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**DHEA ACTION IS MEDIATED BY MULTIPLE RECEPTORS AND
METABOLITES**

By

**Kristy K. Michael Miller, B.S.
Indiana University – Bloomington**

**A Dissertation
Submitted to the Faculty of the
Graduate School of the University of Louisville
in Partial Fulfillment of the Requirements
for the Degree of**

Doctor of Philosophy

**Department of Biochemistry and Molecular Biology
University of Louisville
Louisville, Kentucky**

May 2004

DEDICATION

This dissertation is dedicated to my
"Papaw" Paul Jones whose prayers and example
helped me become the person I am today.

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I have many people to thank for helping me attain my goal of finishing graduate school with a Ph.D. in biochemistry. Foremost, is my research advisor, Dr. Russell A. Prough. He invested a great deal of his time and energy teaching me, guiding me, encouraging and supporting me. Therefore, I would like to express my sincerest gratitude to Dr. Prough who not only has been a wonderful mentor but also a good friend. I will forever be indebted to him for his support and guidance in helping me attain my goals as a scientist and making me a better person.

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Finally, I owe everything to my Lord Jesus Christ who has given me the talents of which I can use. It is only by His grace and love that I am strong and enabled to live the life to which I am called. To Christ be all the glory.

ABSTRACT

DHEA ACTION IS MEDIATED BY MULTIPLE RECEPTORS AND METABOLITES

ADVISOR: RUSSELL A. PROUGH Ph.D.

BY KRISTY K. MICHAEL MILLER

MAY 2004

Dehydroepiandrosterone (DHEA) is a C-19 adrenal steroid and the most abundant circulating hormone in humans. Since circulating levels decline in late adulthood, treatment of humans with DHEA has been suggested to have beneficial health effects. Although the mechanism of action is unknown, DHEA may be metabolized to active metabolites that exert their physiological effects by receptor-mediated processes and cell signaling pathways. The purpose of this study was to investigate the mechanistic processes of DHEA action.

Since DHEA may exert its pleotropic effects by being metabolized to biologically active species, a GC/MS method was developed to quantify the liver microsomal metabolism of DHEA of various species and identify the P450 enzymes responsible for metabolism. 16α -hydroxy-DHEA and 7α -hydroxy-DHEA were formed in rat, hamster, pig and human. CYP3A4 and CYP3A5 formed 7α -hydroxy-DHEA, 16α -hydroxy-DHEA, and the unique human metabolite, 7β -hydroxy-DHEA, while the fetal enzyme CYP3A7 formed only 16α -hydroxy and 7β -hydroxy-DHEA. By using this method to examine the metabolite profiles of various P450s, the developmental expression patterns

of the human cytochrome P4503A forms could be classified and therefore have significant clinical relevance.

Nuclear receptors transduce the effects of hormones into transcriptional responses. DHEA and metabolites were screened in a cell-based assay to determine the interaction with estrogen receptors alpha and beta (ER α and ER β). DHEA, DHEA-S, and androstendiol activated ER α , while DHEA, 7-oxo-DHEA, androstenedione and androstenediol activated ER β demonstrating ER is activated directly by DHEA and some metabolites.

These and other studies from our laboratory demonstrate that DHEA is metabolized into various monohydroxylated metabolites. DHEA and metabolites directly activate ER as well as the pregnane X receptor (PXR). Additionally, DHEA has been shown to activate another nuclear receptor, peroxisome proliferator activated receptor alpha (PPAR α) *in vivo*. This research suggests that DHEA action is mediated by multiple receptors and metabolites with various biological activities, comprising of a complex mode of action of DHEA.

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CHAPTER I

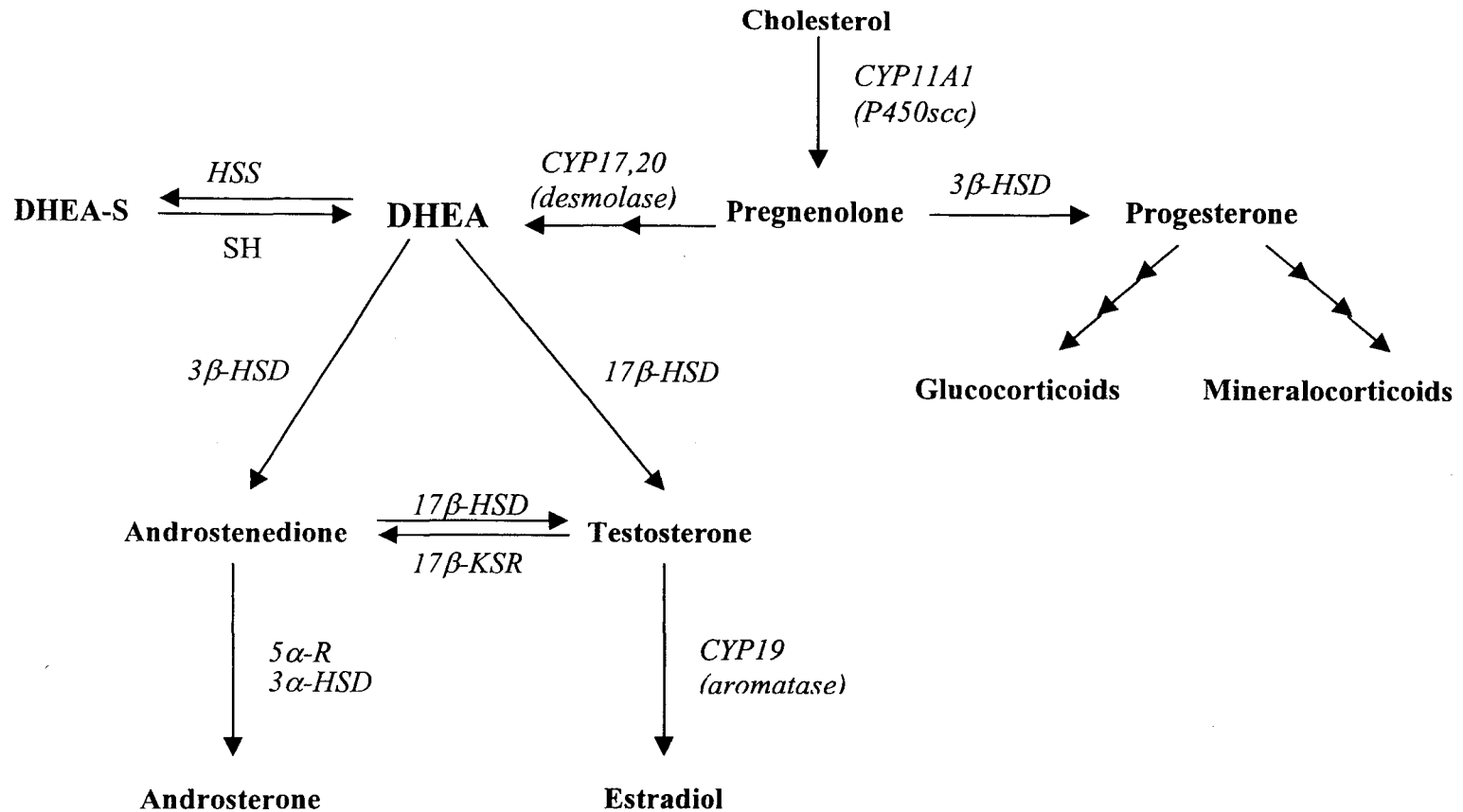
INTRODUCTION

DEHYDROEPIANDROSTERONE

Dehydroepiandrosterone (5-androsten-3 β -ol-17-one) is a naturally occurring C-19 adrenal steroid derived from cholesterol by a series of cytochrome P450 mono-oxygenase and hydroxysteroid dehydrogenase catalyzed reactions (Figure 1).

Dehydroepiandrosterone (DHEA) is secreted primarily by the zona reticularis of the adrenal cortex of humans and other primates. DHEA secretion is controlled by adrenocorticotrophin (ACTH) and other pituitary factors (Nieschlag *et al.*, 1973). The adrenal cortex secretes 75-90% of the body's DHEA, with the remainder being produced by the testes and ovaries (Vermeulen, 1980 and de Peretti and Forest, 1978 and Nieschlag *et al.*, 1973).

Primates produce DHEA by the Δ^5 -steroidogenic pathway in which the double bond at the C-5 and C-6 position is maintained. In this process, the P450 side-chain-cleavage (P450_{scc} or P45011A1) converts cholesterol to pregnenolone. Pregnenolone is then hydroxylated at the C-17 position followed by a two carbon side chain cleavage by P450C17 to form DHEA (Figure 2).



2

Figure 1. Biosynthesis of DHEA and other steroids in humans. The enzymes responsible for conversions are italicized, the listing of more than one enzyme indicates a multisystem process. HSD, hydroxysteroid dehydrogenase; HSS, hydroxysteroid sulfatase; KSR, ketosteroid reductase; R, reductase; scc, side chain cleavage enzyme; SH, sulfohydrolase (Figure adapted from Kroboth *et al. J. Clin Pharmacol.* (1999) 39: 327-348).

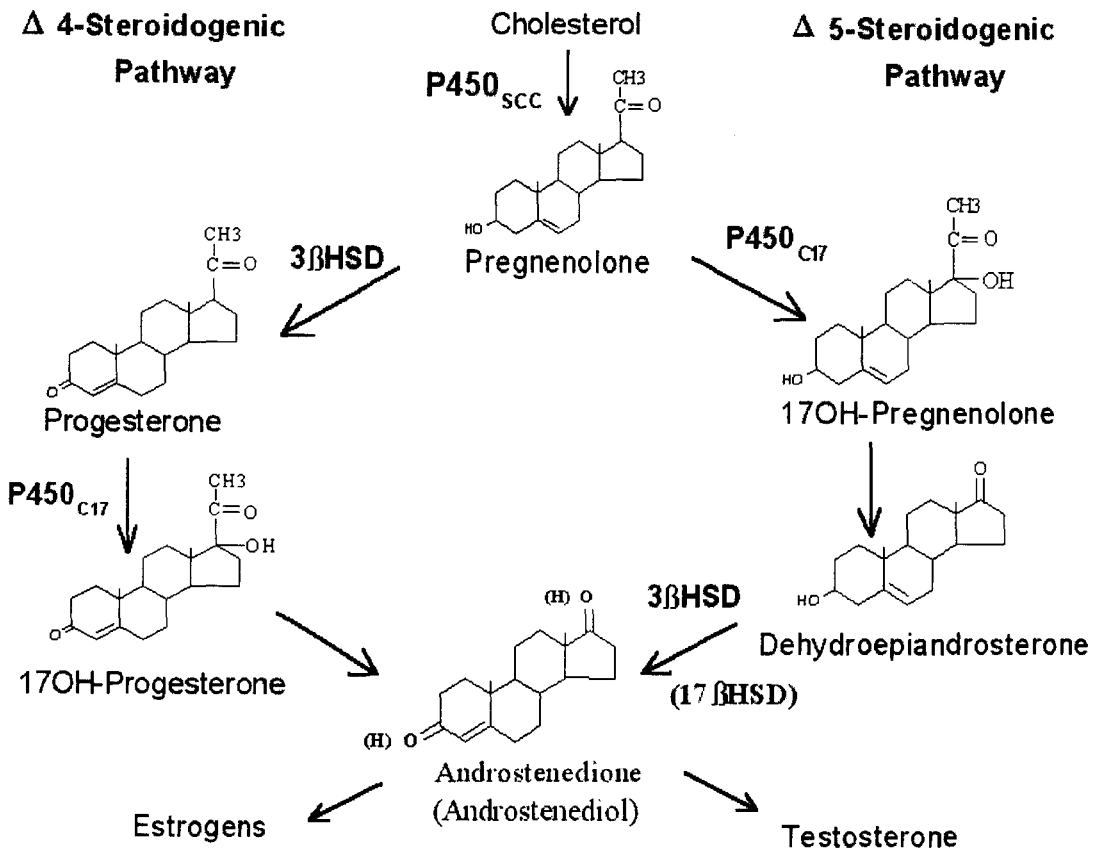


Figure 2. The Δ^4 and Δ^5 Steroidogenic Pathways. (Figure adapted from Conley and Bird. *Biol.Reprod.* (1997) 56:789-799).

Little or no DHEA is produced by the adrenal of nonprimate species, such as mice and rats. Instead, nonprimates produce sex steroids via the Δ^4 -steroidogenic pathway in which cholesterol is converted to pregnenolone by P450_{scc}. Pregnenolone is then converted to progesterone by 3 β -hydroxysteroid dehydrogenase (3 β -HSD) and it is taken up by peripheral steroidogenic tissues and converted to androstenedione by P450C17 (Figure 2).

Although DHEA is the primary sterol in these biosynthetic pathways, DHEA is largely found in circulation in its sulfated form, DHEA 3 β -sulfate (DHEA-S), which can be interconverted with DHEA by DHEA sulfotransferases and hydroxysteroid sulfatases (Regalson *et al.*, 1994). Although DHEA-S is the hydrophilic storage form that circulates in the blood, as stated previously, DHEA is the principle form used in steroid hormone synthesis. Therefore, the differences in tissue-specific expression of DHEA sulfotransferase and steroid sulfatase determine the balance between DHEA interconversions with DHEA inactivation (Allolio and Arlt, 2002).

In humans, plasma DHEA concentrations are found in the range of 1-4 ng/mL (0.003 – 0.015 μ M) (Table I), but circulating DHEA-S concentrations are much greater (Barrett-Connor E *et al.*, 1986 and Hopper and Yen, 1975). Bird *et al.*, (1984) reported that 64% and 74% of the daily production of DHEA is converted to DHEA-S in women and men, respectively, but only about 13% of DHEA-S is hydrolyzed back to DHEA. On a molar basis, circulating DHEA-S concentrations are 250 and 500 times higher (\sim 1 – 10 μ M) than those of DHEA in women and men, respectively (Labrie F *et al.*, 1995). The abundant circulating concentrations of DHEA-S are due in part because DHEA is

Concentration (μM)	Serum Level ($\mu\text{g/dL}$)
0.01	~0.3
0.1	~3
1	~30
5	~150
10	~300
25	~750
50	~1500
100	~3000

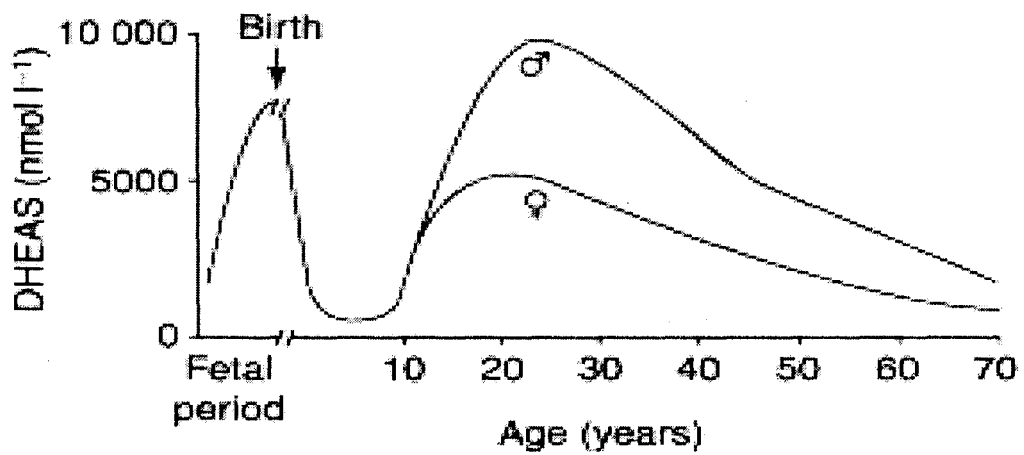
TABLE I. Physiological concentrations of DHEA-S.

cleared from the blood at a rate of approximately 2000 L/day, whereas DHEA-S clearance is about 13 L/day (Lephart *et al.*, 1987). Additionally, DHEA has a half-life in blood of about 1 to 3 hours, while DHEA-S has a half-life of 10 to 20 hours (Rosenfeld *et al.*, 1975). Clearance rate is defined as the volume of plasma that would contain the amount of drug excreted per unit volume. Therefore, clearance expresses the rate of drug removal from the plasma, but not the amount of drug eliminated. The clearance rates of DHEA and its sulfate are also influenced by their protein-binding characteristics. For example, DHEA is weakly bound to albumin, while DHEA-S is strongly bound to albumin.

