levels but then decreased by approximately 2.5 to 3-fold from maximal levels at forskolin concentrations equal to or greater than 10 μM. This increase in HMGR gene expression was accompanied by increases in most other genes and was most evident for StAR and 17β-HSD4. In contrast, at 48 h there was a linear reduction in HMGR expression over the entire dose range (Figure 2.3). This differed from that observed for the other genes evaluated in the study in that there was general increase in their expression from control levels over the exposure range. In animal models, HMGR is the rate-limiting step in cholesterol biosynthesis that is subject to complex regulatory controls (Goldstein and Brown 1990). However, while it is the primary mechanism for controlling cholesterol biosynthesis, other mechanisms are also involved in these processes (Kojima et al 2004). Furthermore, the long term control of HMGR

enzymatic activity and its gene expression is influenced by the presence of cholesterol in the cell where high cholesterol concentrations reduce HMGR expression levels while reduced levels of cholesterol can activate gene expression. However, other non-sterol regulatory mechanisms may also be important in the control HMGR activity.

In figure 2.1, picturing the forskolin dose response analysis, it can be observed that most of the genes reach the maximum expression when exposed to concentration of 10μM during 48h. Based on these results, this concentration and time of exposure were chosen to evaluate the effects of other chemicals on the maximum effects observed by forskolin.

Overall, depending on the mode of action, time can be an important factor in evaluating the effect of chemicals or groups of chemicals on steroidogenic enzymes and genes.

Chemical Treatments

The experiments with individual chemicals and simple binary mixtures in this study clearly indicate the existence of a variety of control mechanisms regulating the expression of these genes that result in responses that would not be easily predicted from the results of studies with the individual chemicals (Table 2.4). However, because of the limitations of the experimental design the occurrence of antagonism, additivity and super-additivity can only be suggested. A most complete study design, including a quantitative definition of summation and individual dose-effect relationships for model chemical 1, model chemical 2 and their mixture (at known

ratio of 1 and 2) is required in order to establish any type of interaction between these chemicals. Here it can only be concluded that for the binary mixtures cumulative effects and possible antagonist behavior were observed.

Aminoglutethimide (AMG), a drug also known as Cytraden, is used as an aromatase inhibitor in patients with breast cancer and adrenal anomalies. In our study, treatment of H295R cells with 300 μM AMG resulted in the inhibition of expression of CYP17, StAR and 17β-HSD4 while no effect on the expression of CYP19 was observed. While this would suggest that this dose of AMG would not adversely affect the production of E2 due to inhibition of degradation to estrone as a consequence of decreased expression of 17β-HSD4 (Figure 2.3), it is important to note that this concentration is relatively high and that at lower concentrations E2 could be decreased due to an inhibition of aromatase (Hecker et al 2005). On the other hand, when H295R cells are exposed to a mixture of forskolin and AMG, CYP19 is inhibited but 17β-HSD4 is up-regulated possibly to compensate for the lack of E2 for aromatase inhibition. The inhibition of aromatase is in agreement with the results of other studies (Bastisda et al 2001), in which the inhibitory action of AMG on protein kinase A was demonstrated. Recently, several aromatase promoter regions have been identified in H295R cells that were shown to be responsive to different stimuli and have implications relative to the regulation of aromatase activities (Heneweer et al 2004). Thus, chemicals or chemical mixtures may have the potential to act on different promoter regions through alterations in second messenger systems such as PKA, PKC or Jak/STAT such that aromatase gene

Table 2.4 Effects and interactions of single and mixture chemical exposures on steroidogenic genes in H295R cell line and hormone production. [a]

Gene	FORS [b]	METY [b]	FORS+METY [c]	AMG [b]	FORS+AMG [c]	KETO [b]	FORS+KETO [c]
CYP11A	↑	-	↑	-	↑	-	↓
CYP17	↑	-	↑	↓	↑	↓	↓
3βHSD2	-	↓	↓	-	-	↓	↓
CYP21	-	-	-	-	-	↓	↓
CYP11B2	↑↑	-	↑↑	-	↑↑	↑↑	↑↑↑↑[e]
CYP19	↑↑	-	↑[d]	-	↓↓[d]	↓	-[d]
17βHSD1	-	↑	↓[d]	-	↓[d]	-	-
17βHSD4	↑	-	↑↑[f]	↓	↑	↑	↑
STAR	↑	-	-[d]	↓	-	-	-
HMGR	-	-	-	-	-	↓	↓
Hormone	**FORS**	**METY**	**FORS+METY**	**AMG**	**FORS+AMG**	**KETO**	**FORS+KETO**
Testosterone	↑	↓↓↓	**na**	↓↓	↓↓[d]	↓↓↓	↓↓↓↓[+d]
Progesterone	-	↓↓	**na**	< MDL	↓↓↓↓[+d]	↓↓	↓↓[d]
Estradiol	↑↑↑↑	↓↓↓	**na**	< MDL	↓↓↓↓[+d]	-	-[d]

[a] Chemicals were Forskolin (FORS), metyrapone (METY), aminoglutethimide (AMG), and ketoconazole (KETO).

[b] Gene expression and hormone production comparisons for single chemicals made to solvent control. [c] Gene expression and hormone production comparisons for mixtures made to forskolin alone. [d] Suggested Antagonism.

[e] Suggested Super-Additivity. na Not analyzed. MDL Minimum Detection Limit. [+] More than 40 fold.

↑ = Up-regulation, ↓ = Down-regulation, ↑ or ↓ = 2 fold or more/significant difference; ↑↑or↓↓ = 5 fold or more, ↑↑↑ or ↓↓↓= 10 fold or more, ↑↑↑↑or↓↓↓↓ = 15 fold or more

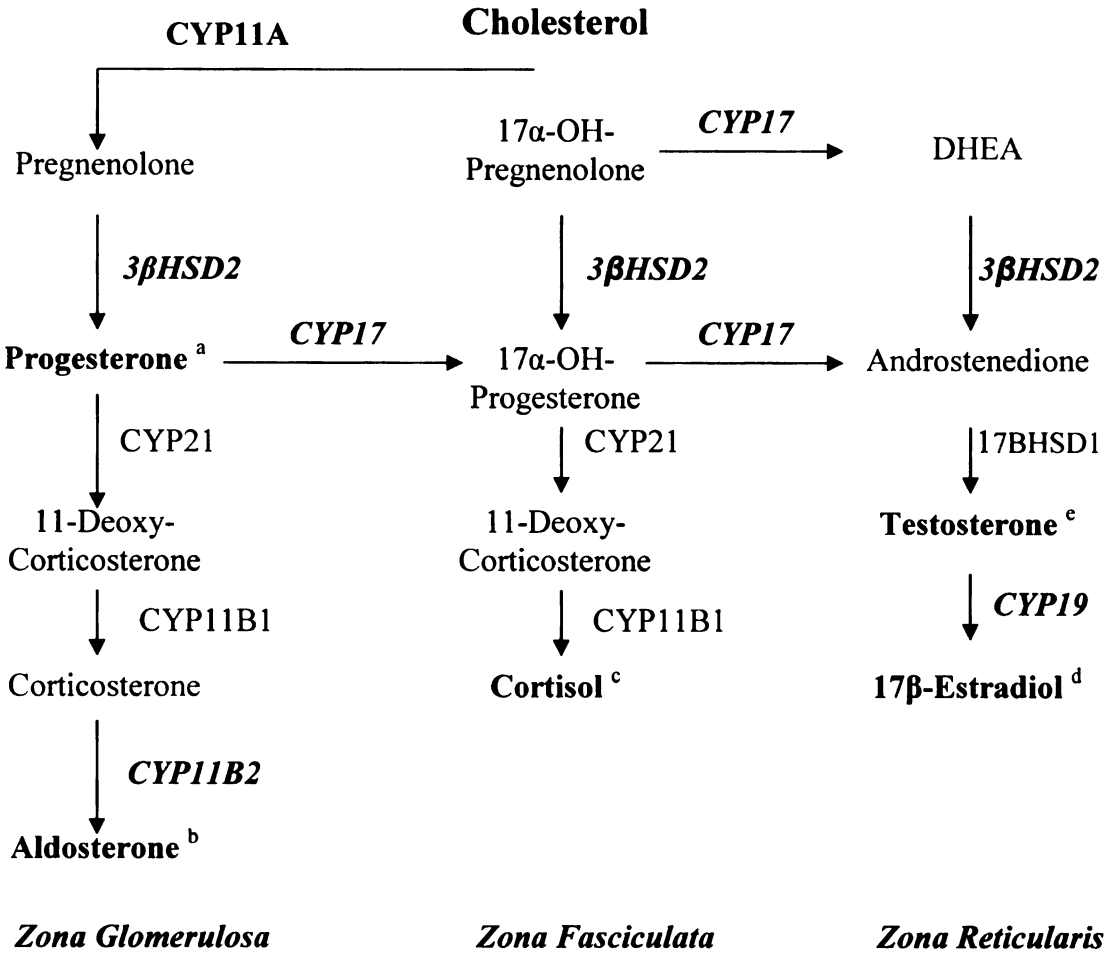

Figure 2.3 Principal pathways in steroid biosynthesis where (a) major progestagen, (b) major mineralo-corticoid, (c) major gluco-corticoid, (d) major estrogen and (e) major androgen.

expression or activities are changed in a manner not predicted based on single chemical exposures. As a result, the mechanism of forskolin-mediated alterations in gene expression may be the action that makes AMG such a potent steroidogenic inhibitor rather than the blockade of the steroidogenesis pathway.

The forskolin related induction of CYP17 and CYP11A gene expression was reduced to almost the same extent by all chemicals; only AMG did not significantly affect the forskolin induced expression of 3β-HSD2 whereas the other chemicals reduced the expression of this enzyme to well below the level of expression seen in either the forskolin only treatment or that of the solvent control. Finally, while forskolin and ketoconazole both caused moderate increases (4 to 6-fold) in the expression of CYP11β2, a binary mixture of these two compounds resulted in a greater increase in expression (37-fold) than would have been predicted from exposures to the individual chemicals. It can be speculated that the reason for this notorious up-regulation may be explained by the interaction of the two known modes of action of the individual chemicals and the fact that these two modes of action come to a critical conjunction which forces the metabolic pathway toward specific products. In contrast, the other chemical mixtures tested had little to no effect on the forskolin-induced expression of CYP11β2 since they did not have modes of action which caused the specific alterations in the metabolic network.

Exposure of H295R cells to forskolin resulted in significant increases in T and E2 production. Although greater than those for E2 and T, differences in P concentrations were not statistically significant, which is likely due to the relatively high variability of control P concentrations. The super production of E2 was most

likely directly related to an increase in the expression of the CYP19 gene, and a subsequent increase in aromatase enzyme concentrations. In addition, the up-regulation of the CYP17 gene expression by forskolin treatment may shift the steroidogenic process to the production of androgenic substrates leading to the formation of testosterone and E2 (Cobb et al 1996).

AMG treatment significantly decreased the production of all three hormones and in particular P and E2 where concentrations were reduced to less than their assay detection limit (Table 2.3). This result may be related to the decrease in CYP17 gene expression that could potentially result in a decrease in the production of androgenic substrates required for the formation of these hormones, but mostly, AMG is an endocrine antihormone that blocks adrenal steroidogenesis by inhibiting the enzymatic conversion of cholesterol to pregnenolone and also blocks the aromatization of androgenic precursors to estrogens by inhibiting aromatase activity. Therefore, it could be speculated that AMG decreased P and E2 production by a mechanism other than gene expression, since 3β-HSD2 and CYP19 gene expression were not affected by this chemical treatment. Because the versatility of the H295R cell line for the evaluation of aromatase activity have been shown before (Sanderson et al 2000, 2001), several experiments are being conducted in our laboratories to evaluate the effects of AMG in the activity of this enzyme in order to search for a more detailed explanation related to the E2 production. However, since all three hormones were greatly decreased by AMG the reduction was most likely due to general stress rather than a specific action of this chemical within the steroidogenic pathway. The powerful antagonism of AMG to forskolin is clearly observed when E2 concentrations dramatically decreased in the

forskolin-AMG treatment compared to the greatest induction by forskolin. In addition, the ten-fold induction in T production by forskolin treatment matches the ten-fold decrement in T production observed in the forskolin-AMG exposure. The ketoconazole-forskolin treatment produced the same effects as those observed with either chemical singly on 17β-HSD1 gene expression resulting in no significant change in E2 concentrations. However, exposure to the mixture resulted in significant decreases in the production of T and P. The decrease in P may have been linked to the down regulation of 3β-HSD2 since no effects were noted on the expression of CYP17 as compared to the solvent controls. Furthermore, the massive increase in CYP11B2 expression could have resulted in an increased synthesis of aldosterone, thus, resulting in the depletion of further upstream precursors including P. A possible explanation for the decrease in T could be a result of increased CYP19 expression in combination with the reduction of precursors such as P, with the lack of a concomitant increase of E2 due to the shift of the E2/estrone balance towards estrone due to increasing 17β-HSD4 gene expression. However, it is difficult to link changes in hormone production with changes in steroidogenic gene expression without the knowledge on how these translate into effects at the enzyme activity levels, and thus, the exact causes for the observed alterations in hormone concentrations remain unclear.

The results from this study underscore the utility of the H295R cell system to investigate the interactions of chemicals on steroidogenic gene expression and hormone production. However, to better understand these interactions it will be necessary to evaluate other endpoints such as steroidogenic enzyme activities, which link the alterations in gene expression to biologically important processes that are controlled by

endocrine systems. Given that environmental exposure to chemical contaminants is almost always in the form of mixtures the use of a system such as the H295R assay is a powerful tool to investigate the effects of single compounds and complex mixtures of xenobiotics, and to investigate the molecular mechanisms of those effects and the molecular mechanisms of chemical interaction.

This study and our previous work (Hilscherova et al 2004) have demonstrated the ability of the H295R assay system to evaluate the effects of xenobiotics on steroidogenesis. By observing the effects of chemical exposure on 10 different steroidogenic endpoints (i.e. the expression of 10 different genes) the assay system has revealed that in general even 'specific' inhibitors affect the expression of multiple genes. This is not surprising given the complex regulatory mechanisms controlling steroidogenesis, but clearly demonstrates that designating any chemical as a "specific inhibitor or inducer" is unwise. However, the responses observed clearly reflect the known mode of action of the various compounds. In the present study the complex non-additive responses observed as a result of exposure to some chemical mixtures can be explained by mechanistic interactions of the known modes of action of the specific chemicals. The additional complexity observed in many of the responses required considerably more effort to be put into interpretation of the gene expression profiles, thus hormone production was also evaluated to observe any correlation to gene expression. The H295R assay together with hormone quantification is a useful in vitro system to investigate regulatory and chemical interaction mechanisms as well as providing a system for screening chemicals for effects on steroidogenesis.

CONCLUSIONS

The H295R assay system is unique among bioassays in that it measures alterations in gene expression and hormone production at the same time. This dual response is particularly significant for chemicals that are able to alter the production of steroid hormones since the ultimate effects of these chemicals are expressed thru the alterations in hormone concentrations in exposed organisms. The results of the H295R assay to date have demonstrated that chemicals maybe grouped alternatively by effects on gene expression or by effects on hormone production. These results clearly show that the chemicals tested have a range of modes of action. Significantly, there are clear examples of interaction between modes of action leading to supra-additive effects. This finding is clearly of significance given that environmental contaminants are most commonly found in complex mixtures. The H295R system is an effective tool for understanding potential mechanisms of action as well as a rapid, sensitive and cost-effective tool for high throughput screening of a range of potential effects of compounds. Furthermore, the H295R system is attractive because it minimizes the use of whole organisms.