PHYSIOLOGICAL AND PHARMACOLOGICAL CONCENTRATIONS OF DHEA

In its sulfated form, DHEA is the most abundant circulating sterol in humans, followed by androstenedione. During fetal development, plasma DHEA-S levels are around 100-200 $\mu\text{g/dL}$ (3-7 μM), but fall rapidly after birth and remain low for the first five years of life. Blood DHEA levels then rise and peak around 300 $\mu\text{g/dL}$ (10 μM) during the second decade of postnatal life, followed by an age-dependent decline. Additionally, there are clear gender differences in circulating levels of DHEA-S with higher levels found in men than women (Figure 3).

Labrie and coworkers (1987) suggest that a decrease in 17,20-desmolase (see Figure 1) activity may be responsible for the dramatic age-related reduction in DHEA and DHEA-S secretion. Regardless, the drastic developmental changes in DHEA



TRENDS in Endocrinology & Metabolism

Figure 3. Variation of circulating DHEA-S levels throughout human life. (Figure adapted from Rainey *et al. Trends Endocrinol. Metab.* (2002) 13:234-239).

secretion are not observed by other steroid hormones, suggesting that the mechanisms regulating DHEA formation are unique (Rainey *et al.*, 2002). In contrast, serum cholesterol levels tend to increase with age, while other steroid hormones, decline more slowly relative to DHEA with age. The decline in circulating levels of DHEA and its sulfate derivative appear to be inversely correlated to the rise in cholesterol and the pathophysiological effects of aging (Barret-Conner *et al.*, 1999).

Because the decline in DHEA is associated with some of the pathophysiological effects of aging, many people supplement their own DHEA levels with exogenous DHEA and even refer to DHEA as the “fountain of youth hormone.” When administered, DHEA is usually in an encapsulated powder in two or three divided doses. Although appropriate physiological doses are not well defined and differ in men and women, many clinical studies have been conducted using 50 mg/day for women and 100 mg/day for men.

Currently, DHEA is available over-the-counter as a dietary supplement and is therefore not regulated by the Food and Drug Administration. However, this has not always been the case. DHEA was once marketed for weight loss and in 1985, the FDA banned over-the-counter sales of DHEA. DHEA is still outlawed by the International Olympic Committee and the National Collegiate Athletic Association, but since the passage of the Dietary Supplement Health and Education Act of 1994, DHEA has again been widely available in health food stores in the US (and elsewhere) where it is marketed as a dietary supplement. There are fewer regulations over the rule of nutritional products than with nonprescription or prescription drugs. For example, expiration dates

are not required and there are no chemical standards for the product, and nutritional supplements, such as DHEA, can be sold unless the FDA proves that they are unsafe.

DHEA ACTION

Since DHEA and DHEA-S have higher serum concentrations than other hormones, DHEA has been viewed as a potential androgen, as a storage repository for androgens and precursor to sex hormones (Ebeling and Koivisto, 1994). However, other than being a precursor to sex hormones and playing a role as such in the development of pubic and axillary hair and the development and maintenance of immunocompetence, a physiological role for DHEA has not been defined to date. DHEA is produced by the adrenal gland in humans and is taken up by several tissues, including brain, liver, kidney, and gonads, and is metabolized to androstenediol, testosterone, estrogen and other biologically active steroids, depending on the tissue. The work of Labrie *et al.* (1987) suggest that more than 30% of total androgen in men and over 90% of estrogen in postmenopausal women are derived from peripheral conversion of DHEA-S to DHEA.

Treatment with high doses of exogenous DHEA has been shown to have beneficial effects on lowering body fat and in modulating the effects of diabetes, atherosclerosis, and obesity in rodent models (Yoneyama *et al.*, 1997). Additionally, DHEA has chemopreventative affects when administered to rodents in low doses (Rao *et al.*, 1992 and Lubet *et al.*, 1998). It is purported that in humans, DHEA may also modify the immune response, alter chemical carcinogenesis, reverse the deleterious effects of glucocorticoids, as well as display neuroprotective and memory-enhancing effects

(Robinson *et al.* 2003, Ben-Nathan *et al.* 1992, and Lapchak *et al.* 2001). However, the mechanism of these processes is not known.

Since DHEA is marketed as a nutritional supplement in the US, allowing companies to bypass the rigorous clinical trials required for FDA approval for medicinal use, DHEA has not been subject to the strict quality control measures applied to other drugs. Although DHEA is purported to have many beneficial effects, there is little evidence to support the use of DHEA and there has been no clinical trial that clearly substantiated the evidence and safety for DHEA supplements. Therefore, with the current utilization of DHEA as a dietary supplement purported to protect against diabetes, atherosclerosis, obesity, lupus and arthritis, the mechanism of action of this sterol and its metabolites is important to study.

DHEA METABOLITES

As stated previously, treatment with exogenous DHEA has been shown to have many beneficial effects. The mechanism by which DHEA exerts its beneficial effects may involve the metabolism of DHEA to multiple biologically active metabolites (Fitzpatrick *et al.*, 2001 and Marwah *et al.* 2002). Miller *et al.* (2004) showed that human liver microsomal metabolism of DHEA produced 7 α -OH-DHEA, 16 α -OH-DHEA as well as 7 β -OH-DHEA.

The hydroxylated metabolites of DHEA have been shown to exhibit biological activity. For instance, Morfin and Starka (2001) showed that 7 α - and 7 β -OH-DHEA were efficient in preventing the nuclear uptake of [³H]dexamethasone-activated glucocorticoid receptor in brain cells demonstrating a key event for the neuroprotection

conferred by neurosteroids. Additionally, 16 α -OH-DHEA is known to be the precursor of fetal 16 α -hydroxylated estrogens which are the main phenolic steroids produced during pregnancy (Hampl and Starka, 2000)

CYTOCHROME P450

Cytochrome P450s (CYPs) are a family of hemoproteins that were named as such because a strong absorption band at 450 nm is observed when CO binds tightly to the ferrous heme of the protein. P450s catalyze the NADPH and O₂-dependent monooxygenation of a wide variety of compounds by incorporating one atom of molecular oxygen into the substrate and one atom into water. P450s are capable of catalyzing an extraordinary range of biochemical reactions, from the synthesis of cholesterol, bile acids, and steroid hormones to the oxidative metabolism of drugs and xenobiotics at carbon, nitrogen, sulfur and phosphorous centers.

There are two different kinds of electron transfer chains observed for mammalian P450s. Some P450s are found in the mitochondrial inner membrane and some are found in the endoplasmic reticulum (ER). Both types of P450s are membrane-bound proteins. In the catalytic cycle for P450 reactions (see Figure 4), NADPH-cytochrome P450 reductase separately donates electrons to the P450. Two electrons are acquired from NADPH and transferred singly from FAD to FMN of the reductase, and then to the P450 heme iron (Nelson, online).

CYP genes are arranged into families and subfamilies based on the percentage of amino acid sequence identity. Currently, there are more than 270 different CYP gene

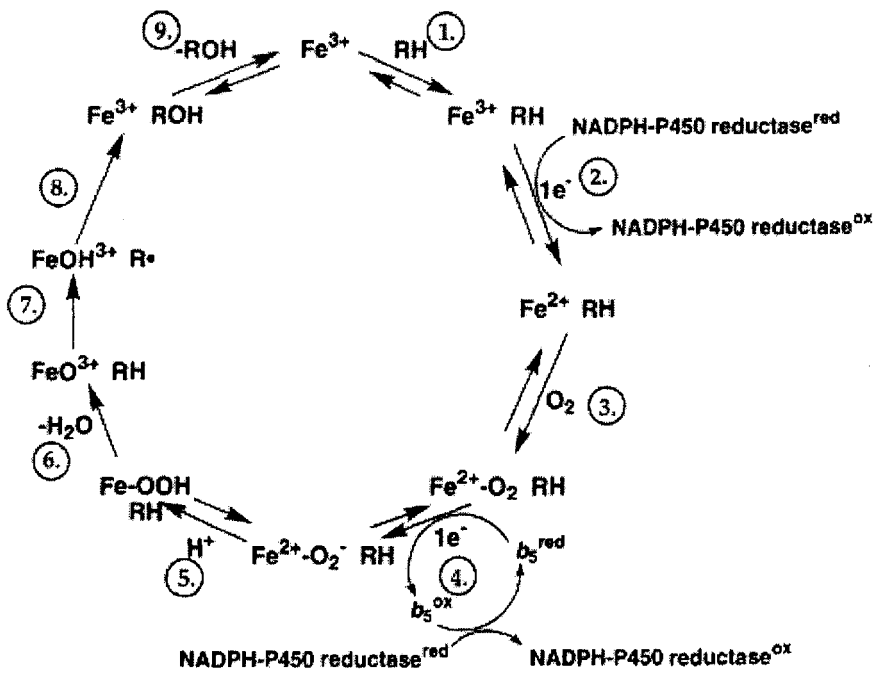


Figure 4. Generalized catalytic cycle for P450 reactions. (Figure adapted from Guengerich *J Biol. Chem.* (1991) 266:10019-10022).

families and 18 recorded in mammals. The human CYP superfamily is composed of 57 genes (Nebert and Nelson, online). These genes code for enzymes that have been known to have toxicological and pharmacological roles involved in metabolizing drugs, xenobiotics, vitamins, steroids, and fatty acids (Table II).

Human P450s that are responsible for the metabolism of toxicological and pharmacological compounds are almost exclusively in the CYP1, CYP2, CYP3, and CYP4 families. However, members of the CYP3A subfamily are the most abundantly expressed P450 enzymes in the human liver and gastrointestinal tract and are known to metabolize more than 120 frequently prescribed drugs, as well as steroids and bile acids (Nebert and Jorge-Nebert, 2002). Four human CYP3A enzymes have been identified; CYP3A4, CYP3A5, CYP3A7, and CYP3A43. CYP3A4 and CYP3A5 are the most abundantly expressed P450 enzymes and are expressed in adult human liver, while CYP3A7 is most prominently expressed in fetal liver. CYP3A43 is expressed at much lower levels in the human liver and its function is not known (Komori M *et al.*, 1989).

Many P450s function as steroid hydroxylases. For instance, members of the CYP7, CYP8, CYP27, CYP39, and CYP46 family of enzymes play a role in bile acid synthesis by hydroxylating cholesterol and subsequently oxidizing the resulting eight carbon side chain to generate water soluble bile acids. Additionally, members of the CYP11, CYP17, CYP19 and CYP21 families participate in steroidogenesis, generating androgens and estrogens from cholesterol. There are other P450s such as the CYP4 family that play a role in the metabolism of fatty acids, arachidonic acid, leukotrienes,

Human P450	Rat Analog	Substrate/Function
1A1	1A1	Foreign chemicals, arachidonic acid, eicosanoids
1A2	1A2	Foreign chemicals, arachidonic acid, eicosanoids
1B1	1B1	Foreign chemicals, arachidonic acid, eicosanoids
2A6	2A2	Foreign chemicals, arachidonic acid, eicosanoids
2B6	2B1	Foreign chemicals, arachidonic acid, eicosanoids
2C8	2C11	Foreign chemicals, arachidonic acid, eicosanoids
2C9	2C12	Foreign chemicals, arachidonic acid, eicosanoids
2C19	2C13	Foreign chemicals, arachidonic acid, eicosanoids
2D6	2D2	Foreign chemicals, arachidonic acid, eicosanoids
3A4	3A23	Foreign chemicals, arachidonic acid, eicosanoids
3A5	3A9	Foreign chemicals, arachidonic acid, eicosanoids
3A7	3A18	Foreign chemicals, arachidonic acid, eicosanoids
4A11	4A1	Fatty acids, arachidonic acid, eicosanoids
7A1	7A1	Cholesterol, bile acid synthesis
8B1	8B1	Prostacyclin synthase, bile acid synthesis
11B1	11B1	Steroidogenesis
17A1	17A1	Steroid 17 α -hydroxylase, 17/20 lyase
19A1	19A1	Aromatase to form estrogen
21A2	21A2	Steroid 21-hydroxylase
27A1	27A1	Bile acid biosynthesis, vitamin D ₃ hydroxylations
39A1	39A1	24-hydroxycholesterol, 7 α -hydroxylase
46A1	46A1	Cholesterol 24-hydroxylase

TABLE II. Substrates and functions of human and rat CYP genes. (Table adapted from Nebert and Russell (2002) *The Lancet*. 360:1155-1162).

prostaglandin, epoxyeicosatrienoic acids (EETs), hydroxyeicosatetraenoic acids (HETEs), and hydroperoxyeicosatetraenoic acids (HPETEs) (Nebert and Russell, 2002).

The products of many P450 reactions function as ligands for nuclear receptors. For example, P450s catalyze both the formation and degradation of many nuclear receptor ligands. Therefore, nuclear receptors play a key role in the regulation of P450 gene transcription by serving as receptors for a diversity of ligands.

NUCLEAR RECEPTORS

The nuclear receptor superfamily consists of an array of transcription factors that transform extracellular and intracellular signals into cellular response by inducing the transcription of nuclear receptor target genes. Unlike hormones for cell surface receptors, nuclear receptors transduce the effects of small, lipophilic hormones, such as glucocorticoids, mineralocorticoids, sex steroids, and thyroid hormones into transcriptional responses (Mangelsdorf and Evans, 1995).

The nuclear receptor superfamily is comprised of steroid nuclear receptors, orphan nuclear receptors and (retinoic X receptor) RXR heterodimers (Figure 5). Steroid nuclear receptors are receptors for which the hormonal ligand has been identified, whereas the term orphan nuclear receptor was coined to describe gene products that appeared to belong to the nuclear receptor family on the basis of gene sequence similarity, or which the ligand(s), if required are unknown. In addition to steroid receptors and orphan receptors, there are the RXR heterodimers which are nuclear receptors that form heterodimers with the retinoid X receptor. The nuclear receptors that are known to heterodimerize with RXR require RXR for DNA-binding. The activation

Nuclear Receptor Superfamily

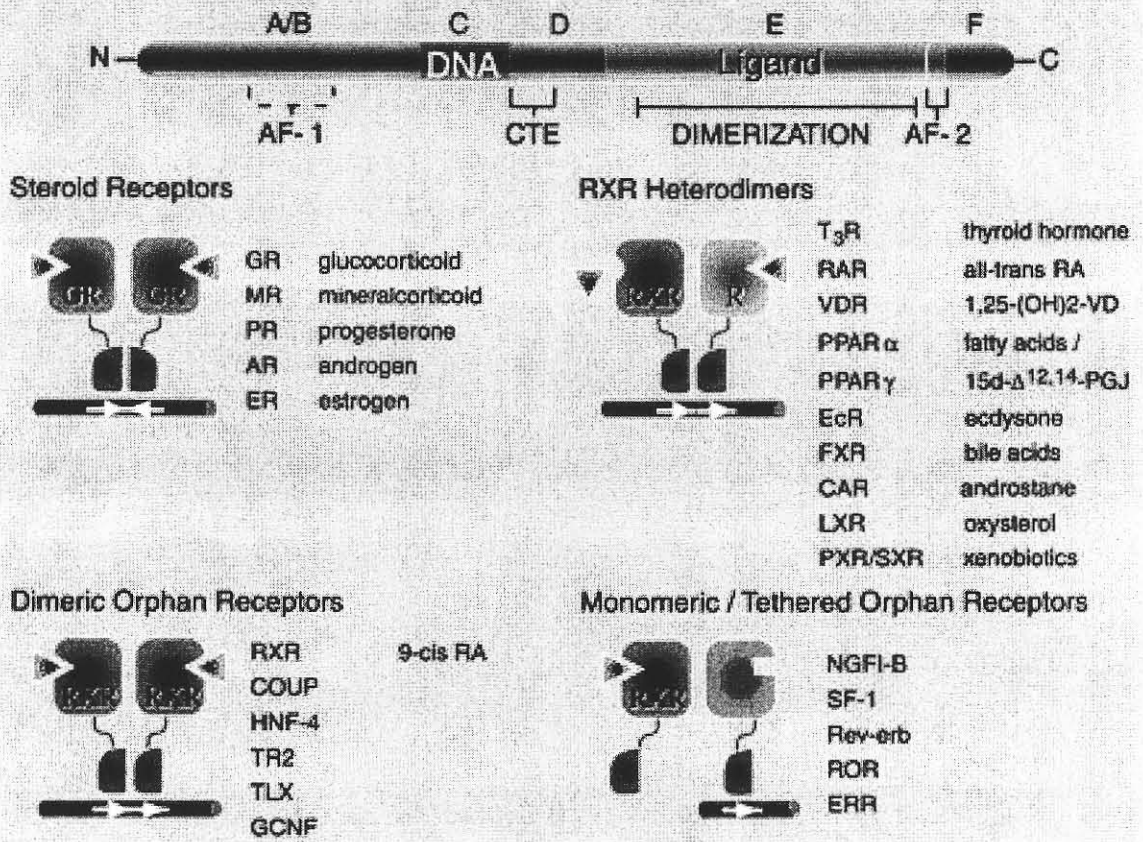


Figure 5. Structure/function organization of nuclear receptors. (Figure adapted from Olefsky (2001) *Journal Biol. Chem.* 276:36863-36864)

state of RXR varies among heterodimers. For instance, RXR can be completely inactive in nonpermissive heterodimers, such as thyroid hormone receptor (TR) and the vitamin D receptor (VDR) (Kurokawa *et al.*, 1994), or be freely active in permissive heterodimers with PPAR (Kliewer *et al.*, 1992).

Nuclear receptors share similarity with classical steroid hormone receptors in their DNA binding domain (DBD), and ligand binding domain (LBD). Nuclear receptors are comprised of certain regions of conserved function and sequence. There are common structural features for all nuclear receptors (Figure 5), such as the DNA binding domain (region C) which is the most highly conserved domain. A variable length hinge domain is located between the DBD and LBD (region D). The N-terminal region contains the Activation Function-1 domain (region A/B) which is a ligand-independent transactivation domain. About 250 C-terminal residues constitute the LBD (region E) that also includes the site for hormone-inducible transcription activating function is present in the LBD (AF-2). Additionally, many receptors contain a variable length C-terminal region (region F) whose function is poorly understood.

A number of molecules that were once thought of as metabolic intermediates are in fact ligands for nuclear receptors, thereby providing a mechanism for coupling metabolic pathways with changes in gene expression. For example, ligands which activate pregnane X receptor (PXR), constitutive androstane receptor (CAR), liver X receptor (LXR), farnesoid X receptor (FXR), and RXR (steroids and xenobiotics, androstanes, hydroxycholesterols, bile acids, and 9-cis retinoic acid, respectively) have been used to identify the biological roles of the receptors and provided insight into the regulation of glucose, lipid and drug metabolism. Additionally, the role of nuclear

receptors in human diseases and their importance as therapeutic targets have implications in human biology, as well as, understanding and development of new drug treatments (Kliewer *et al*, 1999).

ESTROGEN RECEPTOR

The estrogen receptor (ER) is a member of the nuclear receptor superfamily that mediates the biological responses of estrogens and is perhaps one of the most well defined nuclear receptors. Estrogens influence a wide range of physiological processes including growth, differentiation, and the development of reproductive tissues, bone density maintenance, liver, fat and bone cell metabolism, cardiovascular and neuronal activity as well as embryonic and fetal development. Estrogens also influence several pathological processes such as breast, endometrium and ovarian cancers, osteoporosis, atherosclerosis and Alzheimer's disease (Norman and Litwack, 1987). Estrogens have both desirable and harmful effects on certain pathological processes, but the mechanisms of these processes are poorly understood.

The biological actions of estrogens are mediated by estrogen binding as a ligand to one of two specific estrogen receptors (ERs), ER α (NR3A1) and ER β (NR3A2). Although they both mediate the effects of estrogen, the two receptors have unique and distinctly different patterns of expression within the human (Figure 6). 17 β -estradiol (E2) is the typical ER ligand. The classic E2 target tissues have a high ER α content and respond to E2 challenge with increases in transcription of certain genes containing well-documented estrogen responsive elements (EREs) 5'-GGTCAnnnTGACC-3' (n is any nucleotide) within the promoter region or 5'- flanking region of the target gene (Klinge,

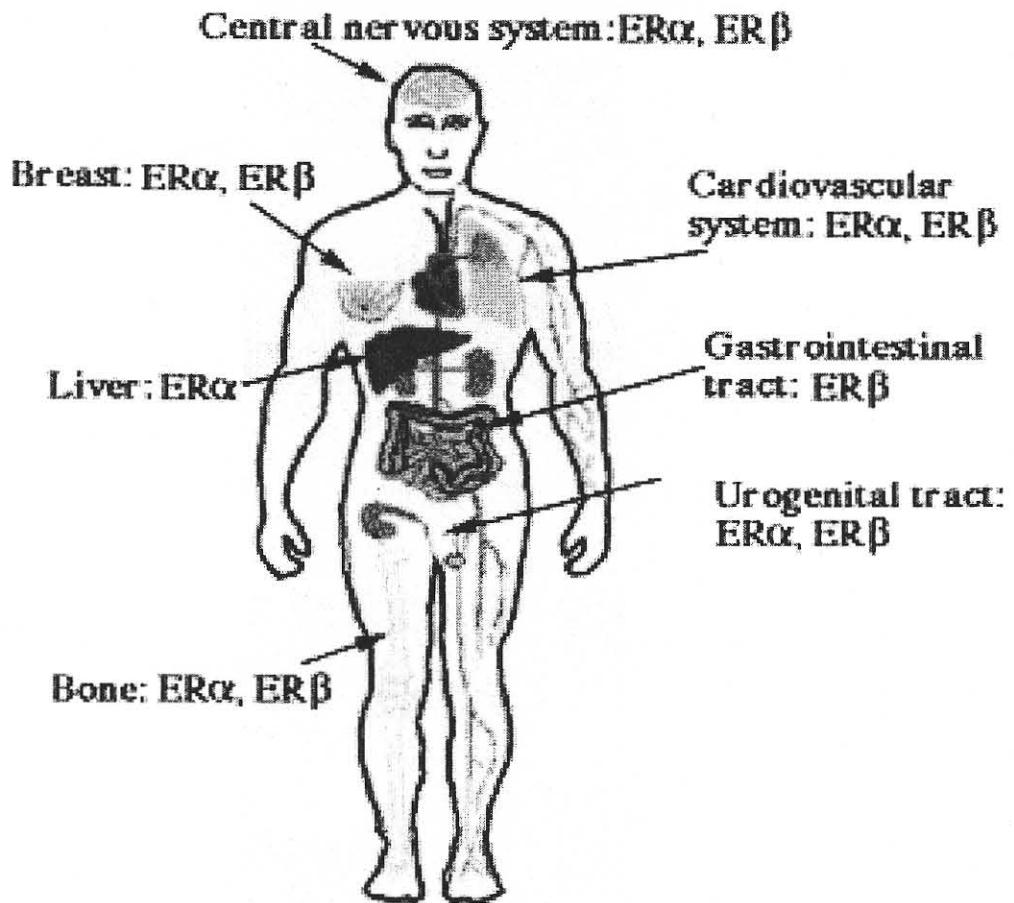


Figure 6. Localization of ER isoforms. ERα and ERβ have distinctly different locations and concentrations within the human (Figure adapted from Gustafsson. (1999) *J. Endocrinol.* 163:379-383).

2001). The classic E2 target tissues defined in the past are the uterus, mammary gland, placenta, liver, central nervous system (CNS), cardiovascular system, and bone. In other target tissues, the expression of ER α is either very low or non-detectable, while ER β is highly expressed. ER β target tissues include prostate, testis, ovary, pineal gland, thyroid gland, parathyroids, adrenals, pancreas, gallbladder, skin, urinary tract, lymphoid and erythroid tissues (Gustafsson, 1999). Since ER α and ER β are differentially expressed among tissues, both subtypes of the receptor are regulated in a tissue- and/or cell-specific manner (Zhou *et. al.*, 2001).

ER α was cloned in 1986 and ten years later, ER β was discovered in rat prostate (Figure 7). There is a 97% amino acid identity between the two receptors in the DBD, suggesting that ER β can recognize and bind to similar EREs as ER α . However, because the LBD homology is only 47% between ER α and ER β , each receptor may have a distinct spectrum of ligands by which they are activated (Kong *et. al.*, 2003 and Paech *et al.*, 1997). Indeed, ER β shows higher affinity for a number of phytoestrogens compared to ER α .

In absence of ligand, ER α is localized within an inhibitory heat shock protein complex. Upon ligand binding to an estrogenic compound, ER changes its conformation, causing displacement of heat shock proteins, recruitment of coregulator proteins and other transcription factors (Rachez and Freedman, 2001). The formation of this preinitiation complex promotes the binding of ER as a homodimer or heterodimer to EREs. Once bound to DNA, transcription is initiated, thereby regulating the activation or repression of ER target genes. In addition to direct binding of ER to DNA, ER can also regulate transcription via a "tethering" mechanism in which ER interacts with other DNA

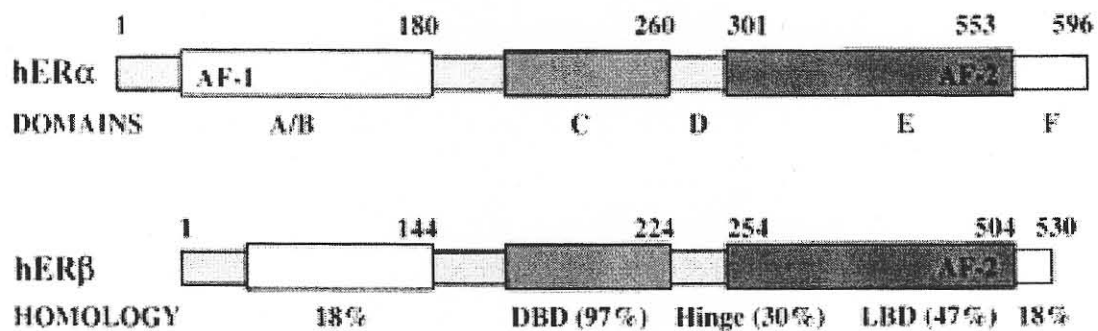


Figure 7. Domain structure representation of human ER α and ER β isoforms.

(Figure adapted from Kong *et al.* (2003) *Biochemical Society Transactions* 31:56-59)

bound transcription factors, i.e. AP-1 (Kushner, 2000), NF- κ B (McKay and Cidlowski, 1998), and SP-1 (Safe, 2001) that stabilize the DNA and recruit other coactivators to the transactivation complex (Webb *et al.*, 1999).

The ligand-dependent transcriptional activity of ER is mediated by various domains within the receptor sequence. Although ER α and ER β share only 59% homology within the LBD, the DBD is highly conserved in both ER α and ER β , and contains two distinct zinc fingers that play a critical role in DNA sequence specific receptor binding and receptor dimerization. The AF-1 domain of ER has been found to be stimulated through phosphorylation by mitogen-activated protein kinase (MAPK) (Kato *et al.*, 1995). However, there is little or no sequence homology between the two receptors within the N-terminal region due to the truncated N-terminal region of ER β receptor (Figure 7) resulting in a lack of ER β AF-1 activity.

Due to the lack of sequence homology in the N-terminal AF-1 and C-terminal AF-2 regions, the two receptors not only exhibit distinctive response to estrogenic compounds, but ER β can function as a dominant inhibitor of ER α transcriptional activity (Hall and McDonnell, 1999). Because their AF domains exhibit distinct properties, AFs regulate ERs in a cell and promoter specific manner (Matthews and Gustafsson, 2003). Therefore, although ER mediates the cellular responses of an estrogenic stimulus, the functional response is dependent on tissue, pathway of regulation, and protein in which the receptor action is mediated.

CHAPTER II

**STEREO- AND REGIOSELECTIVITY ACCOUNT FOR THE DIVERSITY OF
DHEA METABOLITES PRODUCED BY LIVER MICROSOMAL
CYTOCHROMES P450**

(This chapter was published in *Drug Metabolism and Disposition* 32:305-313)

INTRODUCTION

Dehydroepiandrosterone (DHEA) is a 19-carbon steroid derived from cholesterol by a series of cytochrome P450 mono-oxygenase and hydroxysteroid dehydrogenase-dependent reactions (Conley and Bird, 1997). In its sulfated form, DHEA is the most abundant circulating steroid in humans and is a precursor to the sex steroids, estrogen and testosterone. Levels of DHEA-S in the circulation are high during fetal development (1-5 μM), but fall rapidly after birth and remain low for the first five years of life. DHEA and DHEA-S levels in blood then rise and peak during the second decade ($\sim 10 \mu\text{M}$), followed by an age-dependent decline for individuals age 30 or above (Herbert, 1995). The developmental changes in circulating levels DHEA and DHEA-S in the blood are not paralleled by other steroid hormones, suggesting the mechanisms regulating DHEA formation in adrenal are unique (Rainey *et al.*, 2002). In contrast, serum cholesterol levels tend to increase with age, while DHEA levels decline with age. The decline in circulating levels of DHEA and its sulfate derivative appear to be inversely correlated to

the rise in cholesterol and the pathophysiological effects of aging (Barrett-Connor *et al.*, 1999).

Treatment with exogenous DHEA has been shown to have beneficial effects in lowering body fat and modulating the effects of diabetes, atherosclerosis, and obesity in rodent models (Yoneyama *et al.*, 1997). Additionally, DHEA has cancer chemopreventative actions when administered to rodents in low doses (Lubet *et al.*, 1998; Rao *et al.*, 1992). However, at higher doses, DHEA can cause peroxisome proliferation resulting in hepatomegaly (Frenkel *et al.*, 1990) and subsequent development of hepatocarcinomas (Rao *et al.*, 1992). With the current utilization of DHEA as a dietary supplement proposed to protect against diabetes, atherosclerosis, obesity and arthritis, the mechanism of biological action of this sterol and its metabolites have become important to study.

Since the rat adrenal does not express CYP17, the rat does not produce DHEA in the adrenal (Kalimi and Regelson, 1990; Voutilainen *et al.*, 1986). However, DHEA is formed in the human adrenal and is a precursor to sex steroids (Figure 1). In humans, DHEA circulates as the 3 β -sulfate conjugate DHEA-S until taken up by target tissues where it is then converted to DHEA by sulfatases (Burstein and Dorfman, 1963). In steroidogenic tissues, DHEA is metabolized to androgens and estrogens by hydroxysteroid dehydrogenase reactions. However, other oxidative pathways of DHEA metabolism have not been extensively studied.

The beneficial effects resulting from exogenous administration of DHEA may involve the metabolism of DHEA to multiple biologically active species (Fitzpatrick *et al.*, 2001; Marwah *et al.*, 2002). Fitzpatrick *et al.* (2001) used LC/MS to identify 7 α - and

16 α -OH-DHEA as the major metabolites produced by the human along with another mono-hydroxylated DHEA species whose position of hydroxylation was unknown. The purpose of this study was to quantify the liver microsomal metabolism of DHEA by various species and elucidate the P450s responsible for the metabolism of DHEA. A sensitive GC/MS method was developed to identify and quantify all the metabolites produced by the metabolism of DHEA. The results of this study provide a method for quantifying the microsomal metabolism of DHEA and demonstrate the regio- and stereoselectivity of specific CYPs that accounts for the unique DHEA metabolite profiles formed by various species.

MATERIALS AND METHODS

Chemicals. DHEA, 7 α -hydroxy-DHEA, 7 β -hydroxy-DHEA, 16 α -hydroxy-DHEA, androstenedione and etiocholanolone were purchased from Steraloids, Inc. (Wilton, NH). Human liver samples were kindly provided by F. Peter Guengerich (Center for Molecular Toxicology, Vanderbilt University School of Medicine, Nashville, TN). The use of these human tissue samples were approved by the Institutional Review Boards of the University of Louisville and Vanderbilt University. The human P450 baculovirus system used to provide functional CYP preparations was designed to express both CYPs and P450 oxidoreductase using a suspension culture of baculovirus-infected insect cells (Rushmore *et al.*, 2000). Fresh membrane fractions were prepared at Merck Research Laboratories and the metabolic assays were performed at the University of Louisville.