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Chapter 3

MODULATION OF STEROIDOTENIC GENE EXPRESSION AND HORMONE PRODUCTION OF H295R CELLS BY PHARMACEUTICALS AND OTHER ENVIRONMENTALLY ACTIVE COMPOUNDS

ABSTRACT

The recently developed H295R cell bioassay was used to evaluate the potential endocrine disrupting effects of 18 of the pharmaceuticals most commonly used in the United States. Exposures for 48 h with single chemicals and binary mixtures of pharmaceuticals were conducted and the expression of 4 steroidogenic genes, 3βHSD2, CYP11B2, CYP17 and CYP19, was quantified by Q-RT-PCR. Production of the steroid hormones estradiol (E2), Testosterone (T) and Progesterone (P) was also evaluated. Antibiotics were shown to modulate gene expression and hormone production. Amoxicillin up-regulated the expression of CYP11B2 and CYP19 by more than 2-fold and induced estradiol production up to almost 3-fold. Erythromycin not only increased CYP11B2 expression but also increased the production of P and E2 by 3.5- and 2.4-fold, respectively. Production of T was also significantly decreased by erythromycin. The β-blocker, salbutamol, caused the greatest induction of CYP17, 13.64-fold, and significantly decreased E2 production. The binary mixture of cyproterone and salbutamol significantly down-regulated expression of CYP19 while a mixture of ethynylestradiol (EE2) and trenbolone increased E2 production 3.7-fold. Not all the genes measured responded to changes in exposure concentrations. Estradiol

production was significantly affected by changes in concentrations of trenbolone, cyproterone, and EE2.

INTRODUCTION

Based on information obtained from the U.S. Food and Drug Administration (USFDA), approximately 82,000 drugs are registered in the U.S. for human use accounting for more than 3000 active ingredients. Besides the active substances, adjuvants and, in some instances, pigments and dyes are also components of the formulated drug product. After administration to humans and animals, pharmaceuticals are excreted in waste products and many unused medications are disposed of in drains or sewage systems. Sewage treatment facilities, depending on their technology and the chemical's physicochemical properties, are not always effective in removing the active chemicals from waste-water. As a result, pharmaceuticals find their way into the environment, where they can directly affect terrestrial and aquatic organisms and can be incorporated into food chains (Díaz-Cruz et al., 2003; Cecchini and LoPresti, 2006). In recent years, extensive and detailed reports about residues of pharmaceuticals and their metabolites in the environment have been published in the scientific literature (Jorgensen and Halling-Sorensen, 2000; Hereber, 2002; Sanderson et al., 2004; Jones, et al., 2004). Most of these reports concern the situation in Europe, but as yet the potential ecological effects associated with the presence of these compounds have been largely ignored. In Europe, pharmaceuticals in the environment have received regulatory attention through the submission of Environmental Risk Assessments (ERAs) that accompany Marketing

Authorization Approval. Revised draft guidelines for European ERAs were recently reviewed by various stakeholders, and the final guidelines were available in 2004. In Canada, a requirement for environmental assessment is in place and the ERA process is under consideration. Most of the methods used today for the identification and quantification of pharmaceuticals in the environment and the first attempts of eco-toxicity evaluations of these active compounds have been developed in European countries (Commission of the European Communities, 1992). Moreover, the European Union has taken the lead on banning the use of the majority of growth-promoting antibiotics used for livestock on the basis of the "Precautionary Principle" (Casewell, 2003).

Among the frequently detected substances in rivers are beta blockers such as metoprolol at concentrations up to 1.5 μg/l and beta-sympathomimetics (Hirsch et al., 1996; Sedlak et al., 2001), analgesic and anti-inflammatory drugs like diclofenac up to 1.2 μg/l observed in several studies (Ternes, 1998; Stumph et al., 1998; Buser et al., 1998); estrogens such as 17β-estradiol have been found at concentrations up to 13 ng/l (Kuch and Ballschmitter, 2000). In addition, antibiotics such as erythromycin have been reported to occur at concentrations as great as 1.7 μg/l concentrations (Hirsch et al., 1999; Lindsey et al., 2001). Estrogenic compounds have also been identified in rivers of southern and middle Germany (Adler et al., 2001), as well as lipid lowering agents such as the clofibric acid at concentrations as great as 0.2 μg/l (Ollers et al., 2001); and anti-epileptic drugs such as carbamazepine at concentrations as up to 2.1 μg/l (Mohle et al., 1999).

During 1999-2000, the U.S. Geological Survey conducted the first nationwide investigation of the occurrence of pharmaceuticals, hormones and other organic contaminants in 139 streams from 30 states (Kolpin et al., 2002). A total of 95 residues were targeted including antibiotics, prescription, and nonprescription drugs, steroids and hormones, 82 of which were found in at least one sample. Although researchers caution that sites were chosen based on their increased susceptibility to contamination from urban or agricultural activities, a surprising 80% of streams sampled were positive for one or more of the targeted pharmaceutical. Furthermore, 75% of the streams contained two or more of the targeted pharmaceuticals, 54% had more than five, while 34% had more than 10 and 13% tested positive for more than 20 targeted contaminants. Similar reconnaissance studies are ongoing all over the world to evaluate the presence of pharmaceuticals in groundwater and surface water sources of drinking water. Identification of the environmental exposure routes to these drugs is crucial for estimating a realistic environmental assessment of pharmaceuticals because it is the dose of drugs and the duration of treatment that gives a certainty of the environmental loading. The fact that the same drug may be used for several applications and that there are different exposure routes through various environmental matrices, the fate of the drug may also vary resulting in quite different environmental concentrations.

Since they are transformed to some extent in humans, pharmaceuticals are sometimes thought to be easily (bio)degraded in the environment, but it has been established that large proportions of many pharmaceuticals can be excreted from the body un-metabolized and enter wastewater as biologically active substances (Fent et al., 2006; Kummerer, 2001). Thus, human drugs used will be discharged to the sewer

systems in urine and feces and so enter sewage treatment plants (STP) (Loefler et al., 2005; Cleuvers, 2003). Alternatively, for drugs which are metabolized before being released from the body, when exposed to the environment, some can be reactivated to the parent compound (Pickrell, 2002). This has been demonstrated for the glucoronide metabolite of chloramphenicol and the acetylated metabolite of sulphadimidine in samples of liquid manure (Berger et al., 1986). Thus, it is often not only the parent compound which should be the subject for a risk assessment but also the main metabolites. Additionally, drug residues found in the environment, especially in aquatic systems, usually occur as mixtures, not as single contaminants therefore scientific assessment of risk to aquatic life should consider these complex exposure situations.

Since pharmaceuticals are specifically designed to be biologically active they may have unintended effects on non-target organisms in the environment, even at low concentrations. Information concerning possible eco-toxicological risks of pharmaceuticals is rather scarce (van Wezel et al., 2002); unfortunately there is still not only a real lack of information to perform an environmental risk characterization, particularly about data for eco-toxicity, but also a lack of information about the additional effects, other than the original innate function for which the chemical or pharmaceutical was designed and/or produced.

The objective of the present study was to evaluate the potential effects of 18 of the most used human and veterinary pharmaceuticals in the United States (Table 3.1) on steroidogenesis in H295R cells. The effects of antibiotics, growth promoters, hormone

therapy drugs, analgesics, anti-inflammatory medications, anti-lipidics, anti-depressives and β-blockers on the expression of 4 steroidogenic genes encoding for the production of 4 important enzymes in the steroidogenic pathway was evaluated by use of Q-RT-PCR. In addition, the production of the hormones estradiol (E2), testosterone (T), and progesterone (P) were quantified using ELISA methods and related to gene expression. Dose-response curves were also developed to evaluate the effects of chemical concentration on both, gene expression and hormone production.

MATERIALS AND METHODS

Test Chemicals

All chemicals were obtained from Sigma (St. Louis, MO, USA), except for amoxicillin, cephalexin hydrate and erythromycin that were obtained from BioChemiKa (St. Louis, MO, USA). Purity of all test chemicals from Sigma exceeded 98% while chemicals from BioChemiKa exceeded 97%. The chemicals used in this study were selected based on the list of the top 300 prescriptions drugs dispensed in the USA during 2005 (Rx list) and also by their prevalence in surface waters.

Experimental design

The H295R human adrenocortical carcinoma cell line was obtained from the American Type Culture Collection (ATCC # CRL-2128, ATCC, Manassas, VA, USA) and cells were grown in 75 cm^2 flasks with 12.5 mL of supplemented medium at 37°C with a 5% CO_2 atmosphere. Supplemented medium was a 1:1 mixture of Dulbecco's modified Eagle's medium with Ham's F-12 Nutrient mixture with 15 mM HEPES buffer. The

medium was supplemented with 1.2 g/l Na_2CO_3, ITS+ Premix (BD Bioscience, 1 ml Premix/100 ml medium), and 12.5 ml/500ml NuSerum (BD Bioscience, San Jose, CA, USA). Final component concentrations in the medium were: 15 mM HEPES; 6.25 μg/ml insulin; 6.25 μg/ml transferrin; 6.25 ng/mL selenium; 1.25 mg/ml bovine serum albumin; 5.35 μg/ml linoleic acid; and 2.5 % NuSerum. The medium was changed 2-3 times a week and cells were detached from flasks for sub-culturing using trypsin-EDTA (Sterile 1X trypsin-EDTA (Life Technologies Inc.). For exposure, cells were detached from flasks with trypsin/EDTA (Sterile trypsin- EDTA (Life Technologies Inc.) and harvested into a final volume of 10ml of medium. Cell density was determined using a hemacytometer. For dosing, 3 ml of cell suspension containing approximately $1x10^6$ cells/ml were placed in each well. Cells were exposed for 48 h to different groups of pharmaceuticals and several other compounds of relevant environmental importance dissolved in DMSO or methanol using 6-well Tissue Culture Plates (Nalgene Nunc Inc., Rochester, NY, USA). Final concentrations of DMSO and methanol were of 0.1%. H295R cells were exposed for 48 h to individual antibiotics with different spectrums of action (Table 3.1). Amoxicillin, cephalexin, erythromycin, tetracyclines, trimethoprim and tylosin were the antibiotics chosen. A group of drugs used for hormone therapies including cyproterone, dexamethasone, EE2, and trenbolone were also tested. Over the counter drugs, such as the analgesics acetaminophen and ibuprofen were also included. The pharmaceuticals assessed also included other drugs from different therapeutic groups, such as the antidepressant fluoxetine, the antilipidic clofibrate, the β-agonist salbutamol, growth promoters such as trenbolone and zearalanol, and the insect repellant, DEET, which are also frequently found in

Table 3.1. Pharmaceuticals and environmentally active compounds used to expose H295R cells [a,b].

Compound	*Therapeutic Use*	*Conc[a].*
Acetaminophen	Analgesic	300 µg/ml
Clofibrate	Lipid agent	3 µg/ml
Dexamethasone	Corticosteroid	2 µg/ml
Doxycycline	Antibiotic	10 µg/ml
DEET	Pesticide	3 µg/ml
Erythromycin	Antibiotic	3 µg/ml
Ibuprofen	NSAAI	250 µg/ml
Trimethoprim	Antibiotic	3 µg/ml
Tylosin	Antibiotic/growth	3 µg/ml
Compound	***Therapeutic Use***	***Conc[b].***
Amoxicillin	Antibiotic	71 µg/l
Cephalexin	Antibiotic	73 µg/l
Cyproterone	Cancer treatment	62 µg/l
Oxytetracyclin	Antibiotic/growth	81 µg/l
Salbutamol	Asthma/β-agonist	50 ng/l
Trenbolone	Growth prom.	25 µg/l
α-Zearalanol	Hyperestrogen/Nat	2.8 ng/l
Ethynil Estradiol	Oral Contraceptive	1 µg/l
Fluoxetine	Antidepressant	1 µg/l

[a] High concentrations, [b] Environmentally relevant concentrations.

environmental samples. The effects of the target chemicals on gene expression were compared to the effects of exposures to solvent controls at each time interval. Since pharmaceuticals can occur in surface waters as mixtures, a set of 4 binary mixtures of pharmaceuticals were also used as exposure solutions for the H295R cells. The chemicals used in mixture solutions were chosen based on the results of individual exposures. Moreover, dose-response curves were constructed for 3 of the drugs used in hormone therapies to evaluate whether or not changes in gene expression and hormone production were directly related to changes in drug concentration.

Cell viability/Cytotoxicity

Before nucleic acid isolation and hormone analysis, cell viability was determined. Cells were visually inspected under a microscope to evaluate viability and cell number. In addition, to establish the range of chemical concentrations that may be used without producing physical harm to the cells, a Live/Dead cell viability assay kit (Molecular Probes, Eugene, OR, USA) was used. In instances where exposure resulted in cell death or decreased viability the data were not used to evaluate gene expression or hormone production.

RNA Isolation

For nucleic acid extraction, after removal of the medium, cells were lysed in the culture plate, by the addition of 580 µl/well of lysis buffer-β-ME mixture (Stratagene, La Jolla, CA, USA) and RNA was isolated as previously described (Hilscherova et al., 2004). Briefly, lysed cells were mixed and then centrifuged in a pre-filter spin cup and the

mixture centrifuged. The filtrate was diluted with 70% ethanol and vortexed. The mixture was transferred to an RNA spin cup and centrifuged for 1 min. The filtrate was discarded and the spin cup was washed with a low-salt buffer and then centrifuged for 1 min. RNase-Free DNase I solution (Strategene, La Jolla CA, USA) was added to the fiber matrix inside the spin cup and the sample was incubated at 37 °C for 15 min. The sample was then washed with high-salt followed by a low salt buffer. After each wash cycle, the filtrate was discarded. After the final wash, the sample was centrifuged and nuclease-free water was added directly to the fiber matrix inside the spin cup. The tube was incubated for 2 min at room temperature and centrifuged. This elution step was repeated to maximize the yield of RNA. The purified RNA was used immediately or stored at –80 °C until needed. An appropriate dilution of the RNA sample (1:50) was prepared for RNA quantification. The absorbance of the RNA solution was measured at 260 nm and 280 nm and the 260/280 ratio was calculated. The concentration of total RNA was estimated using the A_{260} value and a standard with an A_{260} of 1 that was equivalent to 40 μg RNA/mL.

cDNA Preparation

Total RNA (1-5 μg) was combined with 50 μM oligo-$(dT)_{20}$, 10 mM dNTPs, and diethylpyrocarbamate (DEPC)-treated water to a final volume of 12 μl. RNA and primers were denatured at 65 °C for 5 min and then incubated on ice for 5 min. Reverse transcription was performed using 8 μl of a master mix containing, 5*X* cDNA synthesis buffer (Carlsbad CA, USA) and RNase/DNase free water. Reactions were incubated at 50 °C for 45 min and were terminated by incubation at 85°C for 5 min. Samples were

either used directly for PCR or were stored at –20 °C until analyzed.

Real-time PCR

Real-time PCR (quantitative PCR) was performed by using a Smart Cycler System (Cepheid, Sunnyvale, CA, USA) in 25 µl sterile tubes using a master mix containing 25 mM $MgCl_2$, 1U/µl AmpErase (Applied Biosystems, Foster City, CA, USA), 5 U/µL *Taq* DNA polymerase AmpliTaq Gold, 10*X* SYBR Green (PE Biosystems, Warrington, UK), nuclease free water and between 10 pg and 1 µg of cDNA. The thermal cycling program included an initial denaturing step at 94 °C for 10 min, followed by 25-35 cycles of denaturing (95°C for 15s), primer annealing (at 60-64 °C for 40-60 s), and cDNA extension (72 °C for 30 s); a final extension step at 72 °C for 5-10 min was also included. Melting curve analyses were performed immediately following the final PCR cycle to differentiate between the desired amplicons and any primer-dimers or DNA contaminants. Specifics of the assay parameters such as primers used and annealing temperatures have been published previously (Hilscherova et al., 2004).