Animals. Male Sprague-Dawley rats (225 g, HSD:SD) from Harlan, Indianapolis were maintained on control diet (AIN-76A ICN Biomedicals, Cleveland, OH) for 5 days. Animals were anesthetized with CO₂ and the livers perfused with 0.9% sodium chloride prior to dissection from the body. Livers were cut into small pieces and then homogenized in a Potter-Elvehjem homogenizer containing 4 volumes of 50 mM potassium phosphate buffer, pH 7.4, containing 0.25 M sucrose per gram liver. Microsomal fractions was isolated by differential centrifugation as described by Remmer *et al.* (1966). Microsomal fractions were resuspended in 0.1 M Tris-HCl buffer (pH 7.4), containing 0.25 M sucrose and sedimented a second time. The final preparation was resuspended in Tris-HCl buffer containing sucrose and 10% glycerol and stored at -70°C for up to 3 months without loss of activity. Protein concentrations were determined by measuring formation of bicinchoninic acid Cu¹⁺ complex at 562 nm.

NADPH: cytochrome c oxidoreductase assay. The baculovirus expression system allows coexpression of both P450 and its flavoprotein oxidoreductase (Rushmore *et al.*, 2000) and NADPH: cytochrome *c* oxidoreductase activity was measured to characterize the enzymatic efficiency in this baculovirus-expression system. The reactions were carried out at 25°C in 0.05 M potassium phosphate buffer, pH 7.4 containing 100 μM NADPH, 40 μM cytochrome *c*, and aliquots of the P450 sample being characterized. The absorbance change at 550 nm was monitored at 25° C with a Cary 50 Bio UV-Visible spectrophotometer assuming a molar absorptivity of 21,100 M⁻¹ cm⁻¹ (Masters *et al.*, 1967). The P450/P450 oxidoreductase ratios for CYP3A4, CYP3A5, CYP3A7, CYP2B6, and CYP2B1 preparations are shown in Table III. The ratios for all CYPs

TABLE III

Content of P450 and NADPH:Cytochrome P450 Oxidoreductase in various baculovirus preparations.

Sample	P450 Concentration (nmol/mL)	P450 Oxidoreductase (nmol/mL)	P450/POR Ratio
CYP3A4	2.0	1.9	1.0
CYP3A5	1.0	0.5	2.0
CYP3A7	1.5	0.8	1.8
CYP2B6	1.5	1.4	1.1
CYP2B1	1.0	1.3	0.8

The baculovirus expression system allows co-expression of both P450 and its flavoprotein oxidoreductase. The P450 oxidoreductase activity was used to calculate the concentration of flavoprotein using the factor 1,360 μmol cytochrome *c* reduced per minute per μM of oxidoreductase protein (Yasukochi and Masters, 1976). The ratios for all P450s are approximately equal or more than one, indicating that the content of P450 oxidoreductase is most likely not rate limiting.

prepared were near 1, indicating that the content of P450 oxidoreductase in the preparations was likely not rate-limiting in the reaction.

DHEA metabolism. Hepatic microsomal protein fractions or recombinant CYPs were incubated in 2 mL reaction mixtures containing 0.1 M Tris-HCl buffer, pH 7.5, 1 mM EDTA, 10 mM MgSO₄ and an NADPH-regenerating system consisting of 1 mM β-NADPH, 0.8 mM isocitrate, and 0.1 U/ml of isocitrate dehydrogenase. The samples were oxygenated by blowing pure O₂ into each tube for 15 seconds. The microsomal fractions and regenerating system were preincubated 4 min. at 37°C prior to addition of 50 μM DHEA. After incubation for specified times at 37°C in a shaking water bath, the reactions were terminated at various times by adding equal volumes of chilled ethyl acetate. The rates of product formation were measured in the linear portion of the time course. The metabolites were extracted from the aqueous phase three times with ethyl acetate and dried under a stream of N₂ gas at room temperature.

Derivatization of samples. DHEA and its metabolites were prepared for GC/MS analysis by adding 50 μl of MOX to the dried metabolites overnight at room temperature to derivatize any oxo-functional groups. The sample was dried under a stream of N₂ gas at room temperature, 50 μl of BSTFA-TMS was added, and the solution incubated at 70°C to derivatize hydroxyl groups. An internal standard, etiocholanolone, was added to each sample prior to extraction with ethyl acetate and analysis by GC/MS.

Gas chromatography/mass spectrometric analysis. Single quadrupole GC/MS was utilized to resolve and quantify the DHEA metabolites, using etiocholanolone as an internal standard. Initial experiments assessed linearity of the reaction with time and protein concentration. Reactions were carried out with microsomes from rat, pig and hamster, as well as five different human samples to assess potential inter-individual variability in product formation. Derivatized DHEA and metabolites were analyzed with an HP5890/HP5973 GC/MS system (Hewlett-Packard, Palo Alto, CA). Separation was achieved by using a bonded-phase capillary column (DB-17MS, 15 m x 0.25 mm I.D. x 0.25 μm film thickness) from J&W Scientific (Folsom, CA). The GC injection port and interface temperature was set to 280°C, with helium carrier gas maintained at 14 psig. Injections were made in the splitless mode with the inlet port purged for 1 min following injection. The GC oven temperature was held initially at 100°C for 0.5 minute, increased at a rate of 30°C min^{-1} to 325°C, increased at a rate of 2°C min^{-1} to 325°C, and then held for 5 min. Eluate from GC was analyzed under 70 eV electron ionization (EI) with full mass scan. The mass scan range measured was m/Z 50-550. The peak area of each metabolite standard relative to that of the added internal standard, etiocholanolone, was determined for selected ion retrieval chromatograms to establish a standard curve for quantitating DHEA metabolite formation. An internal standard curve was prepared for each compound of interest spanning the concentrations above and below those observed in the biological samples measured.

Statistical analysis. Experiments were conducted in triplicate and mean \pm standard deviation (SD) was determined. Statistical significance was determined using a two-tailed Student's *t* test with $p \leq 0.05$ as the criterion for significance.

RESULTS

Analysis of DHEA and its metabolites using GC/MS. Fitzpatrick *et al.* (2001) utilized LC/MS to separate and quantify DHEA and its resulting oxidative metabolites. DHEA was found to be converted by human liver microsomal fractions to 7 α -OH-DHEA, 16 α -OH-DHEA and an unknown mono-hydroxylated compound. 7-oxo-DHEA was also observed if longer incubation times were utilized (Fitzpatrick *et al.*, 2001; Robinson *et al.*, 2003). This method was hindered by poor ionization efficiencies of DHEA and its metabolites under conditions of chemical ionization at atmospheric pressure. For the current studies, the possibility of attaining better sensitivity and resolution of DHEA and metabolites using GC/MS was examined. Therefore, a GC/MS method, utilizing derivatization, was developed to separate and quantitate known DHEA metabolites.

DHEA and its metabolite standards contain keto and hydroxyl functional groups that can be derivatized to form stable and more ionizable molecules. In order to stabilize the compounds and improve their separation by GC, MOX was added to the commercial standards or samples to derivatize oxo functional groups (i.e. prevent keto-enol tautomerization) followed by the addition of BSTFA-TMS to derivatize hydroxyl groups (Figure 8A). The standards were then separated by GC/MS after conditions for baseline separation of all metabolites was achieved (Figure 8B). The identity of the compounds produced was determined by co-migration with authentic standards and identical electron

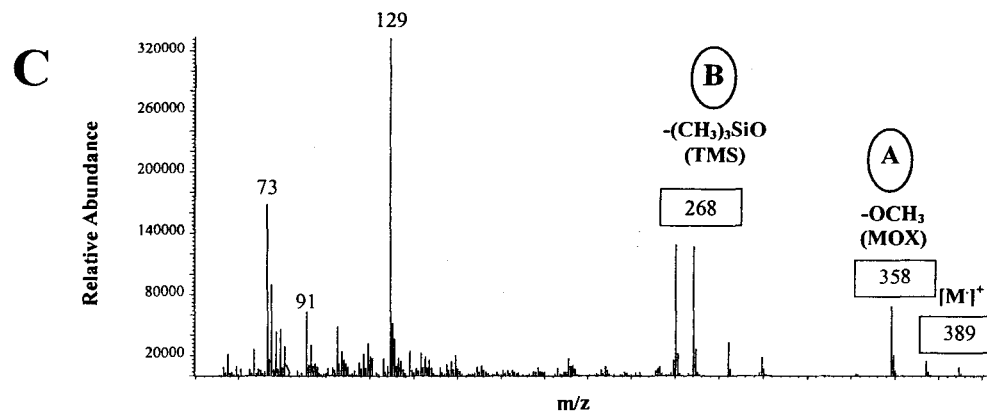
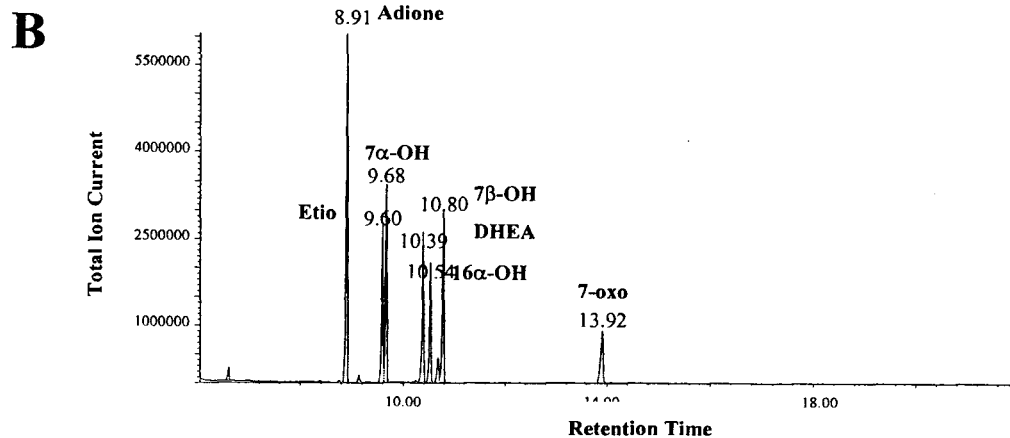
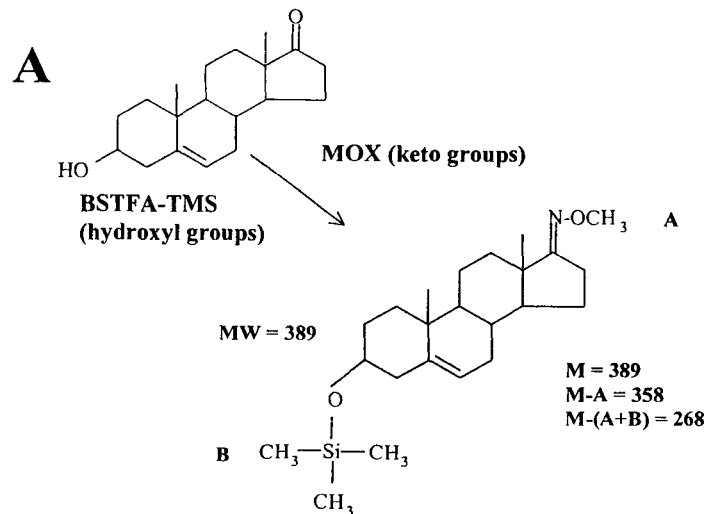


Figure 8. Separation of DHEA and metabolites by GC/MS. A GC/MS method was developed for quantification of DHEA metabolites in various species. **(A)** Schematic representation of the structure and derivatization of DHEA. **(B)** Chromatogram of the separation of DHEA and metabolites. **(C)** Electron ionization mass spectrum of DHEA.

TABLE IV

GC-SIM-MS data for seven steroids.

Steroid	RT ^a	Selected Ion (m/z) ^b	Characteristic Ions (m/z)				a ^c	b ^d	Linearity ^e
		Ions quantified	[M] ⁺	-CH ₃	-CH ₃ O (-MOX)	-(CH ₃) ₃ SiO (-TMS)			
ADIONE	8.91	<u>344</u> , 329	344	329			0.225	0.000461	0.912
Etio	9.60	<u>270</u> , 360			360	270	IS	IS	IS
7 α -OH-DHEA	9.68	<u>387</u> , 356			356	387	0.722	0.0225	0.991
DHEA	10.40	<u>268</u> , 358			358	268	0.172	0.00965	0.985
16 α -OH-DHEA	10.54	<u>446</u> , 356			446	356	0.112	0.0059	0.943
7 β -OH-DHEA	10.79	<u>387</u> , 477	477			387	0.468	0.00984	0.976
7-oxo-DHEA	13.92	<u>432</u> , 401	432		401		0.0124	0.0000345	0.935

IS (internal standard) ^a Retention time in minutes. ^b Ions used for quantitative analysis are underlined. ^c a = Slope = relative mass response = mean peak area ratio of steroid X mass of IS/mass of steroid; b = y-intercept. ^d Linearity is represented by the linear correlation coefficients of the calibration curves for each standard.

ionization mass spectra for each compound as shown for DHEA (Figure 8C). The retention times and MS data are shown in Table IV. Etiocholanolone, which has been previously shown not to be a direct metabolite of DHEA under these conditions, was used as an internal standard. The peak areas of each standard relative to etiocholanolone were used to prepare a standard curve to quantify metabolite production.

Quantification of DHEA and metabolites by GC/MS. In order to study the liver microsomal hydroxylation of DHEA in various species, microsomal protein fractions (0.5 mg/mL) from rat, hamster or pig were incubated with 50 μ M DHEA and an NADPH regenerating system consisting of sodium isocitrate, isocitrate dehydrogenase and $MgSO_4$ for up to 20 minutes. Extracts of the microsomal incubation mixtures were derivatized and then analyzed using GC/MS. In order to quantify and confirm metabolite identities, two or three characteristic ions for each steroid were selected on their basis of their mass fragmentation. The peak areas of the selected ions of each metabolite were obtained and compared to that of the internal standard, and the absolute values were calculated using calibration curves from the standards.

Figure 9 shows a representative chromatogram of the total ion current for rat liver microsomal metabolism of DHEA at 0 minutes. DHEA was metabolized by rat liver microsomes to 7α -OH-DHEA and 16α -OH-DHEA in 10 minutes as indicated by the presence of two metabolite peaks corresponding in retention times to the authentic compounds (Figure 10). Moreover, NADPH was required for microsomal metabolism of DHEA, since no metabolite peaks were formed in the absence of an NADPH regenerating system (data not shown).

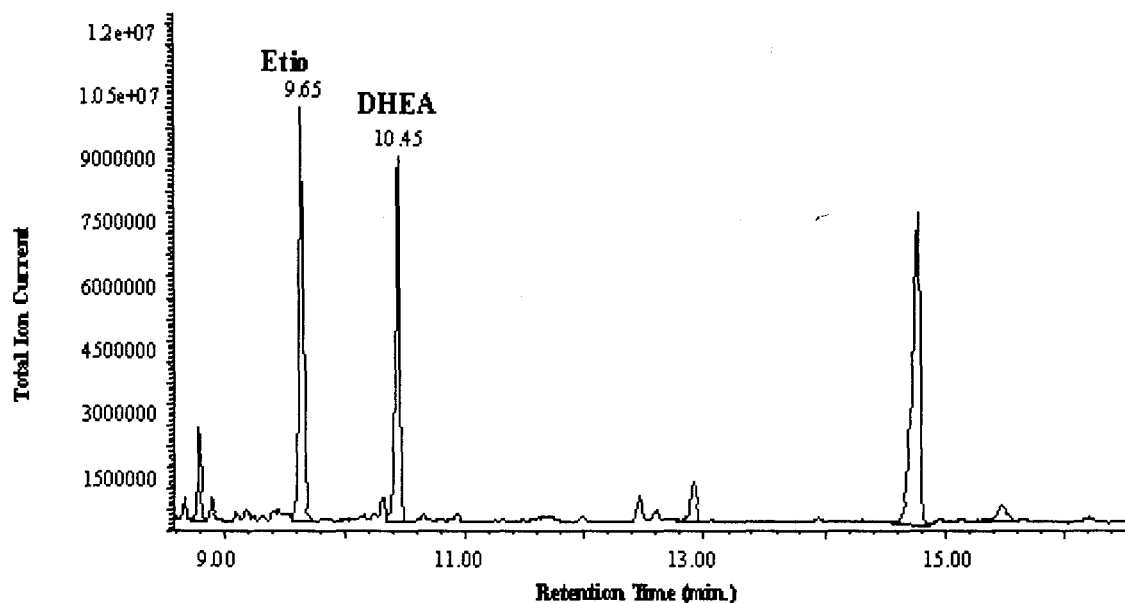


Figure 9. Rat liver microsomal metabolism of DHEA at 0 minutes. Rats were fed control diet for 5 days and then liver microsomal fractions were isolated. Metabolic assays were performed in triplicate with 2 mL reaction mixtures containing microsomal protein (1mg/mL), NADPH regenerating system, and 50 μ M DHEA incubated at 37°C for 10 minutes in a shaking water bath. Reactions were terminated at 0 minutes. Ethyl acetate extracts were examined by GC/MS.

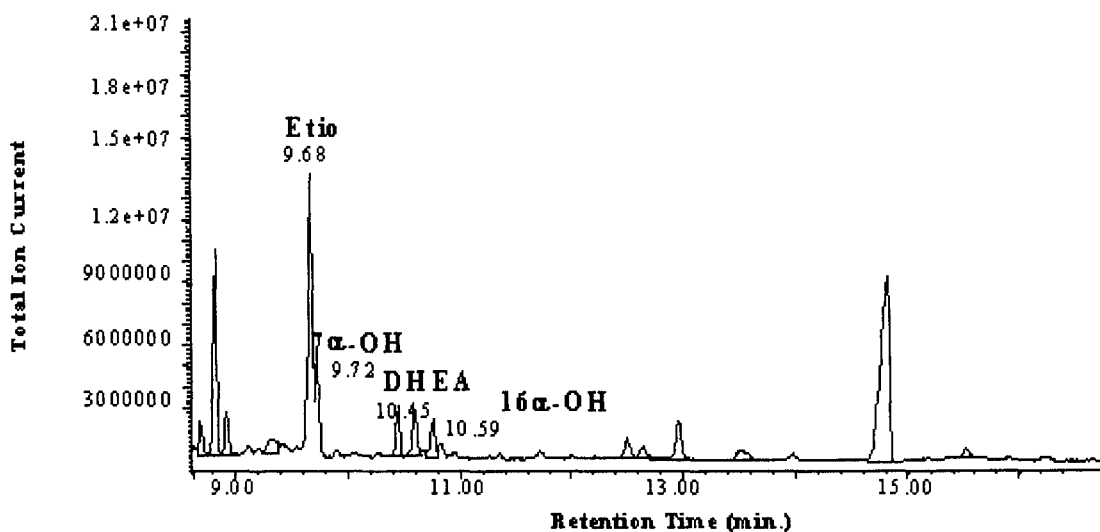


Figure 10. Rat liver microsomal metabolism of DHEA at 10 minutes. Rats were fed control diet for 5 days and then liver microsomal fractions were isolated. Metabolic assays were performed in triplicate with 2 mL reaction mixtures containing microsomal protein (1mg/mL), NADPH regenerating system, and 50 μ M DHEA incubated at 37°C for 10 minutes in a shaking water bath. Reactions were terminated at 10 minutes. Ethyl acetate extracts were examined by GC/MS.

Rat, hamster, pig and human liver microsomal fractions all metabolized DHEA. DHEA was rapidly metabolized in rat (7.2 nmol/min/mg) and hamster (18.9 nmol/min/mg). Rat liver microsomes produced two major monohydroxylated metabolites, 7 α -OH-DHEA (4.6 nmol/min/mg) and 16 α -OH-DHEA (2.6 nmol/min/mg). In the hamster, DHEA was converted to 7 α -OH-DHEA (7.4 nmol/min/mg) and 16 α -OH-DHEA (0.26 nmol/min/mg), as well as 11 unidentified metabolites that accounted for a rate of DHEA conversion of 11.2 nmol/min/mg. Pig microsomal metabolism of DHEA displayed lower rates of conversion than rat and hamster metabolism and produced three metabolites, 7 α -OH-DHEA (0.70 nmol/min./mg), 16 α -OH-DHEA (0.16 nmol/min./mg) and ADIONE (0.26 nmol/min./mg). Although ADIONE has been shown to be formed in the cytosolic fractions of other species with NAD⁺, the formation of ADIONE by pig liver microsomal fractions required NADPH, but not NAD⁺ or NADP⁺ (data not shown), indicating the presence of a 3 β -hydroxysteroid dehydrogenase enzyme activity in not only cytosolic fractions, but also anabolic liver microsomal fractions of the pig (Figure 11 & Table V). Future studies will evaluate the role of CYPs in this reaction.

Upon incubation with 50 μ M DHEA, one human liver microsomal fraction (HL110) hydroxylated DHEA at a rate of 7.8 nmol/min/mg. Like rat, hamster, and pig, 7 α -OH-DHEA (0.66 nmol/min/mg) and 16 α -OH-DHEA (3.6 nmol/min/mg) were produced (Figure 12 & Table V). Unlike the other species, the human also converted DHEA to 7 β -OH-DHEA at a significant rate (3.5 nmol/min/mg). The identity of the unique metabolite, 7 β -OH-DHEA, was established based on its GC retention time and a mass spectrum identical (Figure 13) to 7 β -OH-DHEA standard (Figure 14), but distinct from other DHEA metabolite standards including 11 β -OH-DHEA (data not shown). Not

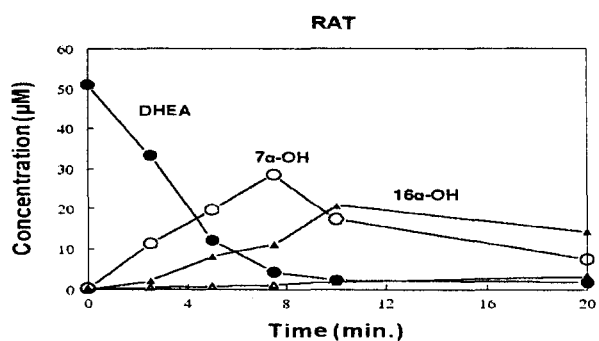
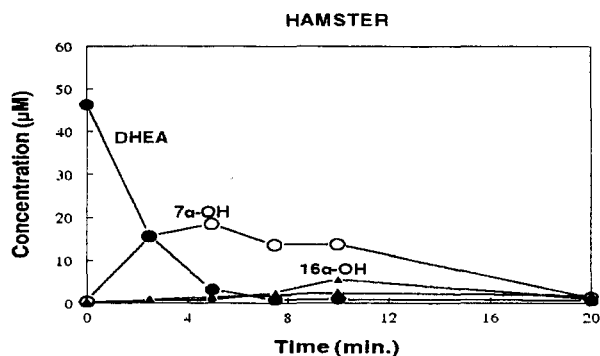
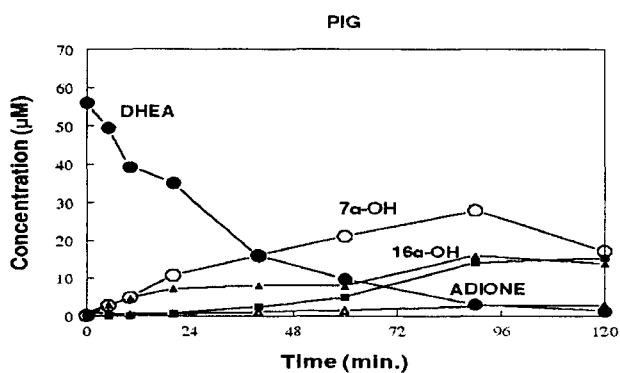
A**B****C**

Figure 11. Time dependent formation of DHEA metabolites by rat, hamster and pig after GC/MS analysis. (A) DHEA metabolite formation from rat liver microsomes. (B) DHEA metabolite formation from hamster liver microsomes. (C) DHEA metabolite formation from pig liver microsomes. (●: DHEA; ○: 7 α -OH-DHEA; ▲: 16 α -OH-DHEA; ■: ADIONE). The results are expressed as the average of triplicate experiments of at least two reactions in which the SD varied by $\leq 5\%$.

TABLE V
GC/MS analysis of DHEA metabolites formed in various species.

	DHEA metabolized (nmol/min/mg)	7α-OH-DHEA formed (nmol/min/mg)	7β-OH-DHEA formed (nmol/min/mg)	16α-OH-DHEA formed (nmol/min/mg)	ADIONE formed (nmol/min/mg)	UNIDENTIFIED metabolites formed (nmol/min/mg)
Rat	7.2	4.6*		2.6*		
Hamster	18.9	7.4*		0.26		11.2*
Pig	1.1	0.70**		0.16**	0.26*	
HL 103	0.45	0.07	0.18	0.20		
HL 110	7.8	0.66**	3.5*	3.6*		
HL 111	0.90	0.08	0.40	0.42		
HL 112	0.76	0.06	0.34	0.36		
HL 113	0.71	0.09	0.30	0.32		

Total metabolite formation was based on amount of DHEA (50 μ M) converted to products during the linear phase of reaction. Known metabolites were quantified by measuring the peak area and comparing to known standards normalized to the internal standard etiocholanolone. The results are expressed as the average of triplicate experiments of at least two reactions in which the SD varied by $\leq 5\%$. The rates of metabolism during the linear portion of the reaction are statistically different from the zero time value (* $p < 0.05$ or ** $p < 0.01$).

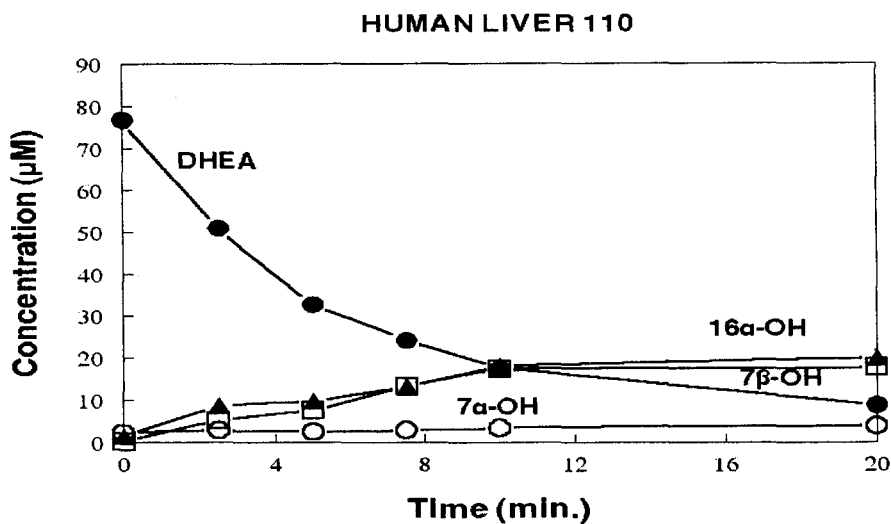


Figure 12. Time dependent formation of DHEA metabolites by human liver microsomal fractions. DHEA metabolite formation from liver microsomes of human subject 110. (●: DHEA; ○: 7α-OH-DHEA; ▲: 16α-OH-DHEA; □: 7β-OH-DHEA).

The results are expressed as the average of triplicate experiments of at least two reactions in which the SD varied by $\leq 5\%$.

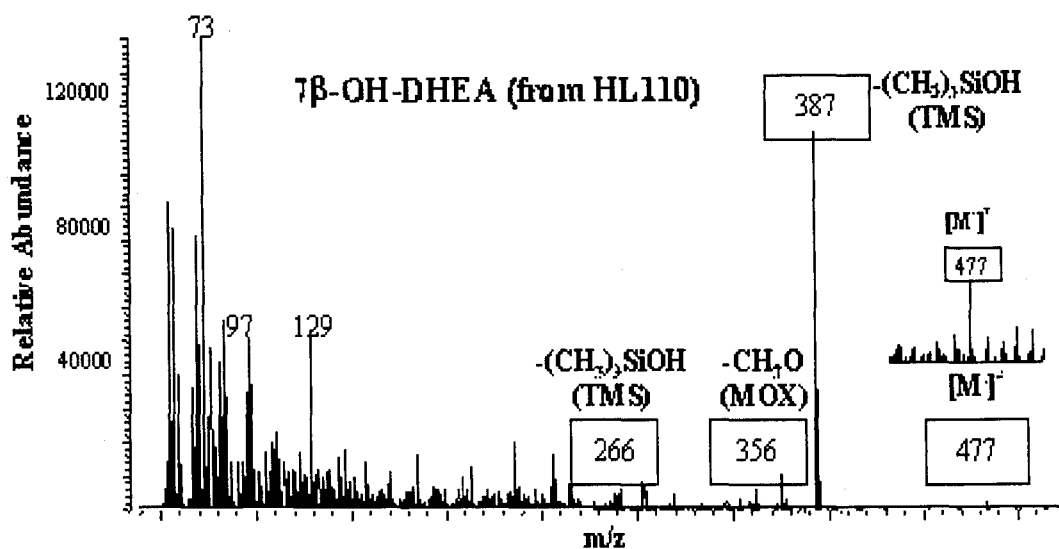


Figure 13. Characteristic mass spectrum of 7β-OH-DHEA from human microsomal fractions. Electron ionization mass spectra of 7β-OH-DHEA from human (110) liver microsomal metabolism of DHEA (**inset: 20X 477 mass spectrum**).

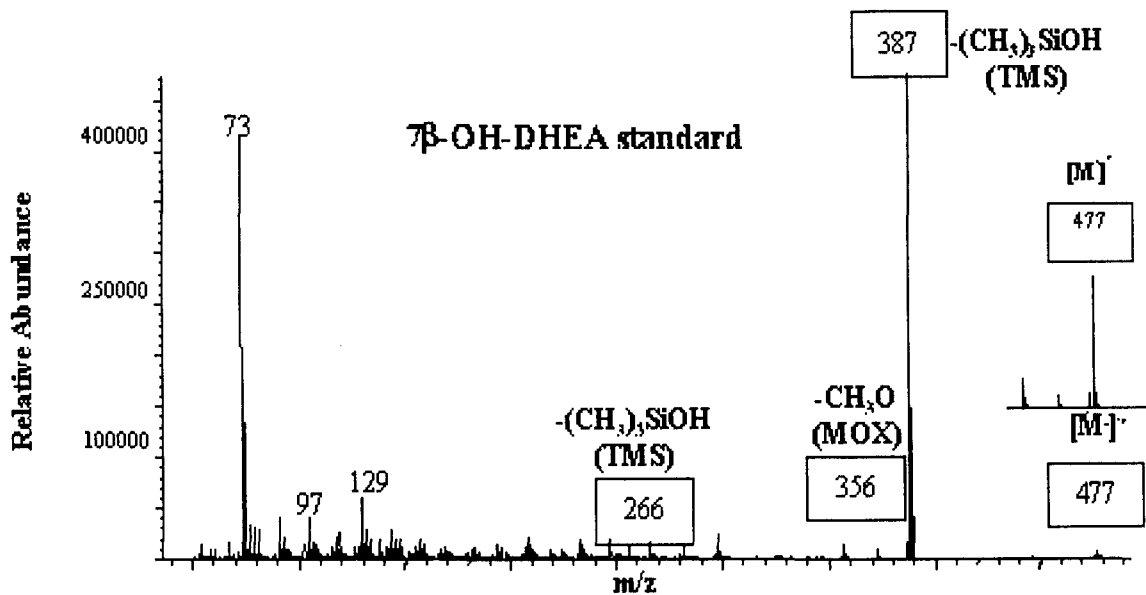


Figure 14. Characteristic mass spectrum of 7β -OH-DHEA standard. Electron ionization mass spectra of 7β -OH DHEA standard (inset: 20X 477 mass spectrum).

all human microsomal fractions tested oxidized DHEA as well as sample HL110. In fact, although four other human liver microsomal fractions displayed the same metabolite profile as HL110, the other human fractions metabolized less than 2 nmol/min/mg of DHEA in 10 minutes (Figure 15), indicating inter-individual variability of DHEA metabolism of the human samples that were measured.