For quantification of PCR results C_t (the cycle at which the fluorescence signal is first significantly different from background) was determined for each reaction. C_t values for each gene of interest were normalized to the endogenous control gene, β-actin. Normalized values were used to calculate the degree of induction or inhibition expressed as a "fold difference" compared to normalized control values. Therefore, all data were statistically analyzed as "fold induction" between exposed and control cultures. Gene expression was measured in triplicate for each control or exposed cell culture and each exposure was repeated at least three times.

Hormone Quantification

Hormone extraction and quantification by ELISA were conducted as previously described (Hecker et al., 2006). Briefly, frozen samples of media were thawed on ice, and the hormones were extracted twice with diethyl ether (5 mL) in glass tubes. To determine extraction recoveries a trace amount of ^{3}H-T was added to each sample prior to extraction. The solvent extract was separated from the water phase by centrifugation at 2,000 x g for 10 min and transferred into small glass vials. The solvent was evaporated under a stream of nitrogen, and the residue was dissolved in EIA buffer from Cayman Chemical Company and either immediately measured or frozen at –80°C for later hormone determination. Concentrations of hormones in media were measured by competitive ELISA using Cayman Chemical® hormone EIA kits (Cayman Chemical Company, Ann Arbor, MI, USA; P [P; Cat # 582601], T [T; Cat # 582701], 17ß-estradiol [E2; Cat # 582251]). Because the antibody to P exhibits cross-reactivity with pregnenolone of 61% and the method does not allow for the separation of these two hormones, P concentrations are expressed as P/ pregnenolone. The working ranges of these assays for the determination of steroid hormones in H295R media were determined to be: P: 7.8 - 1000 pg/ml; T: 3.9 - 500 pg/ml; E2 estradiol: 7.8 - 1000 pg/ml. Media extracts were diluted 1:25 and 1:100 for T while for P and, E2 dilutions were 1:50 to 1:100 and 1:2 to 1:10, respectively.

Statistical Analysis

Statistical analyses of gene expression profiles were conducted using SYSTAT (SYSTAT Software Inc., Point Richmond, CA, USA). Differences in gene expression

and hormone production were evaluated by ANOVA followed by Tukey's Test. Differences with $p<0.05$ were considered significant. Statistical correlations between gene expression and hormone production were established by Pearson correlation analysis followed by Bonferroni probability test. Correlations with $p<0.05$ were considered significant.

RESULTS

Antibiotic Exposure

Gene Expression

Responses of gene expression for the exposures conducted with 7 of the most commonly used antibiotics in human medicine and for veterinary purposes are given (Table 3.2). The responses of gene expression for the blank and solvent control exposures were consistent. Treatment of the H295R cells with environmentally relevant or greater concentrations of the selected antibiotics resulted in significant changes in the expression of several genes. H295R cells when exposed to environmentally relevant concentrations of amoxicillin, cephalexin, oxytetracycline and tylosin significantly altered the pattern of expression of the 4 target genes, relative to that of solvent exposed cells. Amoxicillin significantly increased the expression of CYP17 and CYP19 more than 4-fold compared to solvent control, while cephalexin and oxytetracycline significantly increased expression of CYP19 more than 2-fold. Oxytetracycline was also the only antibiotic studied to affect the expression of the progesterogenic gene 3βHSD2. Tylosin increased expression of the aldosteronogenic gene, CY11B2 approximately 10-fold; although tylosin also decreased the expression of the CYP19, this decrement was not statistically

significant. Erythromycin, oxytetracycline and trimethoprim were used at non-relevant environmental concentrations of 3 to 10μg/mL. Oxytetracycline was not soluble in DMSO and so methanol was used to dissolve this compound. A methanol control was also included among the exposures. Trimethoprim did not affect the expression of any of the genes, while erythromycin increased the expression of CYP11B2 approximately 7-fold and doxycycline induced the expression of CY19 almost 3-fold.

Hormone Production

While concentrations of all the hormones measured were very consistent in the Blank, DMSO and methanol exposures (Table 3.2) some of the pharmaceuticals resulted in changes in production of hormones. Erythromycin increased the production of P and E2 more than 2- and 3-fold respectively, and reduced the production of T by more than 50%. In contrast, the tetracyclines did not significantly affect the production of any of the hormones. Tylosin decreased the production of T and E2, while cephalexin only decreased T production and amoxicillin increased production of E2 more than 2-fold.

Hormone Therapy Drugs

Gene Expression

None of the 4 hormone therapy drugs with hormonal properties that are commonly used in the treatment of cancer, birth control, inflammatory processes and as growth promoters in animal production, significantly affected the expression of 3βHSD2 (Table 3.3). Environmentally relevant concentrations of the cancer therapy drug cyproterone induced the expression of the CYP19 more than 4-fold, but only induced the expression of the

androgenic gene CYP17 3-fold. Dexamethasone and EE2 exposures induced expression of CY11B2 approximately 5-fold. The growth promoter trenbolone only increased the expression of CYP19 by about 3-fold.

Hormone Production

Hormone therapy drugs affected hormone production (Table 3.3). EE2 significantly increased P and E2 production by more than 2-fold and at the same time significantly decreased T production by about 66%. Trenbolone and cyproterone decreased T production by approximately 50% and 66% respectively. However, neither chemical affected E2 or P production.

Other Pharmaceuticals and Environmentally Active Compounds

A non steroidal anti-inflammatory drug, analgesics, an antilipidic, antidepressant, β_2-agonist, a growth-promoter and a commonly used insect repellent (Table 3.4), were also included in this study to evaluate their potential effects on steroidogenesis.

Gene Expression

A significant up regulation of CYP17 was observed for the exposure with the β_2-agonist salbutamol, which increased the expression of this gene for more than 10 fold. None of the other genes studied were affected by this compound. Of the analgesics studied, only acetaminophen significantly affected the expression of CYP11B2 by increasing it approximately 4-fold. CYP11B2 was induced by the antilipidic, clofibrate, the antidepressant, fluoxetine, approximately 5-fold and by the analgesic acetaminophen by

Table 3.2 Gene expression and hormone production in H295R cell exposed to single antibiotics.

	Treatment								
Gene	DMSO 0.1%	MeOH 0.1%	AMOXI 71 ug/L	CEPHA 73 ug/L	ERYT 3 ug/mL	OXYTC 81 ug/L	DOXYC 10 ug/mL[a]	TRIME 3 ug/mL	TYLO 3 ug/mL
CYP11B2	1.00 ± 0.33	1.00 ± 0.19	1.95 ± 0.24	0.77 ± 0.33	6.91 ± 0.97*	1.72 ± 0.31	0.69 ± 0.73	0.64 ± 0.05	9.99 ± 1.09*
CYP19	1.00 ± 0.61	1.00 ± 0.16	4.45 ± 0.55*	2.87 ± 1.13*	0.54 ± 0.14	2.58 ± 0.25*	2.87 ± 0.40*	0.58 ± 0.08	0.49 ± 0.33
CYP17	1.00 ± 0.10	1.00 ± 0.16	4.48 ± 0.35*	1.74 ± 0.56	0.75 ± 0.06	1.89 ± 0.071	0.85 ± 0.04	1.90 ± 0.08	1.03 ± 0.04
3BHSD2	1.00 ± 0.13	1.00 ± 0.16	1.42 ± 0.75	0.72 ± 0.52	0.92 ± 0.25	2.51 ± 0.17*	1.00 ± 0.08	0.60 ± 0.36	1.32 ± 0.27

Hormone	DMSO 0.1%	MeOH 0.1%	AMOXI 71 ug/L	CEPHA 73 ug/L	ERYT 3 ug/mL	OXYTC 81 ug/L	DOXYC 10 ug/mL[a]	TYLO 3 ug/mL
Testosterone	1.00 ± 0.27	1.00 ± 0.34	0.71 ± 0.02	0.11 ± 0.03*	0.44 ± 0.08*	1.23 ± 0.007	1.55 ± 0.085	0.42 ± 0.09*
Progesterone	1.00 ± 0.01	1.00 ± 0.13	0.63 ± 0.13	0.89 ± 0.04	3.55 ± 0.58*	1.32 ± 0.55	1.26 ± 0.20	1.30 ± 0.28
Estradiol	1.00 ± 0.32	1.00 ± 0.60	2.5 ± 0.61*	0.51 ± 0.30	2.46 ± 0.33*	0.15 ± 0.05	2.04 ± 0.60	0.12 ± 0.08*

All exposures were conducted for48 h under standard conditions. All gene expressionand hormone productionvalues for fold change relative to control given as means and standard deviations.DMSO: Dimethylsulfoxide; MeOH: Methanol; AMOXIAmoxicillin; ERYT:Erythromycin; OXYTC: Oxytetracycline;DOXY: Doxycycline;TRIME:Trimethoprim;TYLO: Tylosin.

[a]Compared to MeOH as solvent control.* Indicates statistically significant differences at p 0.05.

Table 3.3 Gene expression and hormone production in H295R cells exposed to single hormone therapy drugs.

	Treatment					
Gene	**DMSO** 0.1%	**CYPROT** 62 ug/L	**DEXAM** 2 ug/mL	**EE2** 1 ug/L	**TRENB** 25 ug/L	**ZEARA** 2.8 ng/L
CYP11B2	1.00 ± 0.33	1.34 ± 0.52	5.39 ± 0.50*	4.88 ± 0.97*	0.81 ± 0.43	0.22 ± 0.03
CYP19	1.00 ± 0.61	4.62 ±1.51*	0.98 ± 0.19	0.54 ± 0.14	2.56 ± 0.55*	0.32 ± 0.10
CYP17	1.00 ± 0.10	2.81 ± 0.5*	0.71 ± 0.04	0.75 ± 0.06	1.79 ± 0.41	0.20 ± 0.04
3BHSD2	1.00 ± 0.13	0.90 ± 0.32	0.64 ± 0.09	0.92 ± 0.25	0.77 ± 0.15	0.14 ± 0.03*

Hormone	**DMSO** 0.1%	**CYPROT** 62 ug/L	**DEXAM** 2 ug/mL	**EE2** 1 ug/L	**TRENB** 25 ug/L	**ZEARA** 2.8 ng/L
Testosterone	1.00 ± 0.27	0.29 ± 0.03*	0.25 ± 0.05*	0.36 ± 0.12*	0.48 ± 0.09*	1.06 ± 0.15
Progesterone	1.00 ± 0.01	1.02 ± 0.30	0.74 ± 0.05	2.71 ± 0.39*	0.72 ± 0.20	1.02 ± 0.21
Estradiol	1.00 ± 0.32	1.25 ± 0.47	1.4 ± 0.26	2.33 ± 0.27*	1.6 ± 0.25	0.17 ± 0.03

All exposures were conducted for 48 h under standard conditions. All gene expression and hormone production values for fold change relative to control given as means and sta ndard deviations.
DMSO: Dimethylsulfoxide; CYPROT: Cyproterone; DEXAM:Dexamethasone; E2:Ethynylestradiol; TRENB:Trenbolone Acetate; ZEARA: -Zearalanol
* Indicates statistically significant differences at $p < 0.05$.

approximately 4-fold. The insect repellent, DEET, also increased the expression of this gene for more than 8-fold. Although the natural phytoestrogen α-zearalanol decreased the expression of all the 4 genes evaluated, only the decrease in 3βHSD2 was statistically significant. The non steroidal analgesic anti-inflammatory drug (NSAAID) ibuprofen did not produce any significant changes in the expression of the steroidogenic genes.

Hormone Production

In the group of drugs with different pharmaceutical applications several changes were observed in hormone production. Ibuprofen and fluoxetine did not produce significant changes in the production of any of the hormones analyzed compared to blank and solvent controls. However, T production was decreased by clofibrate and DEET, while E2 production was significantly inhibited by salbutamol and DEET. Also, the analgesic acetaminophen produced a 2 fold increase in P concentrations (Table 3.4).

Pattern of Responses to Chemical Mixtures

Gene Expression

Exposure of H295R cells to 1μg/L of EE2 for 48 h resulted in statistically significant increases in the expression of the CYP11B2 and in the production of P and E2 by approximately 2-fold. The expression of CYP19, CYP17 and 3βHSD2 were not affected by EE2 but T production was decreased by this environmentally active compound. When H295R cells were exposed to binary mixtures where one of the components was EE2 the gene expression responses were very diverse (Table 3.5).

When exposed to trenbolone, the aromatase gene CYP19 was up-regulated approximately 2.5-fold. Exposure to a mixture of trenbolone and EE2 caused a decrease in CYP19 expression of as much as 50% compared to solvent control. Cyproterone up-regulated expression of CYP19 more than 4-fold, but when exposed to a mixture of cyproterone and EE2, expression of this gene was not significantly different from that of the control. Although tylosin reduced CYP19 expression this reduction was not statistically significant when compared to that of cells exposed to the solvent only. The tylosin-EE2 mixture did not produce changes in the expression of this gene compared to controls. Cyproterone significantly up-regulated the expression of the aromatase gene CYP19 up to 4.6-fold and salbutamol did not produce any effects on this gene, but when these two compounds are mixed together the expression of CYP19 is almost completely inhibited. Salbutamol caused maximum induction of CYP17 which was more than 13-fold; cyproterone on the other hand induced significantly this gene almost 3-fold. Neither tylosin nor EE2 affected the expression of CYP17 moreover none of the binary mixture studied affected significantly the expression of this gene.

3βHSD2 was the gene least affected by any of the treatments. Individual exposures with the chosen chemicals did not produce any significant changes in the normal expression of this gene. However, the cyproterone/salbutamol mixture significantly decreased the expression of 3βHSD2. A variety of responses was also observed for CYP11B2. This gene was increased significantly around 5-fold by EE2. Mixtures of cyproterone and trenbolone with EE2 did not affect the expression of CYP11B2, and although tylosin treatment significantly induced 10-fold the expression of this gene, the tylosin/EE2 mixture only produced a 2-fold induction

Table 3.4 Gene expression and hormone production in H295R cells exposed to single drugs.

	Treatment						
Gene	**DMSO** 0.1%	**ACETA** 300 ug/mL	**IBUPR** 250 ug/mL	**SALBU** 50 ng/L	**CLOFI** 3 ug/mL	**DEET** 3 ug/mL	**FLUOX** 1 ug/L
CYP11B2	1.00 ± 0.33	3.66 ± 0.89*	2.6 ± 0.77	2.00 ± 0.38	4.67 ± 1.24*	8.20 ± 1.30*	5.69 0.77*
CYP19	1.00 ± 0.61	0.88 ± 0.10	0.72 ± 0.01	1.88 ± 0.62	1.30 ± 0.15	0.5 ± 0.04	1.41 ± 0.09
CYP17	1.00 ± 0.10	0.64 ± 0.08	0.55 ± 0.08	13.64 ± 0.98*	1.04 ± 0.09	1.26 ± 0.13	0.90 ± 0.05
3BHSD2	1.00 ± 0.13	0.45 ± 0.20	0.37 ± 0.23	1.05 ± 0.19	0.66 ± 0.03	1.04 ± 0.44	0.69 ± 0.07

Hormone	**DMSO**	**ACETA** 300 ug/mL	**IBUPR** 250 ug/mL	**SALBU** 50 ng/L	**CLOFI** 3 ug/mL	**DEET** 3 ug/mL	**FLUOX** 1 ug/L
Testosterone	1.00 ± 0.27	1.12 ± 0.04	0.88	0.51 ± 0.17	0.68 ± 0.13*	0.39 ± 0.01*	0.75 ± 0.01
Progesterone	1.00 ± 0.01	2.3 ± 0.15*	1.84	0.30 ± 0.30	0.75 ± 0.09	1.68 ± 0.48	1.37 ± 0.08
Estradiol	1.00 ± 0.32	0.50 ± 0.2	0.42	0.32 ± 0.32*	1.55 ± 0.05	0.17 ± 0.10*	1.30 ± 0.30

All exposures were conducted for 48 h under standard conditions. All gene expression and hormone production values for fold change relative to control given as means and standard devi ations. DMSO: Dimethylsulfoxide; ACETA: Acetaminophen; IBU:Ibuprofen; SALBU:Salbutamol; CLOFI:Clofibrate; DEET: N,N-diethyl-3-methylbenzamide; FLUOX:Fluoxetine. * Indicates statistically significant differences at $p < 0.05$.