Cytochrome P450 metabolism of DHEA. To establish which cytochrome P450 was responsible for DHEA metabolite production, 50 μ M DHEA was incubated with membrane fractions from baculovirus-infected insect cells that express both a specific P450 and its flavoprotein oxidoreductase, NADPH:cytochrome P450 oxidoreductase. CYP3A4 and CYP3A5 apparently are responsible for the production of 7 α -OH-DHEA, 16 α -OH-DHEA and 7 β -OH-DHEA, with CYP3A4 exhibiting the highest rate of product formation. CYP3A7 is not expressed in adult liver, but is expressed in fetal liver (Hakkola *et al.*, 1994); it also formed 7 β -OH-DHEA, but no detectable 7 α - or 16 α -OH-DHEA (Table IV). CYP2D1 was the rat P450 that most extensively converts DHEA to 16 α -OH-DHEA. CYP2B1 and CYP2C11 also contributed to 16 α -OH-DHEA metabolite production, while CYP3A23 was the rat P450 apparently responsible for 7 α -OH-DHEA formation.

DISCUSSION

Many animal studies have suggested beneficial effects of DHEA administration in pharmacological dosages. Exogenous DHEA administration to humans has also been suggested to likely also have beneficial effects in cancer prevention, immune function,

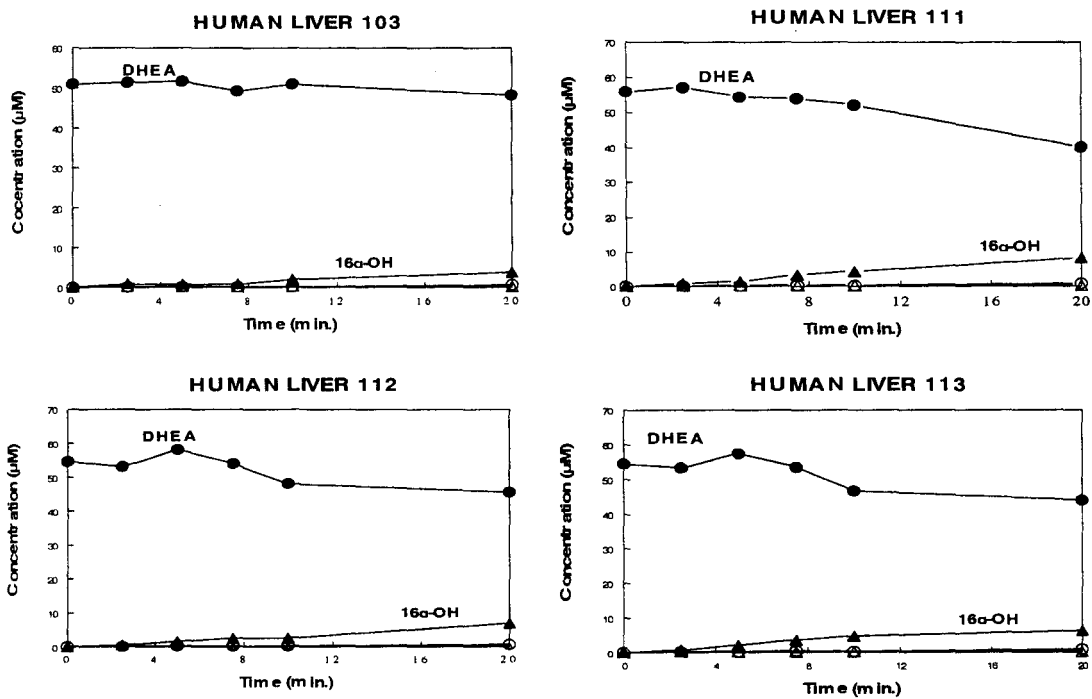


Figure 15. Time dependent formation of DHEA metabolites by human liver

microsomal fractions. Liver microsomal metabolism from 4 human samples. (●:

DHEA; ○: 7α-OH-DHEA; ▲: 16α-OH-DHEA; □: 7β-OH-DHEA). The results are

expressed as the average of triplicate experiments of at least two reactions in which the

SD varied by $\leq 5\%$.

TABLE VI
Rates of DHEA metabolites formed from baculovirus expressed P450

	Rate of Formation (nmol/min/nmol P450)		
	7α -OH DHEA	7β -OH DHEA	16α -OH DHEA
	10 min.	10 min.	10 min.
Human CYPs			
3A4	0.50**	1.4**	1.0**
3A5	0.50**	0.75*	0.25*
3A7	ND	0.75*	ND
2A6	ND	ND	ND
2B6	ND	ND	ND
2C8	ND	ND	ND
2C9	ND	ND	ND
2C19	ND	ND	ND
2D6	ND	ND	ND
Rat CYPs			
3A23	1.0*	ND	ND
2B1	ND	ND	0.63*
2C11	ND	ND	1.9**
2C12	ND	ND	ND
2C13	ND	ND	ND
2D1	ND	ND	2.9*

Metabolic assays were performed in triplicate in 2 mL reactions mixtures containing CYP baculovirus (~0.4 nmol/mL), NADPH regenerating system, and 50 μ M DHEA and incubated at 37°C for 10 minutes in a shaking water bath. Reactions were terminated at 5 minutes and 10 minutes. Ethyl acetate extracts were examined by GC/MS. The results are expressed as the average of triplicate experiments of at least two reactions in which the SD varied by $\leq 5\%$. The rates of metabolism during the linear portion of the reaction are statistically different from a reaction in the absence of baculovirus preparation (* $p < 0.05$ or ** $p < 0.01$). ND, not detected since the rate of product conversion was less than 0.05 nmol/min/nM P450.

diabetes, obesity and cardiovascular disease (Kroboth *et al.*, 1999). Since DHEA is considered a natural product/dietary supplement and is available as an over the counter supplement, the mechanism of action of this sterol and its metabolites become important to study.

DHEA is metabolized to androgens and estrogens in steroidogenic tissues; however, the metabolism of DHEA in other tissues has not been extensively studied. Fitzpatrick *et al.* (2001) utilized LC/MS to identify the metabolites formed by the transformation of DHEA by rodent and human liver microsomal fractions. 16α -OH-DHEA, 7α -OH-DHEA and 7-oxo-DHEA were identified in both species. However, the major metabolite produced in humans was a mono-hydroxylated DHEA metabolite whose position of hydroxylation was unknown. Additionally, Fitzpatrick *et al.* (2001) demonstrated that formation of these products was inhibited by miconazole indicating the role of cytochrome P450s in the metabolism of DHEA. With human liver microsomal fractions, the high levels of DHEA hydroxylation was shown to be due to CYP3A, since its metabolism to several products was strikingly inhibited by troleandomycin (approx. 80% inhibition), while the inhibitor was less effective in inhibiting DHEA hydroxylation in rat liver microsomal fractions (approx. 20% inhibition). Our results demonstrate that human liver microsomal hydroxylation of DHEA is predominantly due to the role of CYP3A, while in rat other CYPs account for significant conversion to 16α -OH-DHEA (CYP2B1, 2C11, 2D1, and others). In addition, α -naphthoflavone (inhibitor of CYP1) and quinidine (inhibitor of CYP2D) also slightly inhibited DHEA hydroxylation by rat liver microsomes (Fitzpatrick *et al.*, 2001) demonstrating that several rat CYPs are involved in DHEA hydroxylation. We have also shown that DHEA and its cytosolic metabolites

induce CYP3A23 (native gene in rat hepatocytes and reporter gene constructs in HepG2 cells) demonstrating that DHEA can induce its own metabolism to the 7 α -OH-DHEA by induction of CYP3A through action of the pregnane X receptor in rats (Ripp *et al.*, 2002). This increase in 7 α -hydroxylase over 16 α -hydroxylase activity is also due to the negative regulation of CYP2C11, a 16 α -hydroxylase, by DHEA (Ripp *et al.*, 2003), demonstrating a complex metabolic scheme when contrasting metabolism across species. The purpose of the current study was to further identify the unknown metabolite formed by the human liver microsomal metabolism of DHEA and identify the specific P450s responsible for production of various DHEA metabolites.

Although LC/MS allowed for the identification of most of the DHEA metabolites, quantification of DHEA metabolism was difficult to attain due to low ionization efficiency of metabolites under conditions of chemical ionization at atmospheric pressure (Fitzpatrick *et al.* 2001). The current study, a GC/MS method was developed to provide a more sensitive method for identification and quantification of the liver microsomal metabolism of DHEA.

The current study examined the oxidative metabolism of DHEA by rodent, hamster, pig and human microsomal fractions. Each species extensively converted DHEA into mono-hydroxylated metabolites. ADIONE was also produced in pig liver microsomal fractions in the presence of NADPH and oxygen. ADIONE is an anabolic steroid that mimics the effects of testosterone to increase growth and development of muscle tissue. Since it has been reported to promote lean muscle growth, ADIONE is used frequently by athletes interested in increasing muscle mass (Ziegenfuss *et al.*, 2002). 3 β -hydroxysteroid dehydrogenases convert DHEA to ADIONE in the presence of NAD.

Pigs are primarily raised for lean muscle production, suggesting a possible role for enhanced levels of an NADPH-dependent microsomal 3 β -hydroxysteroid-dehydrogenase activity in pig liver. Hamster liver microsomal fractions also converted DHEA into 7 α -OH-DHEA and 16 α -OH-DHEA, as well as 11 unidentified hydroxylated DHEA species that are possibly secondary metabolites. These results suggest that several cytochrome P450 enzymes may play a role in the DHEA metabolism in the hamster and demonstrate the significant species differences in the metabolism of DHEA.

Metabolism of DHEA by human microsomal fractions yielded both 7 α -OH-DHEA and 16 α -OH-DHEA; however, the human was the only species to produce 7 β -hydroxy-DHEA. Fitzpatrick *et al.* (2001) previously reported that human liver microsomal metabolism of DHEA resulted in the production of 7 α -OH-DHEA, 16 α -OH-DHEA, 7-oxo-DHEA and an unknown monohydroxylated DHEA accounting for nearly half of total metabolite production. The current study identified 7 α -OH-DHEA and 16 α -OH-DHEA production, as well as 7 β -OH-DHEA which accounts for approximately 44% of total metabolite production. This mono-hydroxylated species, namely 7 β -OH-DHEA, is likely the unknown compound previously reported by Fitzpatrick *et al.* (2001) and was recently shown to be formed by Stevens *et al.* (2003) to be formed by CYP3A4 and 3A5. Not all human microsomal fractions exhibited extensive oxidative metabolism of DHEA. Although one human microsomal fraction (HL110), previously noted by Guengerich and coworkers to contain high levels of CYP3A (Guengerich *et al.*, 1991), metabolized DHEA at a high rate (7.8 nmol/min/mg), fractions from four other humans hydroxylated DHEA at much lower rates (\leq 2 nmol/min/mg of DHEA). Although not all human microsomal fractions formed hydroxylated metabolites at the same rate, all human

microsomal fractions exhibited similar metabolite profiles. The various rates in DHEA metabolism among humans could be attributed to differences in CYP expression or various CYP polymorphisms.

Although 7α -, 16α - and 7β -OH-DHEA were produced in human liver microsomal fractions, 7-oxo-DHEA was also formed, albeit at later time points (Fitzpatrick *et al.*, 2001). Additionally, we have found that upon treatment with 50 μ M of 7-oxo-DHEA, human liver fractions can convert 7-oxo-DHEA into 7α - and 7β -OH-DHEA indicating a complex metabolic pathway for DHEA in the liver that includes 11β -hydroxysteroid dehydrogenase activity (Robinson *et al.*, 2003).

The human CYP3A family plays a dominant role in the metabolic elimination of more drugs than any other biotransformation enzyme (Lamba *et al.*, 2002). Fitzpatrick *et al.* (2001) reported that selective P4503A inhibitors were able to inhibit DHEA metabolite production in the human. The current study utilized insect cells infected with baculovirus expression vectors to examine the CYPs responsible for the liver microsomal metabolism of DHEA. Recombinant CYP3A4 was responsible for the majority of the conversion of DHEA into 7α -OH-DHEA, 16α -OH-DHEA and 7β -OH-DHEA. CYP3A5 also converted DHEA into the same metabolites; however, the hepatic fetal enzyme, CYP3A7 was found to only hydroxylated DHEA to 7β -OH-DHEA. The rat CYP2D1 converted DHEA to 16α -OH-DHEA as did CYP2C11 and CYP2B1. Additionally, CYP3A23, a major constitutive P450 in rat liver, was the CYP responsible for 7α -OH-DHEA production in the rat. This pattern of hydroxylation is strikingly different from the human CYP3A4 or 3A5.

The current study utilized recombinant P450 expressed in insect cells to examine DHEA metabolism. The assay of purified P450s requires that they be reconstituted with NADPH:cytochrome P450 reductase in a complex mixture which includes detergent, phospholipids and reduced glutathione (Gillam *et al.*, 1995). Some *in vitro* reconstitution experiments have shown that for a number of P450s, the inclusion of cytochrome *b₅* can significantly increase substrate turnover by monooxygenase system by improving the coupling between the P450 and NADPH cytochrome P450 reductase (Holmans *et al.*, 1994; Gorsky and Coon., 1986; Bell and Guengerich, 1997). Cytochrome *b₅* is a heme protein whose mechanism of action in reconstituted systems is not clear. It has been suggested that cytochrome *b₅* plays a role in donating electrons from NADPH:cytochrome P450 oxidoreductase to CYP (Bell and Guengerich., 1997; Yamazaki *et al.*, 1996; Morgan and Coon, 1984). Although the authors acknowledge there is evidence that the inclusion of cytochrome *b₅* in bacterial membranes may enhance CYP3A4 activity, inclusion of this cytochrome *b₅* did not enhance DHEA metabolism by recombinant CYP3A4 under the conditions of our assay (K.K. Michael Miller and R.A. Prough, unpublished data).

In conclusion, this study demonstrates that different species exhibit unique DHEA metabolite profiles due to the stereospecificity of hydroxylation by the various CYPs that metabolize DHEA. The unknown major metabolite produced by the human previously reported by Fitzpatrick *et al.* (2001) was shown to be 7 β -OH-DHEA. DHEA and some of its metabolites are known to interact with certain nuclear receptors and activate CYP transcription. This could explain the mechanism of some beneficial effects that have been reported with the administration of DHEA.

CHAPTER III

DHEA AND ITS METABOLITES ACTIVATE ESTROGEN RECEPTOR

INTRODUCTION

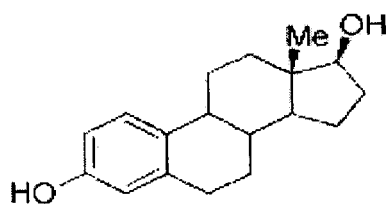
The estrogen receptor (ER) is a ligand-activated transcription factor and a member of the steroid hormone nuclear receptor superfamily. The two subtypes of ER, ER α and ER β , mediate the physiological effects of its primary ligand, 17 β -estradiol (E2) within various tissues (Nillson and Gustafsson, 2002). Binding of ER to an estrogenic ligand induces a conformational change that results in activation of ER and binding of the receptor to specific DNA sequences known as estrogen responsive elements (EREs) (Klinge, 2001). Association with DNA initiates transcription, thereby regulating the activation or repression of ER target genes (Parker *et al.*, 1993).

Estrogens are predominantly synthesized in the ovary and are responsible for cellular growth and differentiation, required for puberty and reproductive processes, as well as maintaining bone density and cholesterol levels. Additionally, estrogen is essential for growth and development of the mammary gland, and therefore has been associated with the promotion and growth of breast cancer (Clark *et al.*, 1992).

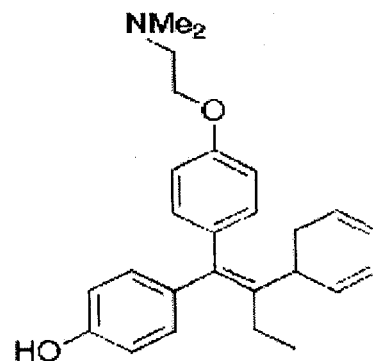
Since estrogens are mitogens in approximately one-third of breast tumors, specific estrogen antagonists have been developed for the treatment of hormone-dependent breast

cancer. (Jordan and Murphy, 1990). Tamoxifen is one of the most widely used antiestrogens that inhibits transcriptional activation by the receptor (Berry *et al.*, 1990). Although tamoxifen serves to stop tumors from proliferating, it also exhibits partial agonist activity (Jordan, 1984). Therefore, the antiestrogen compound ICI 182,780 was developed which does not exhibit agonist activity. The ICI compound is a derivative of E2, but contains an alkamide functional group in the 7 α position of the sterol nucleus (Bowler *et al.*, 1989) (Figure 16). ICI 182,780 binding to ER α results in a conformation of the receptor which is different than that formed with known agonists of the receptor (Pike *et al.*, 2001). Further ICI 182,780 reduces steady-state levels of ER α by increasing the turnover of the protein in the nucleus by targeting ER to the 26S proteasome (Reese and Katzenellenbogen, 1992 and Wijayarathne *et al.*, 1999). In addition to tamoxifen and ICI 182,780, aromatase inhibitors are a family of hormonal treatments that have shown significant activity against breast cancer in post-menopausal woman with estrogen-sensitive tumors (Lonning, 1998). Aromatase, CYP19 (Figure 1), is expressed in breast cancer tissue and catalyzes the conversion of C19 steroids to estrogens. Therefore, aromatase inhibitors reduce the amount of circulating estrogen and thereby inhibit the growth of estrogen sensitive tumors (Geisler *et al.*, 1996).

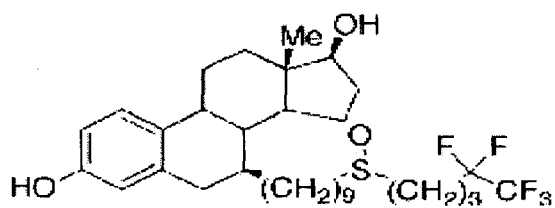
DHEA and its sulfate, DHEA-S, are estrogen precursors whose role in the progression of breast cancer has yet to be clearly defined. Plasma levels of DHEA-S are higher than that of other sterols secreted by the adrenal gland (Ebeling and Koivisto, 1994). The blood plasma levels of DHEA-S are maximal in the middle of the second decade of life and decline thereafter (Figure 3). Treatment of humans with DHEA has



Estradiol



Tamoxifen



ICI 182,780

Figure 16. Structure of estradiol and common anti-estrogenic compounds.

been suggested to have many beneficial effects during this state of decline in DHEA formation. In fact, DHEA has been reported to have anti-carcinogenic effects in the mammary gland of rodents after chemical induction (Feo *et al.*, 2000). However, a role of DHEA in human breast cancer has been debated for years. For instance, DHEA has been reported to be present in normal tissue as well as breast tumors (Brignardell *et al.*, 1995 and Massobrio *et al.*, 1994). Although DHEA has been suggested to have a protective effect in pre-menopausal women, a positive correlation was observed between DHEA plasma levels and breast cancer risk in postmenopausal women (Adams, 1998 and Gordon *et al.*, 1990). Also, it has been reported that DHEA-S levels >90 µg/dL pose a potential risk factor for breast cancer progression in patients treated with tamoxifen (Calhoun *et al.*, 2003). Maggiolini *et al.* (1999) reported that DHEA and ADIOL directly activated transfected ER α reporter genes and stimulated proliferation of MCF-7 cells, an ER α -dependent human breast cancer cell line, as well as, MCF-7SH cells, an estrogen-independent MCF-7 variant. Moreover, Mizokami *et al.* (2004) reported that ADIOL is a major DHEA metabolite formed in human prostate tissue and that ADIOL levels are appreciable in prostate cancer tissue after hormone therapy.

Therefore, the possibility that DHEA and its metabolites activate ER is critical for understanding the biological events of estrogen-mediated gene regulation in normal and diseased tissues. In this study, DHEA and its metabolites were tested to for their ability to activate human ER α and ER β in *in vitro* assays.

MATERIALS AND METHODS

Chemicals. Androstenediol, androstenedione, etiocholanolone, DHEA, DHEA-sulfate, 7 α -hydroxy-DHEA, 7 β -hydroxy-DHEA, 7-oxo-DHEA 11 β -hydroxy-DHEA, 16 α -hydroxy-DHEA, and estradiol were purchased from Steraloids, Inc. (Wilton, NH). ICI 182,780 and 4-hydroxytamoxifen (4-OHT) were purchased from Tocris, Inc. (Ellisville, MO). Miconazole was purchased from Sigma (St. Louis, MO) and exemestane was a generous gift from Pharmacia Upjohn Corp., Kalamazoo, MI. RRTHC, a selective ER β antagonist/ER α agonist was a generous gift from Dr. John A Katzenellenbogen of the University of Illinois (Sun *et al.*, 1999)

Plasmids. The pCMV expression plasmid containing the cDNA for human ER α (Reece and Katzenellenbogen, 1991) was a gift from Dr. Benita Katzenellenbogen (University of Illinois at Urbana). The pSG5 expression plasmid containing the cDNA for human ER β (hER β 1, 530 aa) was a gift from Dr. Eva Enmark (Karolinska Hospital, Stockholm, Sweden). The reporter plasmid ERELUC was constructed by inserting three copies of a consensus oligonucleotide containing the ERE into the KpnI/SacI site of a pGL3 promoter linked to the firefly luciferase reporter gene (Klinge, 1999). The expression plasmid for β -galactosidase (pCMV β) was purchased from CLONTECH (Palo Alto, CA). All plasmids were transformed into DH5 α *Escherichia coli* bacteria, isolated, and prepared for use in transient transfections using QIAGEN plasmid prep kits (QIAGEN, Chatsworth, CA).

Transient Transfections. HEK293, HepG2, CHO-K1 and MDA-MB-231 cells (ATCC) were grown at 37°C in 5% carbon dioxide atmosphere. Cells were plated at 1.5 X 10⁵

cells/well in 12-well plates containing minimal essential medium supplemented with 5% charcoal-stripped fetal bovine serum. Twenty-four hours after plating, cells were transfected using 4 $\mu\text{g/ml}$ LipofectAMINE (Invitrogen, Carlsbad, CA) with hER α or hER β expression plasmid (150 ng/ml), ERELUC reporter plasmid (250 ng/ml), and β -galactosidase expression plasmid in serum free medium. Each well was overlaid with 1 ml of transfection mixture and incubated overnight. After removal of the transfection mixture, cells were supplemented with 5% charcoal-stripped serum. Transfected cells were treated with 500X concentrated stocks of DHEA and metabolites in ethanol, and harvested 24 h later with 100 μl of cell lysis buffer (Promega, Madison, WI). β -Galactosidase and luciferase activities were determined as described by Falkner *et al.* (1998). The data are expressed as luciferase activity relative to β -galactosidase activity to correct for transfection efficiency. All transient transfection experiments were performed in triplicate or quadruplicate, and experiments were repeated at least twice to confirm results.

Estradiol Ligand Binding Assay. Purified recombinant human ER α or ER β were purchased from Panvera (Madison, WI) were incubated in a final volume of 54 μL in TDPK111 buffer (40 nM Tris-HCl (pH 7.5), 1 mM DTT, 0.5 mM PMSF, 111 mM KCl) containing 30 nM [^3H]-E $_2$ (2,3,4,7,-[^3H](N)17 β -estradiol, 74 Ci/mmol, NET-317, NEN) for one hour at 37 $^\circ\text{C}$ prior addition of a 10% hydroxyapatite (HAP) solution in TDPK111 (Pavlik and Coulson, 1976). HAP was added and incubated for 30 minutes. The supernatant was discarded and the pellet was resuspended in TDPK111 buffer and washed two times. After the final wash, the pellet was resuspended in scintillation fluid for

determination of [^3H]E₂. Eight reactions were performed for each concentration, four "cold" reactions and four "hot" reactions. "Cold" reactions contained increasing molar amounts of E₂, DHEA, DHEA-S, ADIOL, ADIONE, and 7-oxo-DHEA as a competitor for ER binding with [^3H]E₂. Ethanol was added in an equal volume to the "hot" reactions containing ER and [^3H]E₂. The percent of binding of the test compounds to ER was calculated by first subtracting the nonspecific binding to provide the specifically bound ligand. The value on the Y axes is expressed as the percentage of [^3H]E₂ bound.

Statistical Analysis. Experiments were conducted in triplicate or quadruplicate and means \pm standard deviations were determined. Statistical comparisons among treatment groups were determined using a two-tailed Student's t test, with $p < 0.05$ as the criterion for significance.

RESULTS

The ability of DHEA and metabolites to activate gene transcription through ER α and ER β was tested in cell-based reporter gene assays. HEK293 (human embryonic kidney) and HepG2 (human hepatoma) cell lines were transiently transfected with luciferase reporter constructs containing a luciferase reporter plasmid constructed by using three copies of a consensus ERE containing oligomer. Cells were also cotransfected with expression plasmids for either human ER α or ER β and treated with 17 β -estradiol, DHEA or its metabolites. Because the expression of ERELUC is induced through ligand-mediated activation of the nuclear receptors ER α and ER β , the ability of 17 β -estradiol to activate gene transcription through ER α and ER β was tested in a number of cell lines

including HEK293, HepG2, CHO-K1 (Chinese hamster ovary), and MDA-MB-231 cells. In our hands, ER α displayed a high basal activity in HepG2 cells, but had a limited response to 17 β -estradiol. In contrast, ER β was not active in regulating ERELUC expression in HEK293 cells and most other cells, but was ligand-activated in HepG2 cells. As shown in Figure 17, both cell lines tested supported activation of the ERELUC reporter in response to the known ER α and ER β agonist, 17 β -estradiol, but ER α was maximally responsive in HEK293 cells, while ER β was maximally responsive in HepG2 cells. It was noted that the response of ER β in HepG2 cells was increased nearly 8-fold by 17 β -estradiol, while ER α was maximally increased 3-4 fold in response to 17 β -estradiol in HEK293 cells.

Subsequently, 5 μ M of DHEA and many of its known metabolites (Miller *et al.*, 2004) were tested for their ability to induce expression of ERELUC in the presence of ER α in HEK293 cells or in the presence of ER β in HepG2 cells, respectively (Figure 18). As a control, an empty expression vector was cotransfected with ERELUC and after treatment with DHEA or its metabolites, there was no additional induction of ERELUC over vehicle in either HEK293 cells or HepG2 cells, indicating that both cell types were a viable null cell-based assay to test ER activation. Although the induction of the expression of ERELUC via ER β was more robust in HepG2 cells than the induction of ERELUC via ER α in HEK293 cells, DHEA, DHEA-S, and ADIOL significantly induced the expression ERELUC via human ER α in HEK293 cells. While 11 β -hydroxy-DHEA, 7 β -hydroxy-DHEA, 7 α -hydroxy-DHEA, 16 α -hydroxy-DHEA, and ETIO exhibited modest activation of the ER β , the cytosolic metabolites ADIONE and ADIOL as well as DHEA and 7-oxo-DHEA significantly induced ERELUC luciferase activity in HepG2

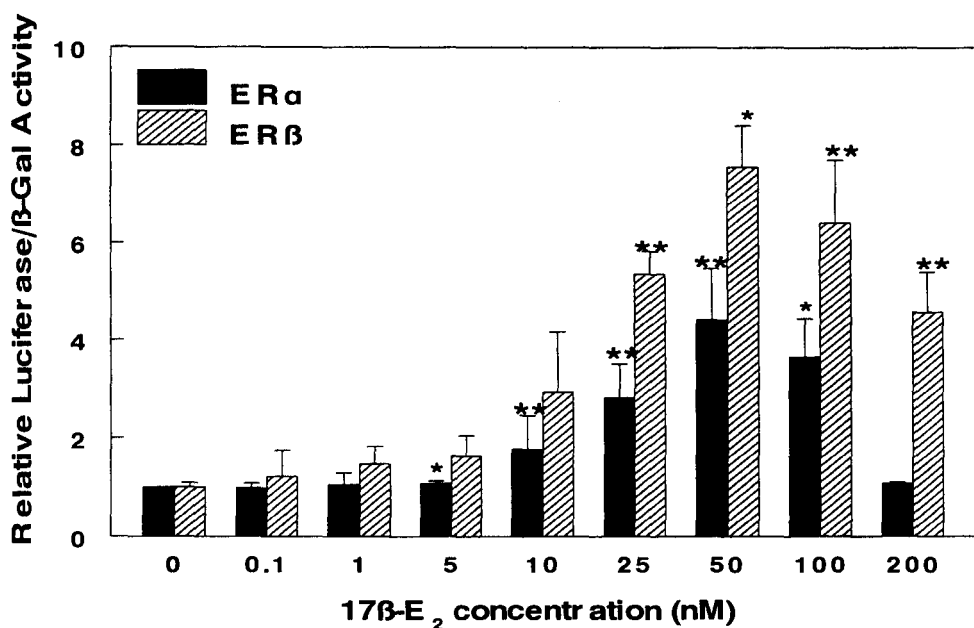


Figure 17. 17β-Estradiol increases expression of 3EREc38-dependent reporter gene activity. HEK 293 cells were transfected with ERELUC reporter plasmid and expression vector for human ERα and HepG2 cells were transfected with ERELUC reporter plasmid and an expression vector for human ERβ. Both cells types were treated for 24 h with 17β-estradiol. The cells were harvested and the lysates were assayed for β-galactosidase and luciferase activities. Data represent the mean ± S.D. of three wells. Experiments were repeated three times with similar results. Statistical significance was determined using analysis of variance followed by Student's *t* tests. * significantly different from cells treated with vehicle, $p < 0.05$, ** $p < 0.01$.

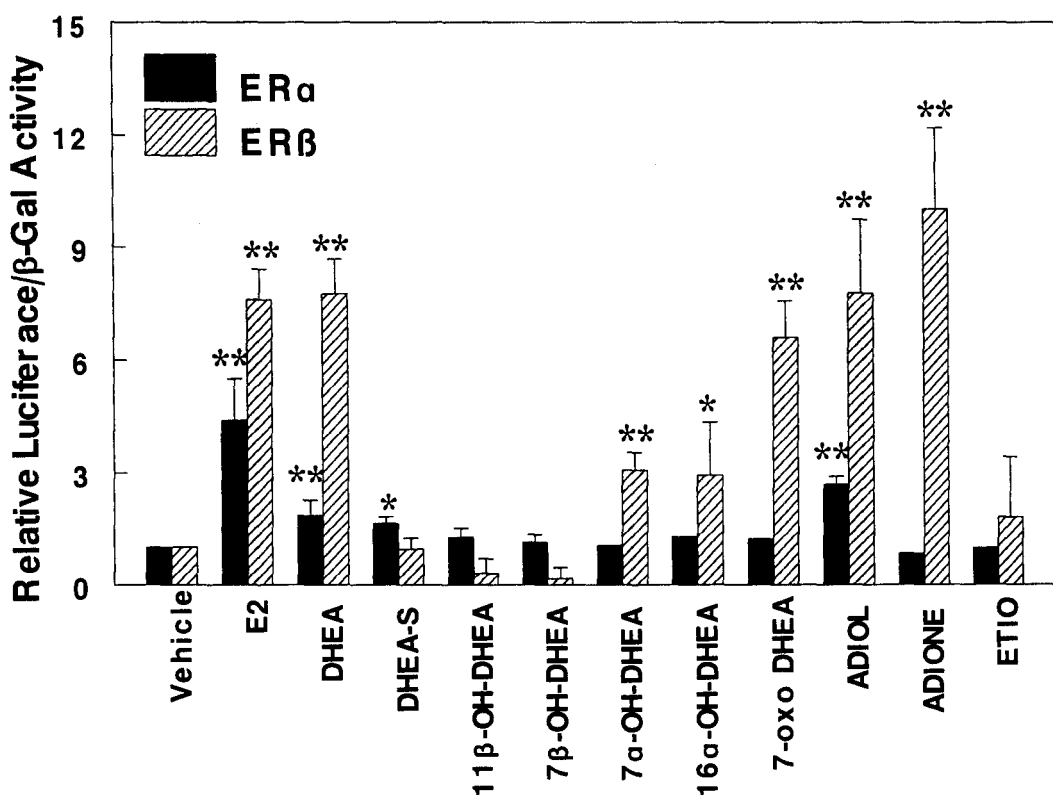


Figure 18. Increased ERELUC reporter activity in response to DHEA and metabolites. HEK293 and HepG2 cells were transfected with ERELUC reporter plasmid and expression vector for human ER α or human ER β respectively. Both sets of cells were treated for 24h with vehicle or 5 μ M of DHEA or its metabolites. Cells were then harvested and lysates assayed for β -galactosidase and luciferase activities. Data represent the mean \pm S.D. of three wells. Experiments were repeated three times with similar results. Statistical significance was determined using analysis of variance followed by Student's *t* tests. *, significantly different from cells treated with vehicle, $p < 0.05$, **, $p < 0.01$. Metabolites tested: 17 β -estradiol (E2) DHEA, DHEA-sulfate (DHEA-S) 11 β -hydroxy-DHEA (11 β -OH-DHEA), 7 β -hydroxy-DHEA (7 β -OH-DHEA), 7 α -hydroxy-

DHEA (7α -OH-DHEA), 16α -hydroxy- expression of ERELUC via ER β was more robust in HepG2 cells than the induction of ERELUC via ER α in HEK293 cells, DHEA, DHEA-S, and ADIOL significantly induced DHEA (16α -OH-DHEA), 7-oxo-DHEA, androstenediol, androstenedione and etiocholanolone.

cells. Due to the difference in induction of ERELUC expression in the different cell lines, Figure 19 shows the induction of luciferase expression through ERELUC by DHEA metabolites normalized to 17 β -estradiol. Concentration-response studies were conducted to evaluate the potency of ER α and ER β mediated induction of ERELUC by DHEA and metabolites (Figure 20 and Figure 21). ADIOL was the most potent inducer of ERELUC, inducing expression by nearly 3-fold with ER α , while DHEA and DHEA-S induced ERELUC expression approximately 2-fold with ER α . With ER β , ADIONE was the most potent inducer, inducing expression of ERELUC by approximately 10-to 12-fold. 7-oxo-DHEA, DHEA, and ADIOL also induced expression of ERELUC by approximately 6- to 8-fold with ER β . This response of different sterols in maximal activation is reminiscent of the differences seen between the rodent vs. the human pregnane X receptor (PXR), where pregnenolone 16 α -carbonitrile activates the rodent receptor, but not the human receptor (Jones *et al.*, 2000). In contrast, rifampacin activates the human pregnane X receptor, but not the murine receptor.