Table 3.5 Gene expression and hormone production in H295R cells exposed to single chemicals and to binary mixtures.[a]

Treatment	CYP17	CYP19	3BHSD2	CYP11B2	PROG	TEST	ESTR
Solvent Control	1.04(0.05)	0.99(0.02)	0.94(0.09)	1.00(0.01)	1.00(0.01)	1.00(0.27)	1.00(0.32)
Ethynylestradiol	1.05(0.18)	1.05(0.18)	0.76(0.1)	4.88(0.88)*	2.71(0.39)*	0.36 ± 0.12*	2.33(0.27)*
Ethynylestradiol + Trenbolone	0.55(0.50)	0.57(0.50)	0.45(0.25)	1.46(1.16)	2.72(1.67)	0.77(0.026)	3.77(1.13)*
Ethynylestradiol + Cyproterone	0.88(0.01)	0.99(0.03)	0.43(0.33)	1.28(0.39)	2.49(0.42)	0.70(0.14)	1.69(0.19)
Ethynylestradiol + Tylosin	0.69(0.43)	1.04(0.44)	0.54(0.47)	2.33(0.27)	1.39(0.95)	0.85(0.17)	0.74(0.09)
Cyproterone + Salbutamol	0.76(0.27)	0.01(0.00)*	0.33(0.06)*	2.53(1.34)	ND	ND	ND
Tylosin	1.03(0.04)	0.49(0.03)	1.32(0.27)	9.99(1.09)*	1.03(0.04)	0.42(0.09)*	0.12(0.08)*
Trenbolone	1.79(0.41)	2.56(0.55)*	0.77(0.15)	0.81(0.43)	0.72(0.20)	0.48(0.09)*	1.60(0.25)
Cyproterone	2.81(0.50)*	4.62 (1.51)*	0.90(0.32)	1.34(0.52)	1.02(0.30)	0.29(0.03)*	1.25(0.47)
Salbutamol	13.64(0.5)*	1.00(0.19)	1.88(0.62)	2.00(0.38)	0.30(0.30)	0.51(0.17)*	0.32(0.32)*

a All exposures were conducted for 48h under standard conditions. All gene expression and hormone production values for fold change relative to control given as means and standard deviations.

[b]Concentrations of single chemicals and mixtures exposures were: Ethynylestradiol (1ug/l), Trenbolone (25ug/l), Cyproterone (62ug/l), Tylosin (3ug/ml), Salbutamol.(50ng/l).

ND No Data.

* Indicates statistically ssignificant differences at $p < 0.05$. All treatment comparisons made to solvent control

that showed to be not statistically significant.

Hormone Production

Responses in hormone production by cells exposed to the mixtures could not be predicted from the results for the individual chemical exposures. Although T production was reduced significantly by all of the five chemicals chosen for the mixture treatments, binary mixtures of these compounds with EE2 did not show significant changes in T production when compared to values from solvent controls.

Production of P was only significantly increased by treatment with EE2 when exposed to compounds individually. However, the cyproterone-E2 mixture increased the production of P by more than 2-fold. E2 production, on the other hand, was significantly increased by more than 3-fold by the trenbolone-EE2 mixture. Exposure to the tylosin-EE2 did not cause significant changes in the production of any of the hormones analyzed.

Dose-Response Analysis

Gene Expression

Dose-response curves were constructed after 48 h of exposure to trenbolone, EE2, and cyproterone in the ranges of 0-39 μg/l, 0-150 μg/L and 0-45 μg/l respectively. Relative expression of 3βHSD2, CYP11β2, CYP17 and CYP19 values normalized to β-actin were compared to solvent controls. 17BHSD1 responses were also analyzed for cyproterone and trenbolone only.

In the EE2 exposure (Figure 3.1) 3βHSD2, CYP17 and CYP19 were not affected by the different concentrations of this chemical, however, CYP11B2 expression values

tend to start increasing at 1.5 μg/l and be constant up to 75 μg/l where expression levels started to rise again. For the cyproterone exposure (Figure 3.2), 3βHSD2, CYP19 and

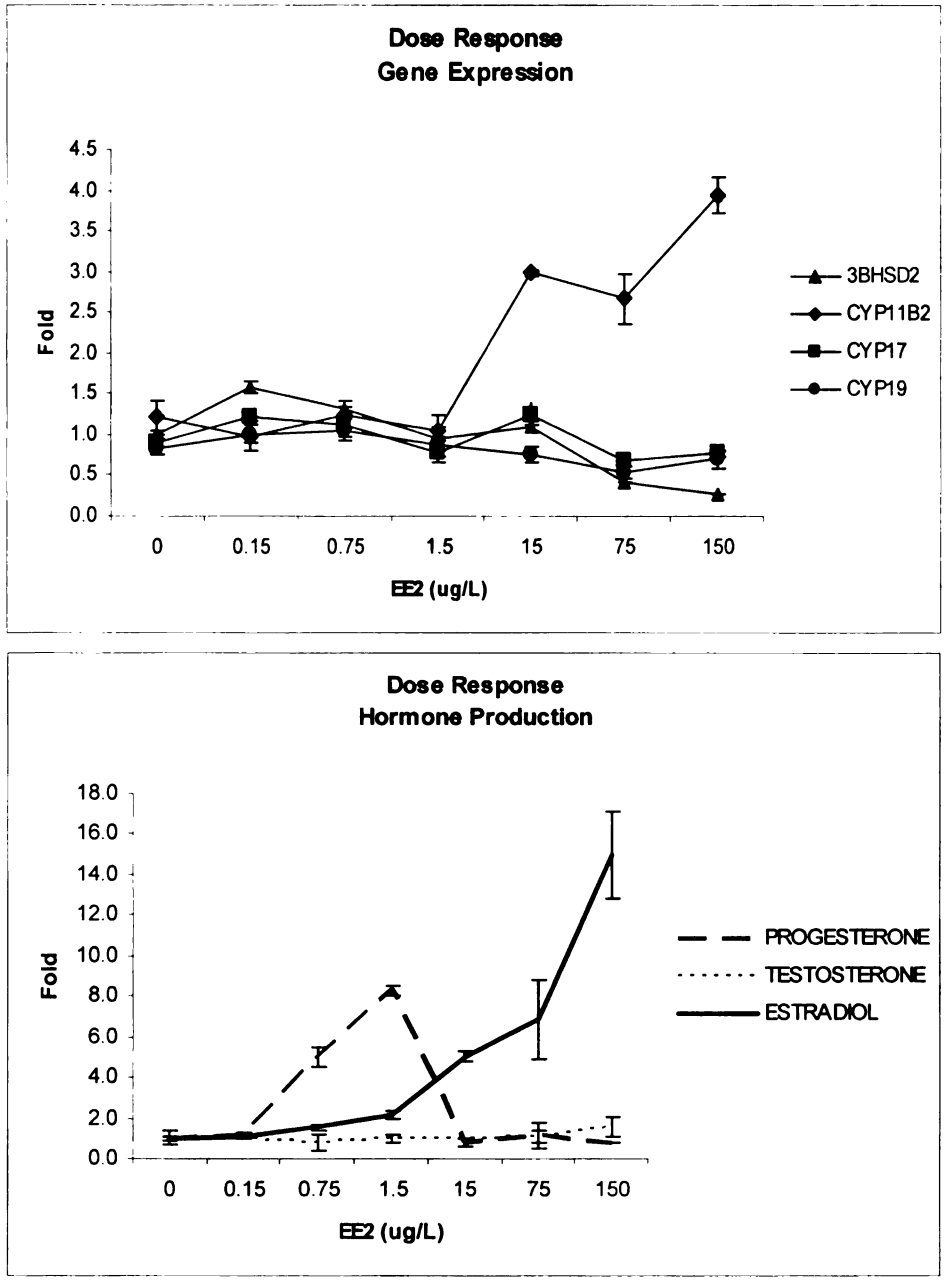
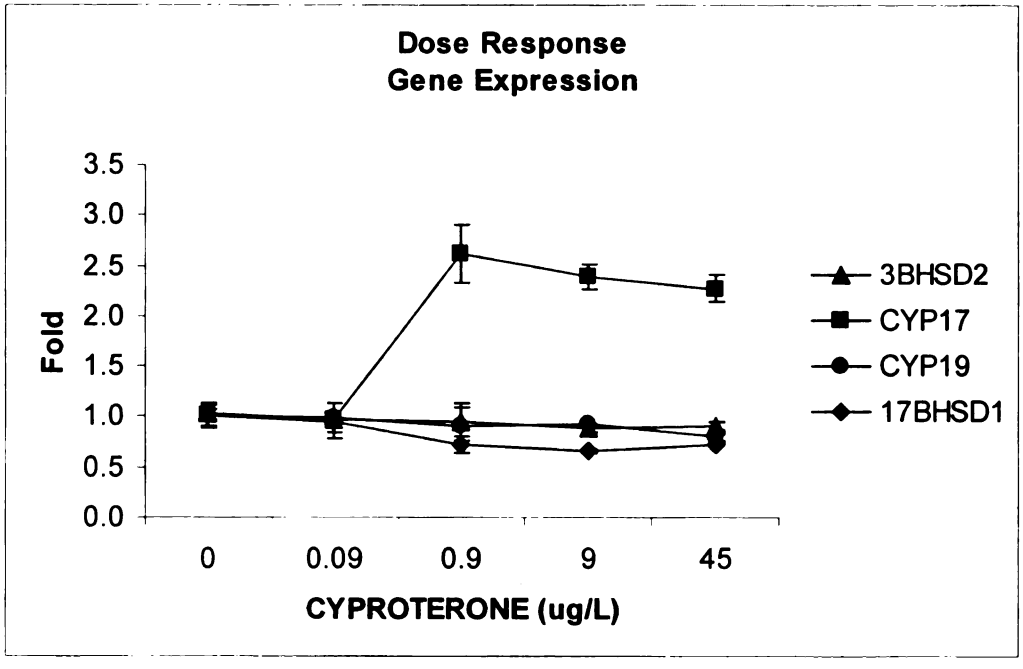


Figure 3.1 Dose-response curve for the expression of steroidogenic genes and hormone production after 48h exposure with different concentrations of Ethynylestradiol.



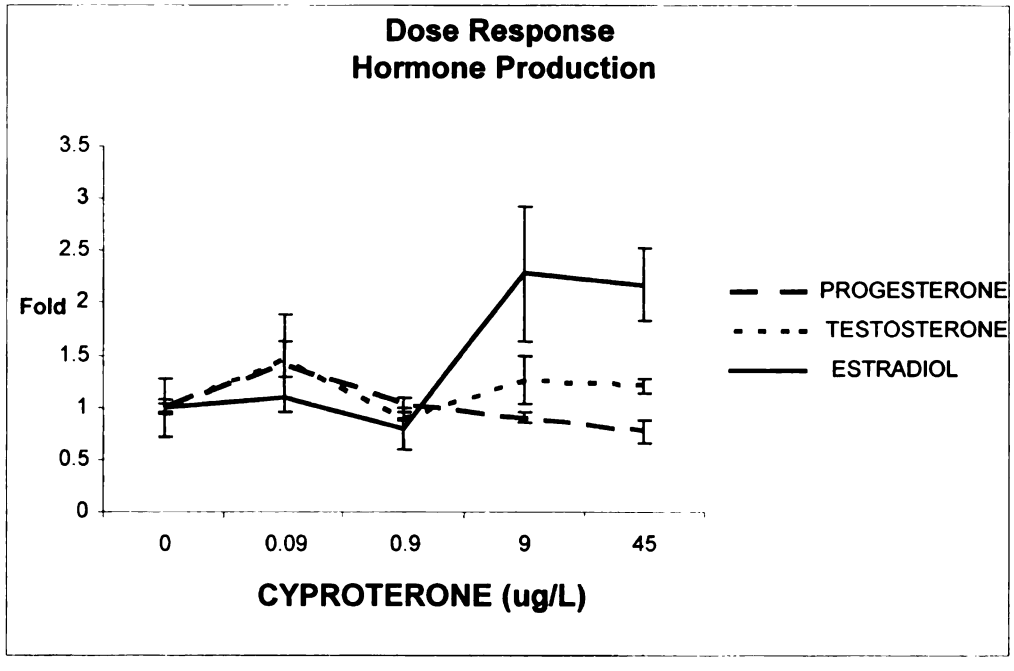


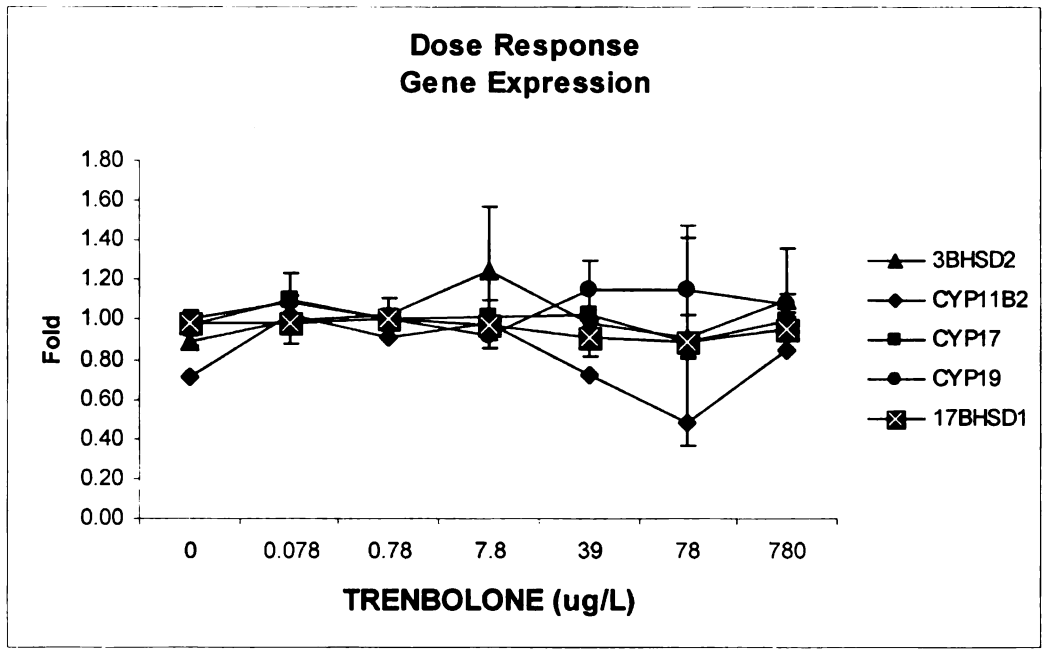
Figure 3.2 Dose-response curve for the expression of steroidogenic genes and hormone production after 48h exposure with different concentrations of Cyproterone.

17BHSD1 expression remained at basal levels, while the androgenic gene CYP17 increase in expression to approximately 3-fold to a concentration of 0.9 μg of cyproterone/l. Expression of none of the 5 genes studied in the dose-response exposures, changed when exposed to trenbolone at concentrations ranging from 0 to 780 μg/l (Figure 3.3). Most of the genes fluctuated positively around basal values of expression, except for CY11B2 which showed a decrease in expression at 78 μg/l but then at greater concentrations, such as 780 μg/l, expression levels returned to basal values.

Hormone Production

Production of P and T remained at control concentrations for exposures of trenbolone concentrations ranging from 0 to 39μg/l. In contrast, E2 concentrations increased rapidly and significantly at trenbolone concentrations greater than 7,8 x 10^{-2} μg/l (Figure 3). The 6-fold induction at this concentration was kept up to 0.78 μg/l then started to rise again at 7.8 and 39 μg/l reaching a maximum of almost a 10-fold increase. The dose-response curve for the production of T was constant and did not change due to EE2 exposure. Moreover, P production started to increase at 0.15 μg/l of EE2 concentration reaching an 8-fold maximum induction when at 1.5 μg/l EE2 concentrations. P production then returned to basal at concentrations of 15 μg/l of EE2.

The dose-response curve for hormone production in response to cyproterone exposure was bimodal through the concentration range of 0 to 45 μg/l (Figure 3.2). P and T production were slightly greater than control when exposed to 0.09 μg/L of cyproterone, then decreased at 0.9 μg/l and it was raised again at 9 μg/l remaining constant up to 45 μg/l cyproterone. E2 production followed the same pattern as P and T



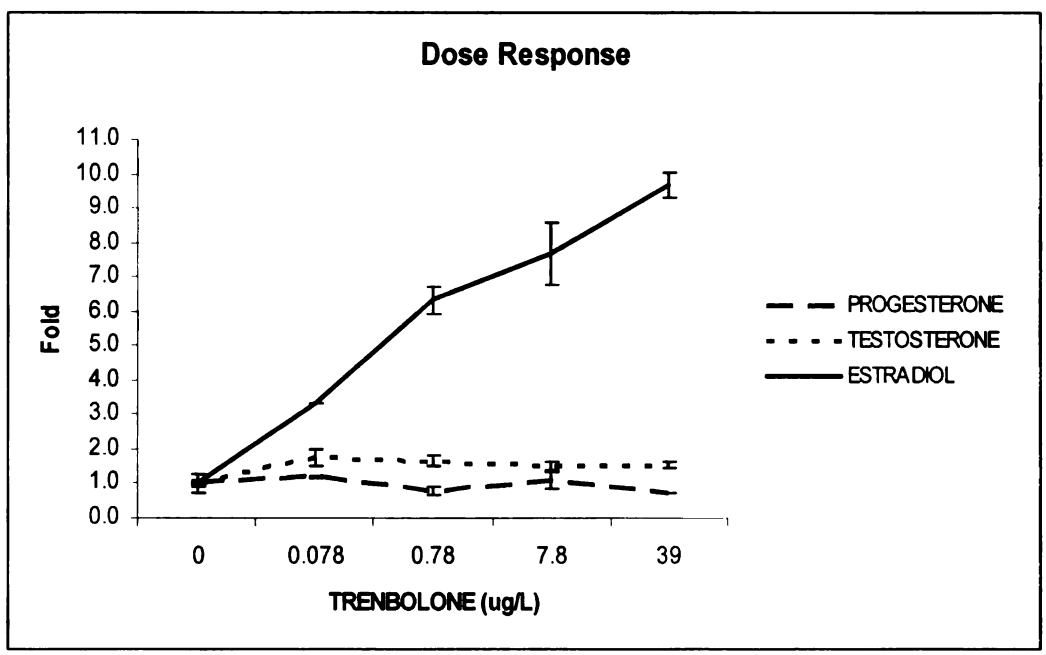


Figure 3.3 Dose-response curve for the expression of steroidogenic genes and hormone production after 48h exposure with different concentrations of Ethynylestradiol.

but at greater fold values. Maximum increments of E2 production were around 2.5-fold, while for P and T it reached less than 1.5-fold.

Relationship between gene expression and hormone production

The relationships between gene expression and hormone production were investigated by correlation analyses with all the chemicals and by group, that is, for the antibiotic group and the hormone therapy group separately (Tables 3.6-3.8).

A Pearson correlation matrix for all data from individual exposures showed small positive and negative correlations between the 4 genes studied and between the 3 hormones analyzed. To ascertain the validity of these correlations Bonferroni probabilities were also calculated. The results of these analyses indicated that by pooling all treatments together only one correlation was statistically significant and it was the negative correlation between the responses of the aromatase gene CYP19 and the aldosterone gene CYP11B2. No statistically significant correlations between hormone production and gene expression were observed for this group of treatments.

Correlations for the group of antibiotics not only showed the correlation established before between CYP19 and CYP11B2 but also a positive correlation between the CYP19 and CYP17; both correlations were of statistical significance. Again, no significant correlations between gene expression and hormone production were observed. A single correlation was observed between expression of CYP19 and CYP17 for the group of chemicals used for hormone therapy, but the one correlation between CYP11B2 and CYP19 was not observed.

DISCUSSION

Previous studies have demonstrated the utility and effectiveness of the H295R assay in identifying the potential harm that compounds may exert at different points in the steroidogenic pathway (Hilscherova et al., 2004; Hecker et al., 2006; Zhang et al., 2005; Gracia et al., 2006; Blaha et al., 2006). With the H295R cell culture system not only is it possible to analyze gene expression and hormone production, but also to evaluate enzyme activity. Moreover, this cell system has proven to be useful in identifying chemical mechanisms of action, in establishing patterns of gene and hormone responses, and also in the analysis of different interactions between chemicals when present in complex mixtures. Results from the experiments conducted in the present study confirm once more the effectiveness of the H295R screening system and its capacities when examining steroid production at environmentally relevant chemical doses.

Pattern of Responses by Group of Chemicals

Antibiotics

The results from the present study demonstrate that pharmaceuticals have the potential to negatively interfere in the normal pathway of steroid production. Particularly, antibiotics showed to have a broad range of effects on steroidogenesis.

Table 3.6 Pearson correlation matrix for steroidogenic genes and steroid hormones for all chemical treatments.

	CYP11B2	CYP17	CYP19	3βHSD2	TEST	PROG	ESTR
CYP11B2	1.000						
CYP17	-0.136	1.000					
CYP19	-0.395*	0.276	1.000				
3βHSD2	0.061	0.173	0.336	1.000			
TEST	-0.367	-0.148	-0.040	0.127	1.000		
PROG	0.254	-0.079	-0.285	-0.042	0.239	1.000	
ESTR	-0.065	0.193	0.191	-0.008	-0.207	0.088	1.000

Testosterone:TEST, Progesterone:PROG, Estradiol:ESTR.

* Indicates statistically significant correlations at $p < 0.05$.

Table 3.7 Pearson correlation matrix for steroidogenic genes and steroid hormones for the antibiotic treatments.

	CYP11B2	CYP17	CYP19	3βHSD2	TEST	PROG	ESTR
CYP11B2	1.000						
CYP17	-0.353	1.000					
CYP19	-0.803*	0.747*	1.000				
3βHSD2	-0.078	0.272	0.191	1.000			
TEST	-0.408	-0.052	0.299	0.404	1.000		
PROG	0.452	-0.511	-0.656	-0.159	-0.132	1.000	
ESTR	-0.107	0.121	0.133	-0.382	0.189	0.376	1.000

Testosterone:TEST, Progesterone:PROG, Estradiol:ESTR.

* Indicates statistically significant correlations at $p < 0.05$.

Table 3.8 Pearson correlation matrix for steroidogenic genes and steroid hormones for the hormone therapy treatments.

	CYP11B2	CYP17	CYP19	3βHSD2	TEST	PROG	ESTR
CYP11B2	1.000						
CYP17	-0.758	1.000					
CYP19	-0.677	0.913*	1.000				
3βHSD2	-0.255	0.567	0.728	1.000			
TEST	-0.504	0.123	0.032	-0.023	1.000		
PROG	0.430	-0.222	-0.254	0.111	0.062	1.000	
ESTR	0.385	0.369	-0.297	0.259	0.191	0.783	1.000

Testosterone: TEST, Progesterone: PROG, Estradiol: ESTR.

* Indicates statistically significant correlations at $p < 0.05$.

The semisynthetic β-lactam antibiotics amoxicillin and cephalexin, although they have the same therapeutic mechanism of action, their effects on both steroidogenic gene expression and hormone production, were quite different. In the case of the semi-synthetic macrolide antibiotics, erythromycin and tylosin, both caused the same gene expression profile, but they differed in the hormone production profile. Amoxicillin and cephalexin have in common the β-lactamic ring in their chemical structures, while erythromycin and tylosin both have a macrolide ring; all of these chemicals are the result of small structural modifications of their main structures that have been made in order to improve their therapeutic properties. These small differences in chemical structure between chemicals of the same group may be responsible for the differences observed in gene expression and hormone production profiles since changes in chemical structure translate into changes in physico-chemical properties that at the end are going to define their different endocrine disruptive potential.

Since the effects of antibiotics on steroid production have not been previously studied, the mechanisms by which these compounds exert their effects on steroidogenesis are unknown. Based on the extensive use of antibiotics and their loadings to the environment it therefore seems that endocrine effects may also need to be considered along with the promotion of antibiotic resistance and the potential of these compounds to influence growth in humans (Ternak, 2004) and other non-target organisms.

Hormone Therapy Group

Drugs employed as hormone therapy agents have a broad range of medical uses. Pharmaceuticals of this group are used in cancer treatment, birth control, for diagnostic,

and as growth promoters, among other uses. Cyproterone is a steroidal anti-androgen with weak progestagenic activity used in the treatment of prostate cancer (Wirth et al, 2006). This drug exerts its functions by suppressing androgen action both by binding directly to the androgen receptor and by inhibiting the positive feedback of androgens on the pituitary ultimately resulting in reductions in the production of sex steroids (Sharpe et al., 2004). The anti-androgenic properties of cyproterone were observed in the results for hormone analysis where concentrations of T were reduced by up to one third. It is noteworthy that the expression of CYP19 and CYP17 were increased, probably in response to depletion of T in the medium. Induction of CYP17 would drive steroidogenesis towards the production of androgens while increments in CYP19 activity would ensure that E2 was produced despite small concentrations of substrate. Statistical correlations for these two genes showed a great degree of significance for this group of pharmaceuticals supporting the idea of a coordinated expression system.

EE2 is the most common and most potent estrogenic compound found in sewage effluents (Sarmah, 2006). This synthetic E2 analog is used in combination with other estrogenic substances in the manufacturing of contraceptive pills. Studies have demonstrated the different effects of EE2 on the survival, sex ratio, gonadal growth, spawning and sexual differentiation of aquatic organisms especially fish (Scholz and Gutzeit, 2000). In H295R cells exposed to 1μg/l of EE2 the production of P and E2 in H295R cells were doubled, while T production was greatly reduced. The observed decrease in T production may be a reaction to the increased production of E2 since T production may be substrate limited.

Trenbolone acetate (TBA) is a synthetic steroid hormone commonly used to enhance growth in beef cattle. TBA is quickly metabolized to the potent androgen 17β-trenbolone (Durhan et al., 2006). Decreases in T production in the H295R cells may be explained by the high affinity of 17β-trenbolone for the human androgen receptor, which has been established to be similar to that of dihydrotestosterone (Bauer et al., 2001). Because of this interaction between 17β-trenbolone and the androgen receptor it is possible that cells responded to an apparent lack of T and induced the expression of the aromatase gene CYP19 trying to keep E2 concentrations at normal levels.

α-zearalanol is the estrogenic equivalent of 17β-trenbolone; this chemical is the active metabolite of the mycotoxin, zearalenone, that is obtained from *Fusarium sp* (Sheehan et al., 1984). α-zearalanol is also used in veterinary medicine as a growth promoter. Exposure of H295R cells to zearalenone caused reduced E2 production, due perhaps to the interaction of this estrogenic compound with the estrogen receptor, mimicking a high abundance of E2. T and P production were not affected by this chemical nor was the expression of the steroidogenic genes studied, except for 3BHSD2.

Together these results indicate that extensive attention must be directed to the use and fate of pharmaceuticals with hormonal properties since this is a group of chemicals that will surely produce significant effects when reaching non-target organisms. This is especially the case for compounds used for veterinary purposes which may be excreted in their active forms by treated animals and then reach aquatic ecosystems via runoff (Lange and Dietrch, 2002).

Other Pharmaceuticals

Over the counter analgesics and anti-inflammatory drugs such as acetaminophen and ibuprofen did not produce significant changes in gene expression or hormone responses. Acetaminophen increased P production 2-fold, which may be responsible for the induction of CYP11B2 since P is a precursor for this enzyme. No steroidogenic effects have been demonstrated for acetaminophen and its exact mechanism of action as an analgesic is unknown. Antilipidic drugs, such as clofibrate, are commonly used to treat hyperlipidemia, a condition considered a major risk factor of cardio and cerebro-vascular diseases. Despite being withdrawn from the market in most countries in Western Europe, clofibrate concentrations in the ng/l to μg/l range have been reported in several sewage treatment plant effluents (Koutsouba et al., 2003). In H295R cells clofibrate concentrations of 3 μg/ml significantly reduced T production. Reduction of T levels by this drug has also been observed in rat studies where it was suggested that clofibrate may exert its action through direct effects on the microsomal enzyme systems responsible for steroid metabolism (Xu et al., 2002).

Of interest where the results from the exposure with the drug salbutamol, a short-acting, β_2-adrenergic receptor agonist used to treat broncho-spasm and in some cases is used in obstetrics as a tocolytic to relax the uterine smooth muscle and delay premature labor (Blanchard et al., 1993). Its mode of action is to bind β_2-adrenergic receptors with greater affinity than β_1-receptors; the activation of β_2-adrenergic receptors results in relaxation of smooth muscles. Salbutamol is also used in combination with other drugs as a growth promoter in livestock. This β_2-adrenergic enhances lipolysis and the rate at which fatty acids are oxidized producing leaner animals (Hernández-Carrasquilla, 2003). Thus, we hypothesized that the lipolytic effects of salbutamol could be responsible for

the significant decreases of almost 50% in E2 production compared to solvent controls. The most obvious effect of this agonist compound was the increase in expression of the androgenic gene CYP17 by more than 10-fold. More specific studies need to be designed in order to know if this increase in the expression of CYP17 is in some way linked to depletion of E2.

Effects of Drug Mixtures

From the beginnings of their use, pharmaceuticals have been entering the environment and have been constantly detected at measurable concentrations; mostly they are ordinarily found in mixtures of active ingredients with a variety of biological activities. Thus, non-target organisms are being exposed to different biological actions at the same time. Few toxicological studies have been conducted to address chronic mixture exposure issues and the risks associated with the presence of combinations of biologically active contaminants (Crane et al., 2006). Developing an understanding of the effects of complex mixtures of compounds acting together must become a priority when evaluating the potential risks of pharmaceuticals in the environment. One of the major difficulties in analyzing effects of complex mixtures is the understanding of the different ways in which compounds in the mixture will interact to produce the final effects.

H295R cells were exposed to 4 binary mixtures of different pharmaceuticals. EE2, the most common component in birth control pills was a common component for 3 of the 4 binary mixtures prepared. Because of the effects of individual compounds on the 4 genes studied, cyproterone, trenbolone and tylosin were chosen as the second

component in the mixtures with EE2. In addition, due to the effects of cyproterone on gene expression and salbutamol on hormone production, H295R cells were also exposed to a mixture of these two compounds. Gene responses suggested that the chemicals present in these mixtures interact mostly by antagonistic mechanisms, although agonism was also observed in some cases. The dominant effects of EE2 were observed when in mixtures with trenbolone, cyproterone, and tylosin. In particular, when expression values produced by individual exposures of EE2 where greater than those produced by the second component in the mixture, the joint effects observed were expression values similar to those caused by the second component or the one who produced lower fold inductions.

Different to gene expression, several type of interactions were observed for the hormone production responses to the mixture treatments. For instance, for P the binary mixtures produced the same effects produced by EE2, which was more than a 2 fold induction in the production of this hormone, an indication that the EE2 effects prevail in the mixture. On the other hand T production was down-regulated by all the individual treatments but the mixtures did not produce significant changes in the concentration of this hormone showing that these chemicals block each other's antagonistic effects respect to T production. On the contrary, the results of E2 showed that an additive effect was produced by the mixture of EE2 and trenbolone; such a response is due to the affinity that both of these compounds have for the ER. From these results it can be observed that the steroidogenic effects exerted by the binary mixture exposures could not be predicted from the results of exposures of the individual chemicals in question. As for an example, cyproterone significantly up-regulated the expression of the aromatase gene while

salbutamol did not produced significant changes in the expression of this gene, when together in a mixture, the expression of aromatase was completely down-regulated. This corroborates the belief that not only do interactions between compounds occur but also that their effects are usually different to individual responses. The results show that pharmaceuticals and their mixtures act through additional unknown modes of toxic action that have to be understood in order to truly assess their potential effects as environmental contaminants.

Dose-Response Analysis

Three pharmaceuticals used in hormone therapy were selected to conduct dose-response studies. The results showed that the dose-dependent changes in gene expression behaved differently for each chemical. Exposure to EE2 affected only CYP11B2 expression. Of the hormones, only T production was not affected by exposure to EE2. E2 concentrations were proportional to the concentration of EE2. Changes were observed even at EE2 concentrations as small as 0.15 μg/l. The positive relationship between E2 production and EE2 in the medium is consistent with the great affinity of EE2 to for the ER. Despite the continuous induction of CYP11B2 by EE2, P production was not affected in the same manner. Increases in the production of this hormone were only observed between 0.15 and 1.5 μg/l before returning to basal levels.

The anti-androgen cyproterone prevents dihydrotestosterone, the active form of T in mammals, from binding to receptors in carcinoma cells. Thus, induction of CYP17 may be a response to the presence of the anti-androgen that results in an increase in the production of active T to compete for the receptors. E2 was the only hormone to increase

proportionally with cyproterone concentration. The mechanisms by which this process occurred are unknown.

Changes in trenbolone concentrations did not produce mayor effects on the expression of any of the steroidogenic genes, or hormone production except for its effects in E2. The production of E2 was greater than that in the control at all trenbolone concentrations tested, although the expression of the aromatase gene CYP19 was not increased, instead, T production stayed within the basal concentration range. These results suggest the possibility that trenbolone induced the activity of the aromatase enzyme by the activation of other pathways.

Chemicals with the same "mechanism of action" may have different effects on the expression of steroidogenic genes and the production of steroid hormones. For instance, although cyproterone and trenbolone both interact with the androgen receptor, each caused different effects on gene expression and hormone production. Thus, it appears that each of these chemicals, in addition to it's interaction with the androgen receptor, may induce, or inhibit other points in the pathway resulting in the observed differences in the effects.

CONCLUSIONS

The versatility of the H295R assay system was proven to be useful in the evaluation of the potential effects that commonly found environmental contaminants may exert on steroidogenesis. For the first time the H295R cell system has shown how several biologically active compounds such as pharmaceuticals, even at trace concentrations have the potential to significantly affect the normal functioning of the endocrine system in

non-target organisms. Also, this system allowed the evaluation of the joint effects produced by combinations of bioactive compounds in binary mixtures, mostly antagonistic interactions were observed after exposures with binary mixtures of hormonal therapy drugs. The construction of dose-responses curves provided valuable information to identify genes and hormones that may be susceptible to changes in concentrations of chemical exposure. It may be concluded, that the H295R cell bioassay is a very quick, practical, and sensitive pre-screening method by which the endocrine disruptive effects of environmentally relevant chemicals may be evaluated.

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Chapter 4

IN VITRO MODULATION OF ENDOCRINE FUNCTION IN H295R CELLS BY EXTRACTS OF WATERS FROM THE VICINITY OF HONG KONG, S.A.R., CHINA

ABSTRACT

Recently Hong Kong has installed a sewage interceptor system for areas adjacent to Victoria Harbor and treats the sewage and surface runoff at the Stonecutter's Island treatment facility. As part of an on-going evaluation of the effectiveness of this treatment system, the H295R cell bioassay was used to evaluate the effects of methanol extracts of marine waters taken at 22 points of suspected pollution in Hong Kong, China. Treated and non-treated effluents from 2 major wastewater treatment plants were also assessed. Modulation of expression of the steroidogenic genes CYP19, CYP17, 3βHSD2 and CYP11B2 and production of the hormones progesterone (P), estradiol (E2) and testosterone (T) were evaluated. No significant modulation of gene expression or hormone production was caused by extracts of water collected from the wastewater treatment plant (WWTP) effluents. Thus, it was determined that the waste water effluents were treated to a level that should no result in endocrine modulation of marine animals, including fish. Extracts from 2 fish culture sites were toxic to the H295R cells. The expression of CYP19 was the most sensitive steroidogenic response observed. CYP19 was down-regulated by extracts of water samples collected from areas receiving effluents from older WWTPs and public filling areas.

INTRODUCTION

A broad variety of detrimental reproductive disorders has been observed in wildlife and possibly humans during the past decades. These occurrences have been linked to the increasingly broad spectrum of environmental contaminants including both, natural and synthetic compounds (Kavlock et al., 1996; EDSTAC Final Report, 1998). Although there is still insufficient data to demonstrate specific associations between environmental exposure and endocrine-mediated adverse effects, the continuous presence in the environment of such compounds may represent a threat to the normal development of organisms living in exposed aquatic ecosystems (Committee on Restoration of Aquatic Ecosystems, 1992). For example, PAHs, pesticides, phthalate plasticizers, PBDEs, dioxins, alkylphenols and steroids can be considered potential endocrine disrupting chemicals (EDCs). Thus, there is a considerable body of evidence suggesting that most of the contaminants frequently found in Hong Kong waters have the potential to produce adverse effects on endocrine functions. EDCs can exert their disrupting effects by different mechanisms but at present most of the bioassays to evaluate endocrine disruption are designed to analyze only the interaction of individual chemicals with the different hormone receptors. The need to evaluate endocrine disruption exerted by pathways other than receptor-mediated mechanisms and the effects of EDCs mixtures has resulted in development of new bioassays such as the H295R cell system (Gazdar et al., 1990; Hilscherova et al., 2004). This in vitro system has been proved to be effective in characterization of endocrine disruptive effects of individual chemicals, pharmaceuticals, pesticides and their mixtures (Zhang et al., 2005; Gracia et al., 2006; Sanderson et al., 2000; Blaha et al., 2006; Xu et al., 2006). As well as evaluating the expression of

steroidogenic genes, this assay also permits evaluation of hormone production and enzyme activity.

Some of the causes of deterioration of water quality of the coastal marine environment are lack of adequate sewage infrastructure, growing population, untreated livestock waste, discharges of untreated sewage, industrial effluents and agricultural runoff. Relatively great levels of pollution have been identified in Asian waters (Connell et al., 1998; Monfils et al., 2006). In Hong Kong, SAR, China, for example, water quality has significantly deteriorated over the last decade as a consequence of the growing industrial activities and population increases (Wong et al., 1995; Yung et al., 1999). Large amounts of bacteria, total nitrogen and poor levels of dissolved oxygen have been identified in the most polluted spots in Hong Kong such as Victoria Harbor, where 65% of the regions population live. Furthermore, pollution from the harbors seems to be spreading to adjacent sensitive water bodies. The lower sections of the East River (Dongjiang), which provides approximately 80% of the drinking water for Hong Kong, is contaminated by organic and inorganic pollutants (Ho et al., 2003), including metals such as cadmium, copper and zinc (Ip et al., 2003; Hung, et al., 2006) and persistent organic pollutants including polybrominated diphenyl ethers (PBDEs) (Liu et al., 2005; Ramu et al., 2005), organochlorine pesticides and dioxin-like compounds (So et al., 2005). Low levels of contamination of PBDEs have been also found in Hong Kong marine waters, the concentrations found in a recent study ranged from 31 to $1.2x10^2$ pg/l (Wurl et al., 2006). It has been suggested that the presence of these compounds may arise from the disposal of electronics industry waste in southern China, as well as the discharge of untreated wastewater of local origin. Moreover, residual levels of DDTs and

polycyclic aromatic hydrocarbons (PAHs) have been found in freshwater and marine fish sold in Hong Kong markets (Cheung et al., 2006).

Everyday in Hong Kong a population of almost 7 million people produces some 2.2 million cubic meters of sewage. Hong Kong has about 1,320 kilometers of sewerage network running through it which all feed into some 200 sewage treatment plants. These plants range from preliminary (screening) treatment plants to secondary (biological) treatment plants treating sewage from residential, commercial and industrial premises in the territory prior to disposal to the sea for dilution and dispersion through submarine outfalls. About 95% of Hong Kong's population is now served by the public sewerage system with over 98% of the sewage produced being collected and treated. All new towns in the New Territories have been designed and developed with modern secondary sewage treatment works, however older urban areas still have a very poor sewage infrastructure that does not cater for their needs and has in the past not provided world-class sewage treatment. In order to meet and manage development demands throughout Hong Kong and the rise in people's living standards, Hong Kong's sewage infrastructure is gradually being upgraded under a scheme referred to as the Strategic Sewage Disposal Scheme (SSDS) - a sewerage rehabilitation and improvement program (Water Quality Report, 2005). The whole scheme comprises a series of deep tunnels to collect and transfer sewage from the central urban areas of Hong Kong and Kowloon to a centralized treatment works at Stonecutters Island, were samples for this study were collected, and then after treatment waters are sent to a submarine outfall south east of Lamma Island, a point also sampled.

The present study was conducted to measure the potential for coastal marine waters and effluents of the WWTPs of Hong Kong to contain mixtures of EDCs that can modulate steroidogenesis in H295R cells (Figure 4.1). The areas selected for study were considered to be impacted by contamination from human, industrial, and agricultural wastewater. The sampling points for this study included STW influents and effluents, fish culture zones, marine disposal and STW discharge areas, and public filling areas which are very common in Hong Kong; these areas are designated as part of a development project that accepts public fill for reclamation purposes and disposal of public fill in a public filling area which requires a license issued by the director of civil engineering department. The methodology involved the extraction of the water samples using SPE techniques and exposure of the H295R cells to the extracts. After exposure the expression of the steroidogenic genes CYP19, CYP17, 3βHSD2 and CYP11B2 was evaluated using PCR protocols. Furthermore, the production of the hormones progesterone (P), estradiol (E2) and testosterone (T) was evaluated using ELISA methods.

MATERIALS AND METHODS

Sample Collection

Water samples were collected in July 2005 from 22 coastal marine areas and from 2 major waste water treatment plants in Hong Kong (Figure 4.2; Table 4.1). In addition to areas known to receive sewage effluents, areas with intensive aquaculture and marine

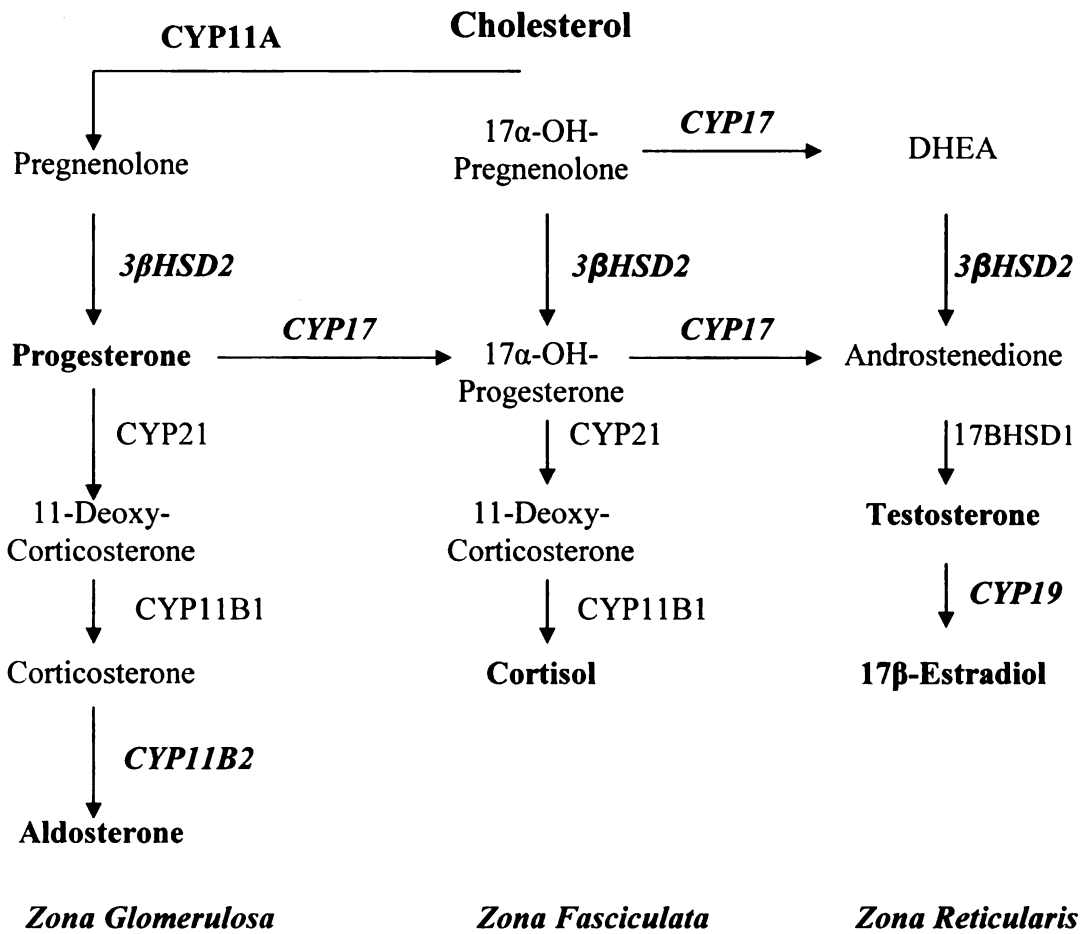


Figure 4.1 Enzymes, substrates and products of the steroidogenic pathway in the different zones of the human adrenal cortex.

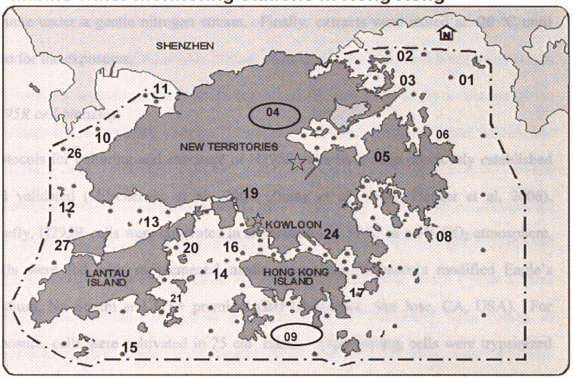
disposal activities were also included. Water samples were collected using standard depth and width integrating techniques designed to obtain a representative sample (Shelton, L.R., 1994). Composite samples collected at each site were split into two pre-cleaned amber glass bottles. Measurements of the collected water samples included pH, dissolved oxygen (DO), conductivity, and temperature and were conducted with calibrated instruments. Samples were immediately chilled and sent to the laboratory where they were stored at 4 °C until they were filtered and prepared for extraction, within 24 h to keep degradation to a minimum and to avoid the need for addition of chemical preservatives.

Extract preparation

Prior to extraction, each sample was vacuum filtered through a 70 mm glass fiber GF/F filter (Whatman, Maidstone-UK) with a pore size of 0.7 μm and the retained particulate material was washed with 0.5 mL of methanol which was added to the aqueous sample. Following filtration, 1 mL of 200mg/mL Na_2EDTA was added to each liter of sample to prevent compounds (such as the tetracycline antibiotics) from forming complexes with divalent ions such as Ca^{2+} and Mg^{2+}. The pH of water samples was then adjusted to 3 using glacial acetic acid. To determine extraction efficiencies a trace amount of ^3H-testosterone was added to each one liter sample prior to extraction. Quantities of the ^3H-testosterone recovered were measured by scintillation counting. Solid phase extraction cartridges, 200 mg Strata X (Phenomenex, Torrance, CA,USA) were washed twice using 2 mL of methanol, followed by conditioning with 3 aliquots of 2mL distilled water. One liter of the prepared samples was pulled through the each washed and conditioned

Figure 4.2

Marine water monitoring stations in Hong Kong



Sampling points in a circle are those producing high toxicity to H295R cells

☆ Water treatment plants

cartridge at 10mL/min using a vacuum pump. The cartridges were taken to dryness and the retained compounds were eluted with 3 portions of 2 mL of methanol. In a thermostatic bath set at 30 °C the extracts were then concentrated up to 1 mL final volume under a gentle nitrogen stream. Finally, extracts were stored at -20 °C until used for the exposures.

H295R cell bioassay

Protocols for culturing and exposure of H295R cells have been previously established and validated (Hilscherova et al., 2004; Zhang et al., 2005; Hecker et al, 2006). Briefly, H295R cells were cultivated in an incubator at 37 °C in a 5% CO_2 atmosphere. Cells were fed with supplemental media containing Dulbecco's modified Eagle's medium, Nu Serum and ITS+ premium (BD Bioscience, San Jose, CA, USA). For exposure, cells were cultivated in 25 cm^2 flasks. After rinsing, cells were trypsinized with 1.2 mL of trypsin per flask and incubated for 3 min maximum, then harvested and mixed with supplement media to deactivate trypsin; 3 ml of cell suspension, containing approximately $1x10^6$ cells/ml, were placed in each well of a 6 well test plate.

To establish the range of extract concentrations that could be used without causing cytotoxicity to the cells, a Live/Dead cell viability kit (Molecular Probes, Eugene, OR, USA) was used. In instances where exposure resulted in cell death or decreased viability the data were not used to evaluate gene expression or hormone production. Exposures to the extracts were done at least 24 h of plating. Dosing solutions of the extracts were prepared in medium by adding 10 µL of extract to 10 mL medium to make methanol concentrations of 0.1%. A solvent control dose solution was prepared adding 10 µL of methanol into 10 mL of medium. Cells were checked

under a microscope to assure good cell condition prior to dosing. The culture medium was removed and cells were exposed to 3 mL of dosing solutions.

After 48 h of exposure, medium was removed and saved for hormone analysis. RNA was extracted from the cells using Absolutely RNA RT-PCR miniprep kits (Stratagene, La Jolla, CA, USA). Before nucleic acid isolation and hormone analysis, cell viability was determined. Cells were visually inspected under a microscope to evaluate viability and cell number. Quantity and quality of the extracted mRNA was measured using spectrophotometric methods. After RNA extraction, reverse transcription was conducted with the Cloned AMV First-Strand cDNA synthesis kit (Invitrogen, Carlsbad, CA, USA). cDNA dilutions were prepared and qPCR conducted using previously published methods by Hilscherova et al. in a Smart Cycler System (Cepheid, Sunnyvale, CA, USA) in 25 μL sterile tubes using SyBr Green as quantification dye. The steroidogenic genes analyzed by PCR were 3βHSD2, CYP11B2, CYP17 and CYP19. For quantification of gene expression Ct values from the PCR reactions were used to express the results as fold difference with respect to the appropriate solvent control. Gene expression data was standardized to the expression of β-actin.

Hormone quantification

Hormone extraction and quantification by ELISA were conducted as previously described (Hecker et al., 2006). Briefly, frozen media samples were thawed on ice, and the hormones were extracted twice with diethyl ether (5 ml) in glass tubes. To determine extraction efficiencies a trace amount of ^{3}H-testosterone was added to each sample prior to extraction. Concentrations of hormones in media were measured by

competitive ELISA using Cayman Chemical® hormone EIA kits (Cayman Chemical Company, Ann Arbor, MI, USA) for progesterone [Cat # 582601], testosterone [Cat # 582701], estradiol [Cat # 582251]. Because the antibody to progesterone exhibits cross-reactivity with pregnenolone (P) of 61% and the method does not allow for the separation of these two hormones, progesterone concentrations are expressed as the sum of progesterone and pregnenolone. The working ranges of these assays for the determination of steroid hormones in H295R media were determined to be: progesterone (P): 7.8 - 1000 pg/mL; testosterone (T): 3.9 - 500 pg/mL; 17ß-estradiol (E2): 7.8 - 1000 pg/mL. Extracts were diluted 1:25 and 1:100 for T while for P and E2 dilutions were 1:50 to 1:100 and 1:2 to 1:10, respectively.

Statistical Analysis

Statistical analyses of gene expression and hormone production were conducted using SYSTAT (SYSTAT Software Inc., Point Richmond, CA, USA). Differences in gene expression were evaluated by ANOVA followed by Tukey's Test. Differences with $p<0.05$ were considered significant

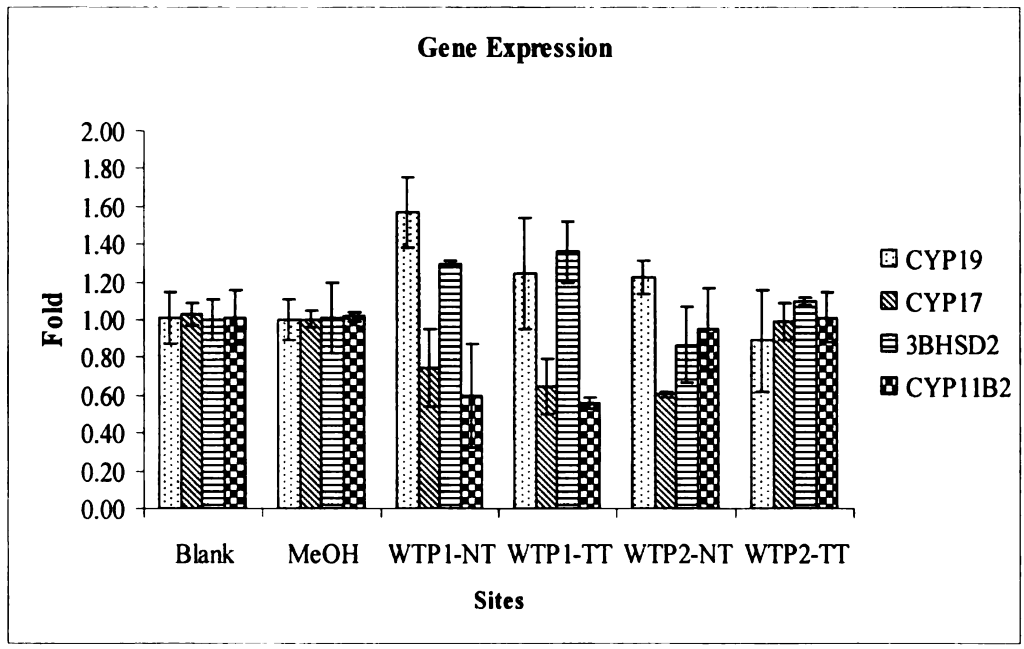
RESULTS

Values for the physical and chemical characteristics varied among locations (Table 4.1). In general, the lowest pH values were measured in waters from the STPs, where values were approximately 7.5. However, untreated waters from the Sha Tin treatment plant had a pH of 8.34. The greatest pH value (8.76) was measured in the vicinity of the effluents from San Wa system treatment works (STW). Salinity values ranged from

11.5 to 36 ‰, among locations. The minimum and maximum salinities were observed in samples of treated effluents from the Sha Tin WWTP and the public filling area Tseung Kwan (HK17), respectively. Dissolved oxygen ranged from a minimum of 0.2 mg/L in untreated sewage from the Sha Tin WWTP to 0.8 mg/L in treated waters from the Stonecutters Island WWTP. Dissolved oxygen concentrations in coastal marine waters ranged from 3.8 to 9.6 mg/L.

Extracts from neither the Sha Tin nor Stonecutters Island WWTP produced significant changes in the expression of any of the 4 steroidogenic genes (Figure 4.3) and only extracts from the non-treated water from the Sha Tin WWTP affected the production of E2. Treatment with the extract of effluent from the Sha Tin WWTP caused a 30% reduction in E2 release compared to the solvent control. P production was increased significantly by extracts of non-treated waters from the Stonecutters Island treatment plant. T production was not affected significantly by any of the extracts from the treatment plants. None of the extracts from water samples taken after treatment significantly affected the expression of the steroidogenic genes nor the production of any of the hormones studied.

Extracts from samples taken from 2 of the major fish culture zones, Yim Tin Tsai (HK04) and the Lo Tik Wan open-water fishing zone (HK09) were very toxic to H295R cells even at 10^4 – 10^5 dilutions, to the point of inhibiting cell growing and proliferation with consequent death. This effect suggests the presence of extremely toxic compounds.



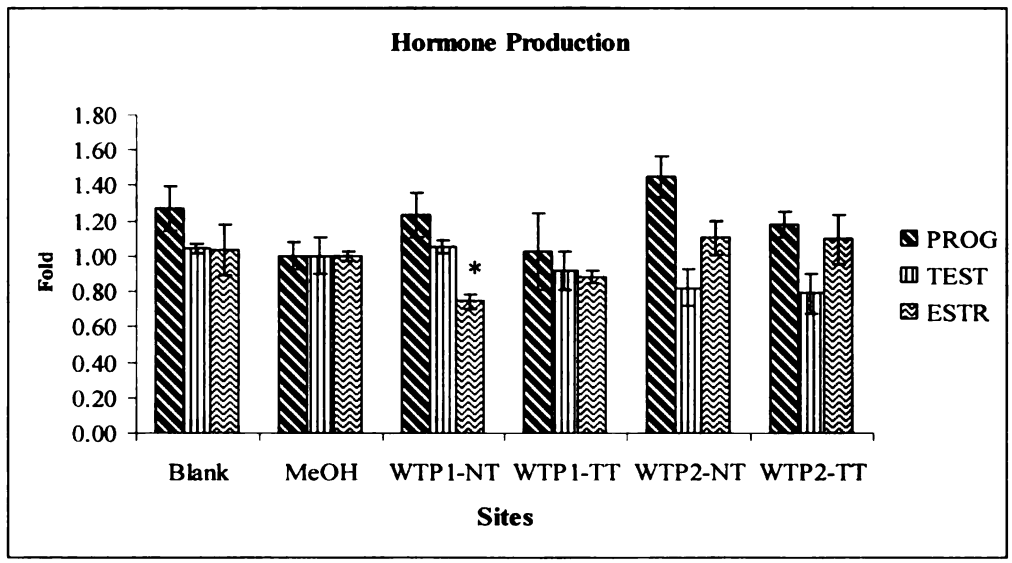


Figure 4.3. Gene expression and hormone production for methanolic extracts from water samples from major water treatment plants in Hong Kong, SAR, China. WTP1: Shatin; WTP2: Stonecutters; NT: Before treatment; TT: After treatment. All exposures were conducted for 48 h under standard conditions. All values are expressed as fold change relative to control. * Indicates statistically significant differences at $p < 0.05$.

Table 4.1. Sampling points for marine waters in Hong Kong, SAR, China

Sample	Name	Activity	Temperature °C	pH	DO [a] mg/l	Salinity %
HK01	Tung Ping Chau	Reference Site	29.6	8.29	6.7	16.0
HK02	Ap Chau, Kat O	Fish culture	28.3	8.60	8.1	34.0
HK03	Sai Lau Kong	Fish culture	29.1	8.63	8.6	35.0
HK04	Yim Tin Tsai	Fish culture	27.8	8.63	8.0	30.8
HK05	Young Shue Au	Fish culture	26.6	8.46	7.2	34.0
HK06	Tap Mun, Kau Lau	Fish culture	27.2	8.44	6.9	34.2
HK07	Ma Nam wat	Fish culture	29.5	8.38	6.6	35.2
HK08	Leung ShuenWan	Fish culture	26.5	8.39	6.6	33.0
HK09	Sok Kwi Wan	Fish culture	26.8	8.17	5.4	31.2
HK10	Oyst	Oyster Production	29.4	8.04	5.4	16.0
HK11	Mai Po	Marine Reserve	24.8	8.33	6.5	28.5
HK12	Tuen Mun	Public filling area	28.6	8.61	8.7	23.2
HK13	Sha Chau (East)	Marine disposal	26.2	8.43	6.0	28.0
HK14	Tsin Yi (South)	Marine disposal	24.4	8.40	5.5	30.2
HK15	Cheung Chau (South)	Marine disposal	26.0	8.56	8.0	31.8
HK16	Wan Cha (East&East)	Disch. STW [b]	24.2	8.25	5.5	25.0
HK17	Tseung Kwan	Major reclamation s.	26.8	8.37	5.3	36.0
HK19	Stonecutters Island	Disch. STW	24.7	8.36	6.4	28.2
HK20	Pennys Bay	Major reclamation s.	25.5	8.52	7.5	33.2
HK21	Mu Wo	Disch. STW	27.0	8.59	8.3	32.2
HK22	Aberdeen & Ao Lei	STW & Fish culture	26.6	8.21	4.9	30.0
HK23	Tolo Harbor	Disch.. STW	26.7	8.37	5.7	34.0
HK24	North Poing	Disch.. STW	26.1	7.97	3.8	27.2
HK25	San Wa	Disch.. STW	27.6	8.76	9.6	31.0
HK26	Pilaf Port	Disch.. STW	29.0	8.60	8.6	20.8
HK27	Cogeneration Plant	Power plant	29.6	8.29	6.7	16.0
HK28	Shatin - untreated	STW	29.3	8.34	0.2	13.2
HK29	Shatin - treated	STW	30.2	7.48	6.7	11.5
HK30	Stonecutters- untreated	STW	28.8	7.94	1.4	13.8
HK31	Stonecutters- treated	STW	28.8	7.56	0.8	13.8

[a] DO: Dissolved Oxygen; [b] STW: Sewage Treatment Works

The expression of the aromatase gene (CYP19) was the most affected of the 4 studied, genes (Table 2). CYP19 expression was significantly decreased by sample extracts from 2 of the 8 fishing culturing zones sampled (HK05 and HK08). A sample from a small fish culture zone, known as Wong Wan (HK03), did not produce significant changes in the expression of the aromatase gene but increased the expression of 3BHSD2 and CYP11B2. Only increments in CYP11B2 were statistically significant and no changes in hormone production were exerted by this sample. The extract from one particular sample, HK22, taken close to the Aberdeen and Ao Lei Chao sewage treatment works, not only decreased significantly the expression of the aromatase gene but also the expression of the androgenic gene CYP17. Although extracts from this sample also increased by more than 2-fold the production of P and E2 hormones, the increases were not statistically significant when compared to solvent control values.

Extracts of waters from the Central reclamation site (HK16) located between Kowloon and Hong Kong Island, not only suppressed the expression of the aromatase gene, but also induced by almost 10-fold the expression of the aldosterogenic gene CYP11B2. Sample HK25 corresponding to the extract of waters receiving effluents from the old STW from San Wa, with a flow of 36000 x 1000 m^3/yr and 7300 ton/yr of suspended solids, not only suppressed the expression of CYP19 but also increased significantly, by almost 4-fold, the production of E2 (Table 3). CYP19 was also decreased by several sample extracts taken from sites affected by effluents of major public sewage treatment works. Despite the decrease in the expression of the aromatase gene, no consequent significant alterations on E2 production were observed for samples affecting CYP19 expression.

Table 4.2 Gene expression results for methanolic water extracts.

	Gene							
	CYP19		CYP17		3BHSD2		CYP11B2	
Sample	Mean	STD	Mean	STD	Mean	STD	Mean	STD
Blank	1.08	0.57	1.37	1.38	1.20	0.18	1.03	0.03
MeOH	1.00	0.02	0.96	0.05	1.07	0.10	1.00	0.01
HK01	1.04	0.12	1.36	0.21	1.87	0.28	1.56	0.32
HK11	0.03*	0.00	0.68	0.14	0.92	0.11	2.29	0.19
HK02	1.13	0.10	1.09	0.06	2.08	0.27	4.53	1.67
HK03	1.03	0.04	0.97	0.13	2.60	1.34	5.31*	0.10
HK05	0.04*	0.01	0.76	0.21	0.80	0.07	3.52	0.36
HK06	1.16	0.09	1.48	0.35	0.89	0.41	2.64	0.35
HK07	0.97	0.10	0.97	0.03	ND	ND	ND	ND
HK08	0.04*	0.00	0.59	0.05	0.96	0.05	2.30	0.21
HK10	0.93	0.02	0.95	0.14	1.55	0.69	4.57	0.28
HK12	1.02	0.01	0.92	0.03	1.72	0.41	1.19	0.38
HK13	1.08	0.20	1.02	0.11	1.31	0.44	2.80	0.52
HK14	0.02*	0.01	0.35	0.43	0.64	0.52	2.28	0.40
HK15	0.96	0.01	0.89	0.01	0.73	0.05	1.80	0.39
HK16	0.01*	0.00	0.53	0.05	0.40	0.00	9.87*	0.54
HK17	0.01*	0.00	1.10	0.07	0.38	0.03	3.48	0.33
HK19	1.12	0.05	0.94	0.14	0.87	0.30	1.89	0.44
HK20	0.01*	0.00	0.90	0.01	0.32	0.02	3.90	0.35
HK21	1.07	0.08	1.02	0.06	1.11	0.32	3.02	0.82
HK22	0.01*	0.01	0.09*	0.07	0.37	0.18	2.48	0.19
HK23	0.17*	0.18	0.42	0.05	0.34	0.13	0.62	0.09
HK24	0.01*	0.00	0.66	0.02	0.41	0.00	3.29	0.65
HK25	0.03*	0.03	0.48	0.62	0.57	0.58	2.81	0.28
HK26	0.01*	0.00	0.78	0.02	0.36	0.03	2.58	0.18
HK27	2.53	0.28	1.82	0.01	0.62	0.09	1.93	0.16

All exposures were conducted for 48 h under standard conditions. All gene expression values for fold change relative to control given as means and standard deviations. * Indicates statistically significant differences at $p<0.05$

Table 4.3 Hormone Production in H295R cells exposed to methanolic extracts of marine waters from Hong Kong. [a]

	Hormone					
	PROG		TEST		ESTR	
Sample	Mean	STD	Mean	STD	Mean	STD
Blank	1.88	1.13	0.95	0.37	1.24	0.51
MeOH	1.00	0.21	1.00	0.13	1.00	0.02
HK-01	2.57	0.66	0.96	0.46	2.29	1.05
HK-02	2.86	1.24	1.10	0.24	2.97	0.90
HK-03	1.58	0.33	1.01	0.23	2.43	0.04
HK-05	1.88	0.43	1.34	0.23	2.76	1.06
HK-06	1.13	0.42	0.73	0.10	0.92	0.05
HK-07	2.90	1.92	1.01	0.08	2.48	0.15
HK-08	1.31	0.34	0.91	0.09	2.67	0.12
HK-10	0.77	0.02	0.68	0.09	0.83	0.18
HK-12	1.66	0.88	0.69	0.14	2.22	0.81
HK-13	1.72	1.10	0.80	0.04	1.03	0.25
HK-14	3.01	0.72	1.04	0.07	2.32	0.13
HK-15	0.58	0.38	0.32	0.06	0.84	0.07
HK-19	1.96	0.83	0.77	0.15	1.15	0.16
HK-21	1.86	1.15	0.99	0.19	1.14	0.38
HK-22	2.26	1.29	0.99	0.20	3.04	0.57
HK-25	3.11	1.30	1.32	0.15	3.61*	0.57
HK-27	1.14	0.05	0.95	0.08	0.89	0.10
HK-16	0.93	0.15	0.87	0.06	0.84	0.12

[a] All exposures were conducted for 48 h under standard conditions. All hormone production values are expressed as fold change relative to control given as means and standard deviations. PROG:Progesterone; TEST:Testosterone; ESTR:Estradiol

* Indicates statistically significant differences at $p < 0.05$.

DISCUSSION

Results from this study strongly suggest that treatment used in both plants, Sha Tin and Stonecutters Island are effectively removing most of the compounds and metabolites present in raw waters that may exert significant changes in endocrine functions such as steroid production. Moreover, it may suggest that effluents from these water treatment plants reaching the aquatic environment may not represent a threat for endocrine activities of living organisms in the surrounding areas.

The cause of the cytotoxicity provoked by 2 of the extracts from marine waters from areas used as fish culture zones (HK04 and HK09) remains unknown. The compounds causing the toxicity of these extracts may be of industrial origin; moreover their presence in such a complex mixture may provoke interactions that may be responsible for the extreme toxicity caused in the H295R cells. Further evaluation of these 2 samples must be conducted because the high toxicity, and probable unwanted effects, may be transmitted through the food chain and may be reaching microorganisms and wildlife from other ecosystems and most importantly, humans.

The aromatase gene CYP19 regulates the production of the aromatase enzyme responsible for converting T to E2. Several of the Hong Kong sample extracts were capable of decreasing significantly the expression of the CYP19 gene although no significant changes were observed in E2 production. Moreover, some samples (HK25) even showed an increase E2 production. This probably indicates the presence of mixtures of compounds that may affect aromatase gene expression but do not affect the activity of the aromatase enzyme *per se*, or that are capable of increasing E2 production by other unknown mechanisms.

Studies conducted in the northwestern waters of Hong Kong have indicated the continued presence of polychlorinated biphenyls (PCBs) and organochlorine pesticides together with considerable bioaccumulation of metals in marine mammals native to the region (Ip et al., 2003; Tam and Yao, 2002). Results of exposure of H295R cells to PCBs have previously demonstrated the down-regulation of the androgenic gene CYP17 by these chemicals (Li and Wang, 2005), thus, the presence of these compounds in the samples collected in the Aberdeen and Ao Lei area (HK22) may be responsible for the observed decreases in the expression of this gene.

To assess realistically the significant up-regulation of the CYP11B2 expression and E2 production observed in these results, and most of all, the significant decreases in the expression of CYP19 and CYP17 and the severe toxicity caused by several of the water extracts further investigation must be conducted. Chemical analysis is required to formally link the effects to known EDCs.

CONCLUSIONS

The results from this study confirm that the H295R cell bioassay is a reliable method to identify the potential endocrine disruptive effects of environmental contaminants. Although the constant upgrade in the infrastructure of the newly constructed WWTPs in Hong Kong together with the new strategies established for sewage disposal in the region provide good results in terms of the elimination of EDCs from waste waters, the significant endocrine disrupting effects caused by complex environmental samples from marine and other surface waters are a clear indication of the presence of pollutants in the different aquatic ecosystems in Hong Kong waters. These pollutants possibly come

from sources other than the effluents coming from the modern WWTPs. Chemical analysis to identify and quantify compounds present in the marine water extracts may provide more specific information about the chemical complexity of the samples to which cells were exposed. Based on results from this study, additional *in vitro* and *in vivo* experiments should be conducted to determine the physiological effects that may indicate the molecular changes of gene expression and hormone production observed after exposure with compounds extracted from the water samples.

The development of tools such as the H295R bioassay helps considerably not only to identify the potential effects that these contaminants may exert on living organisms, but also to elucidate some aspects of their chemical origin when it is unknown. The H295R cell bioassay may be a useful tool for the identification of areas where the presence of environmental pollutants is suspected. Moreover, this rapid, sensitive, and cost-effective cell bioassay may be also used to evaluate the endocrine toxicity of residual waters, industrial or STW effluents entering aquatic environments.

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SUMMARY AND CONCLUSIONS

The studies in this dissertation are centered on the validation and evaluation process of the novel H295R cell bioassay recently developed as a pre-screening system for the analysis of the potential endocrine disruptive effects of compounds. Chapter 1 described briefly the functions of the endocrine system and more in detail the steps of the steroidogenic pathway; it also described the different mechanisms by which thousands of compounds may interfere with the normal functions of the endocrine system in humans and wildlife, emphasizing the importance of the continuous development of practical tools for the early detection of the different endocrine disruptive properties of compounds. Since hormones, the main executors of endocrine functions, exert their actions through receptor mediate mechanisms, most of the assays available for the evaluation of endocrine disruptive properties of compounds are based on chemical-receptor interactions; since several studies have demonstrated that endocrine disruptive effects are not only exerted via receptor interaction, but others such as inhibition of hormone synthesis, transport or metabolism, new methodologies are urgently required to complete the evaluation of the potential endocrine disruptive properties of substances. The H295R cell line, a human cell line derived from carcinogenic tissue of the adrenal glands, represents the ideal system for the evaluation of chemical effects on steroidogenesis. The ability of this cell line of produce most of

the steroidogenic hormones synthesized in the 3 different zones of a normal adrenal gland, allows the evaluation of the potential modulative effects of compounds on steroidogenesis at the level of gene expression, enzyme activity and steroid production.

The versatile method developed during the execution of this thesis uses the H295R cell line as the exposure system. Exposure and Q-RT-PCR methods were developed for the analysis of the expression of genes encoding for 10 of the most important enzymes controlling the steroidogenic pathway. Chapter 2 of this dissertation focused on the validation of the developed molecular methods using model chemicals which are well known for their inductive or down-regulative properties. Dose response curves and time course experiments using the developed exposure and PCR methods were conducted to evaluate their validity and versatility in identifying concentration changes and exposure times. The model chemical with inductive effects chosen for the validation study here described was forskolin, a compound capable of resensitizing cell receptors by activating the enzyme adenylcyclase and increasing the levels of cyclic AMP in cells, which is an important signal carrier that is necessary for the proper biological response of cells to hormones. Among the inhibitory chemicals analyzed were aminoglutethimide, because of its properties as an aromatase inhibitor; ketoconazol and metyrapone were also included for its inhibitory effects on the enzymes 14-α-desmethylase, and 11-β-Hydrolase which is responsible for the conversion of deoxycortisone to corticosterone. In addition, the H295R cell bioassay was tested in its effectiveness identifying interactive effects when exposed to mixture of inducers and inhibitory chemicals, as the real EDCs are present in the environment when they exert their disruptive effects. Furthermore, quantification of hormone

production after exposure to individual chemicals and binary mixtures was conducted. Progesterone, testosterone and estradiol were the steroidogenic hormones analyzed by the ELISA methods described in Hecker, et al. The results from the dose response and time course experiments, proved the versatility of the H295R cell bioassay to show that not only genes respond differently to the changes of chemical concentration, but also that groups of genes react in similar manner to these concentration changes, which may suggest a mechanistic linkage in the gene regulation process. Gene expression data also showed that the most affected genes were those encoding for enzymes regulating final hormone production rather than those involved in substrate production which may be an indication of the sensitivity of the final reactions leading to the final products. The differences in effects on gene expression at different exposure times indicate that time is an important factor when evaluating steroidogenic gene expression. The data collected after the exposures with mixture of chemicals with inductive and inhibitory activity showed the complexity of the mechanistic effects taking place when chemicals with such difference in properties interact. Moreover, it was clearly observed that individual responses can not always predict interactive effects. One important conclusion reached after correlating gene expression and hormone data was that hormone production is not necessarily directly affected by changes in gene expression.

After the results from the validation process detailed in chapter 2, the effectiveness of the H295R cell bioassay was tested through exposures with real environmental contaminants with high potential for endocrine disruptive effects. Because of their uncontrolled and demanding use, and their continuous presence in aquatic environments, 18 of the pharmaceuticals most used in the United States where

chosen for the evaluation process. Prescription and over the counter drugs of different therapeutic groups and of known and unknown endocrine disruptive properties were chosen for the evaluation process of the H295R cell system. Antibiotics, growth promoters, analgesics, anti-inflammatory compounds, anti-lipidics, cancer therapy drugs were among the chemicals evaluated. The H295R cells were exposed to high and environmentally relevant concentrations of the pharmaceuticals chosen. Gene expression and hormone production was evaluated by therapeutic groups and statistical correlations between gene expression and hormone data were established. Dose-response curves were constructed and interactive effects were evaluated through exposures to several binary mixtures of drugs. Results demonstrated that compounds different to those already classified as EDCs may produce changes in steroidogenic gene expression and hormone production. Although compounds with the same mechanism of action may show similar gene expression profiles, the hormone production effects may be different. Not all the genes evaluated reacted to changes in concentrations of the drugs. No statistical correlations between gene expression and hormone production were observed, but correlations among genes were shown to be significant. Perhaps the main conclusion of this part of the study was that the H295R cell bioassay is capable of detecting the steroidogenic effects of real environmental contaminants, individually and when present in complex mixtures.

The applicability of the H295R cell bioassay was proven by the results detailed in chapter 4. The endocrine disruptive properties of marine water samples collected all around the Hong Kong, SAR, China were evaluated using the H295R cell bioassay. Knowing the high levels of pollution found during the last decade the Hong Kong

government through the Environmental Protection Department has established a marine water quality monitoring program that yearly collect data that allows establishing the pollution levels of Hong Kong waters. In total 30 water samples were collected, 4 of which correspond to influents and effluents from 2 of the most important STW in Hong Kong. After extracting 1 liter of each of the samples using phase extraction cartridges, the H295R cells were exposed to the methanolic extracts. The responses of gene expression and hormone production after the exposures confirmed the presence of EDCs in Hong Kong marine waters. Moreover, areas with possible presence of pollutants lethal to the cells were identified since. Extracts from effluents of the 2 water treatment plants sampled did not produce significant changes on gene expression or hormone production suggesting the effectiveness of the treatments used in the plants.

Overall, results from the experiments conducted in this dissertation corroborate once more how this novel pre-screening cell system, the H295R cell bioassay, which allows simultaneous analysis of the expression of genes encoding for key enzymes involved in steroidogenesis, is a powerful, practical, sensitive and integrative tool for environmental toxicology and risk assessment studies of endocrine disruptive effects. The H295R cell bioassay is now being validated for use in a tiered screening approach by the US-EPA in order to be considered to replace several currently used assays for determination of endocrine effects.