The ER is a member of the nuclear receptor superfamily that acts as a ligand-dependent transcription factor. Upon ligand binding, the receptor binds to the response elements of the target genes to activate transcription. Since estrogens are mitogens in approximately one-third of breast tumors (McGuire, 1976), specific estrogen antagonists have been developed for the treatment of hormone-dependent breast cancer. The non-steroidal compound, tamoxifen is one of the most widely used antiestrogens (Jordan, 1984). Tamoxifen binds with high affinity to ER (Katzenellenbogen *et al.*, 1983), but inhibits transcriptional reporter activity by DHEA and metabolites when the cells were

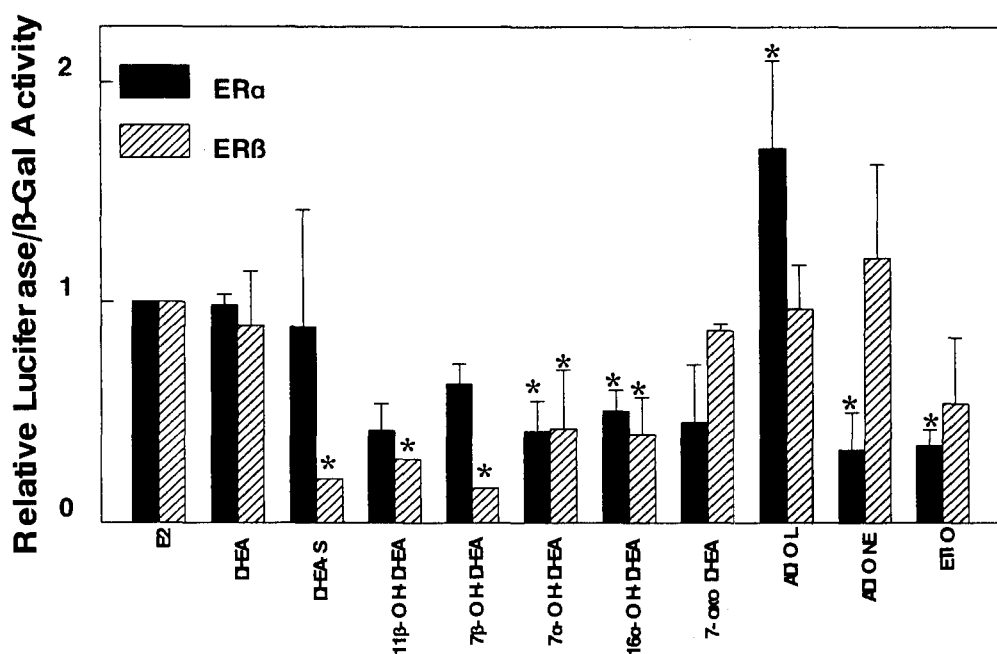


Figure 19. Normalized ERELUC reporter activity in response to DHEA and metabolites. HEK293 and HepG2 cells were transfected with ERELUC reporter plasmid and expression vector for human ER α or human ER β respectively. Both sets of cells were treated for 24 h with vehicle or 5 μ M DHEA metabolites. Cells were then harvested and lysates assayed for β -galactosidase and luciferase activities. Data are normalized to the optimal E2 concentration which was set to 1. Data represent the mean \pm S.D. of three wells. Experiments were repeated three times with similar results. Metabolites tested: DHEA, DHEA-sulfate (DHEA-S) 11 β -hydroxy-DHEA (11 β -OH-DHEA), 7 β -hydroxy-DHEA (7 β -OH-DHEA), 7 α -hydroxy-DHEA (7 α -OH-DHEA), 16 α -hydroxy-DHEA (16 α -OH-DHEA), 7-oxo-DHEA, androstendiol, androstenedione and etiocholanolone. Statistical significance was determined using analysis of variance followed by Student's *t* tests. *, significantly different from cells treated with vehicle, $p < 0.05$.

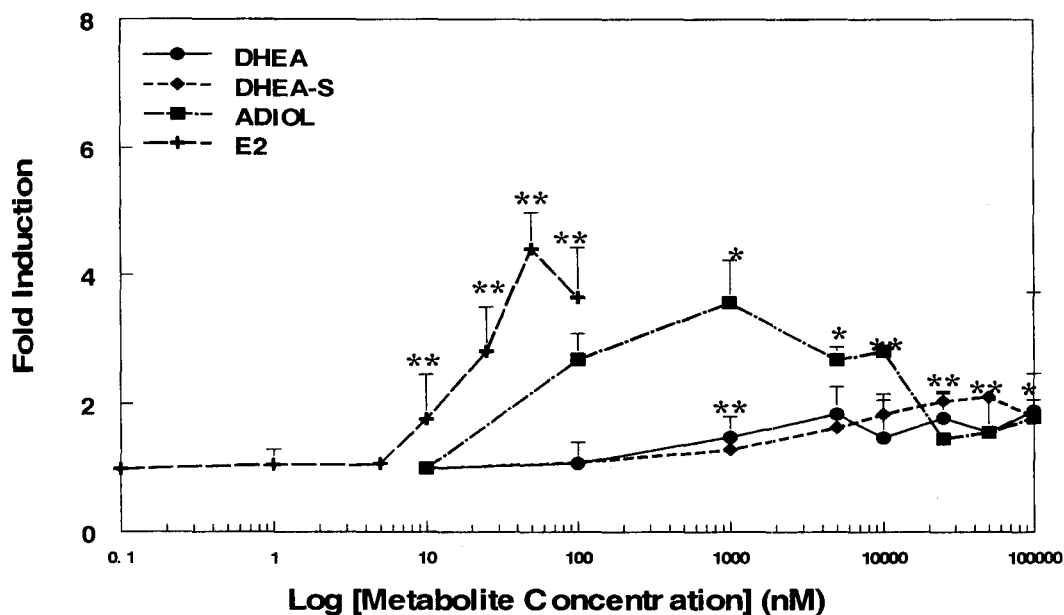


Figure 20. Concentration-dependent activation of ER α by DHEA, DHEA-S, and ADIOL in HEK293 cells. HEK293 cells were transfected with ERELUC reporter plasmid and expression vector for human ER α . Cells were treated for 24h with varying concentrations of E2, DHEA, DHEA-S, and ADIOL. Cells were harvested and lysates were assayed for β -galactosidase and luciferase activities. The data represent the mean \pm S.D. of three wells. Experiments were repeated at least twice with similar results. Statistical significance was determined using analysis of variance followed by Student's *t* tests. *, significantly different from vehicle-treated cells, $p < 0.05$, **, $p < 0.01$. +, E2; ●, DHEA; ◆, DHEA-S; ■, ADIOL.

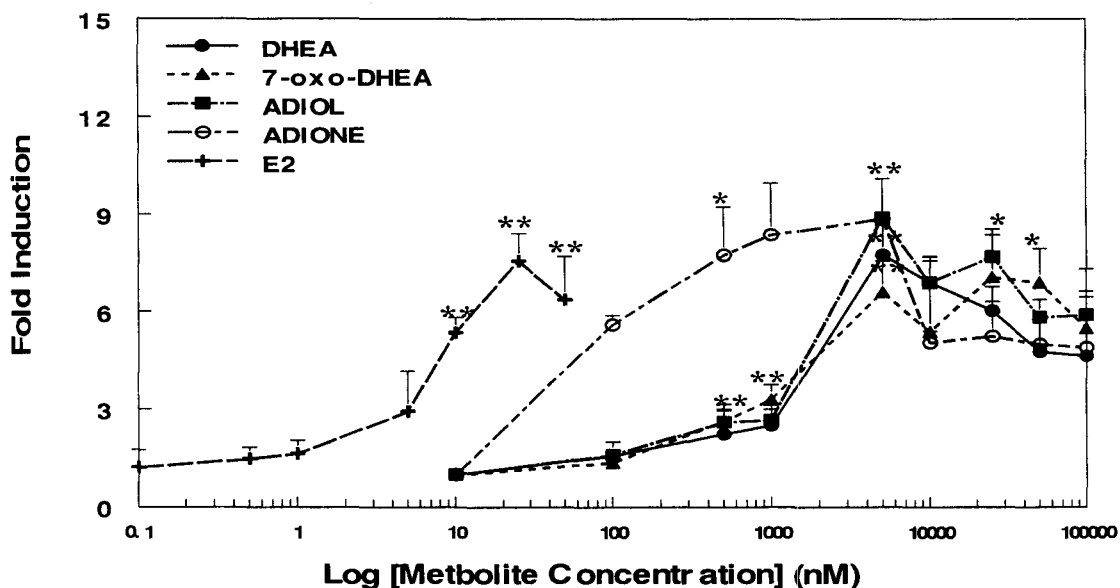


Figure 21. Concentration-dependent activation of ER β by DHEA, 7-oxo-DHEA, ADIOL, and ADIONE in HepG2 cells. HepG2 cells were transfected with ERELUC reporter plasmid and expression vector for human ER β . The cells were treated for 24h with varying concentrations of E2, DHEA, 7-oxo-DHEA, ADIOL, and ADIONE. Cells were harvested and lysates were assayed for β -galactosidase and luciferase activities. Data represent the mean \pm S.D. of three wells. Experiments were repeated at least twice with similar results. Statistical significance was determined using analysis of variance followed by Student's *t* tests. *, significantly different from vehicle-treated cells, $p < 0.05$, **, $p < 0.01$. +, E2; ●, DHEA; ▲, 7-oxo-DHEA; ■, ADIOL; ○, ADIONE.

treated with 5 μ M DHEA metabolites in combination with 100 nM 4-hydroxytamoxifen (4-OHT), 1 μ M ICI 182,780 (ICI), 1 μ M R,R,-THC and 50 nM 17 β -estradiol (E2) in the presence of cotransfected ER α or ER β (Figure 22 and Figure 23). The ER inhibitor, ICI 182,780 as well as the ER antagonist, 4-hydroxytamoxifen inhibited the ER α -mediated induction of ERE by 17 β -estradiol, DHEA, DHEA-S, and ADIOL and also the ER β mediated induction of ERE driven reporter activity by 17 β -estradiol, DHEA, 7-oxo-DHEA, ADIOL, and ADIONE. The ER α agonist/ER β antagonist R,R,-THC significantly inhibited the ER β mediated induction of luciferase activity by 17 β -estradiol, DHEA, 7-oxo-DHEA, ADIOL, and ADIONE, but did not inhibit ERE activation mediated by ER α . In addition, 17 β -estradiol did not act synergistically with DHEA metabolites in ER α - or ER β -mediated induction of ERELUC.

Androgens are converted to estrogens by the aromatase enzyme complex, which consists of the ubiquitous non-specific flavoprotein, NADPH-cytochrome P450 reductase, and a specific microsomal form of cytochrome P450. A majority of breast cancers are estrogen sensitive, because they require the presence of estrogen in order to proliferate (Brodie *et al.*, 1990). Aromatase inhibitors have recently been shown to have significantly greater activity against breast cancer in post-menopausal women with estrogen-sensitive tumors compared to tamoxifen (Smith, 2003). Their mode of action is in preventing the conversion of androgen precursors into active estrogens. In order to examine whether the ER α and ER β ligand-mediated activation of ERELUC is mediated by direct ligand activation of DHEA metabolites, cells were pretreated with 5 μ M miconazole, a general P450 inhibitor and 100 nM exemestane, an aromatase inhibitor.

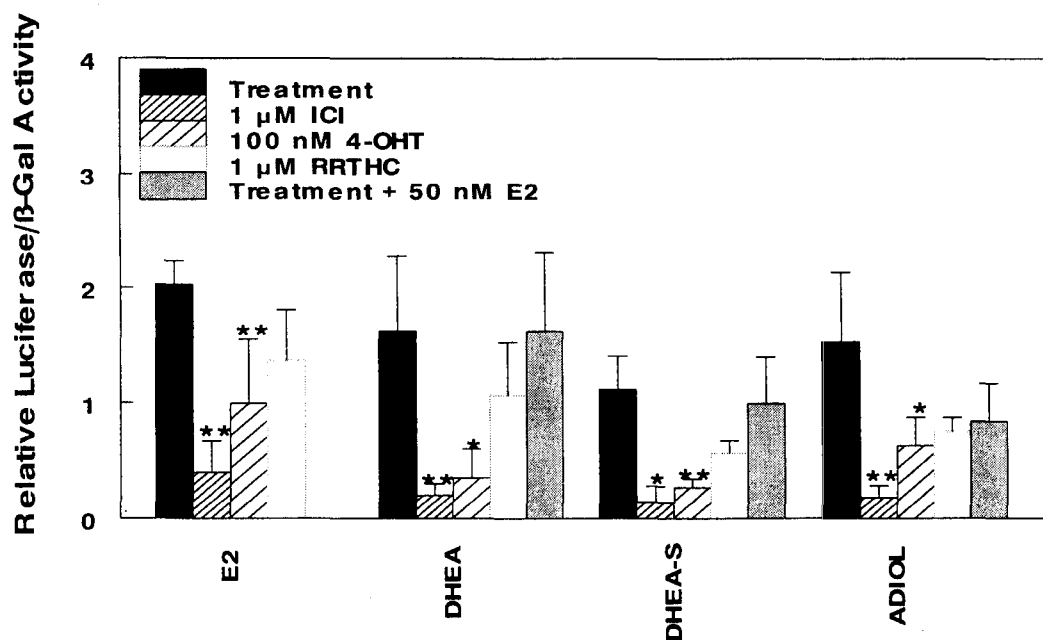


Figure 22. Inhibition of ERELUC reporter activity in the presence of cotransfected ER α in HEK293 cells. HEK293 cells were transfected with an ERELUC reporter plasmid and an expression vector for human ER α . Cells were treated for 24 h with 5 μ M DHEA metabolite, 1 μ M 182,780 ICI, 100 nM 4-hydroxytamoxifen (4-OHT), 1 μ M R,R,-THC, or 50 nM 17 β -estradiol (E2). Cells were then harvested and lysates assayed for β -galactosidase and luciferase activities. Data represent the mean \pm S.D. of three wells. Experiments were repeated three times with similar results. Statistical significance was determined using analysis of variance followed by Student's *t* tests. *, significantly different from treated cells, $p < 0.05$ *, or ** p , 0.01.

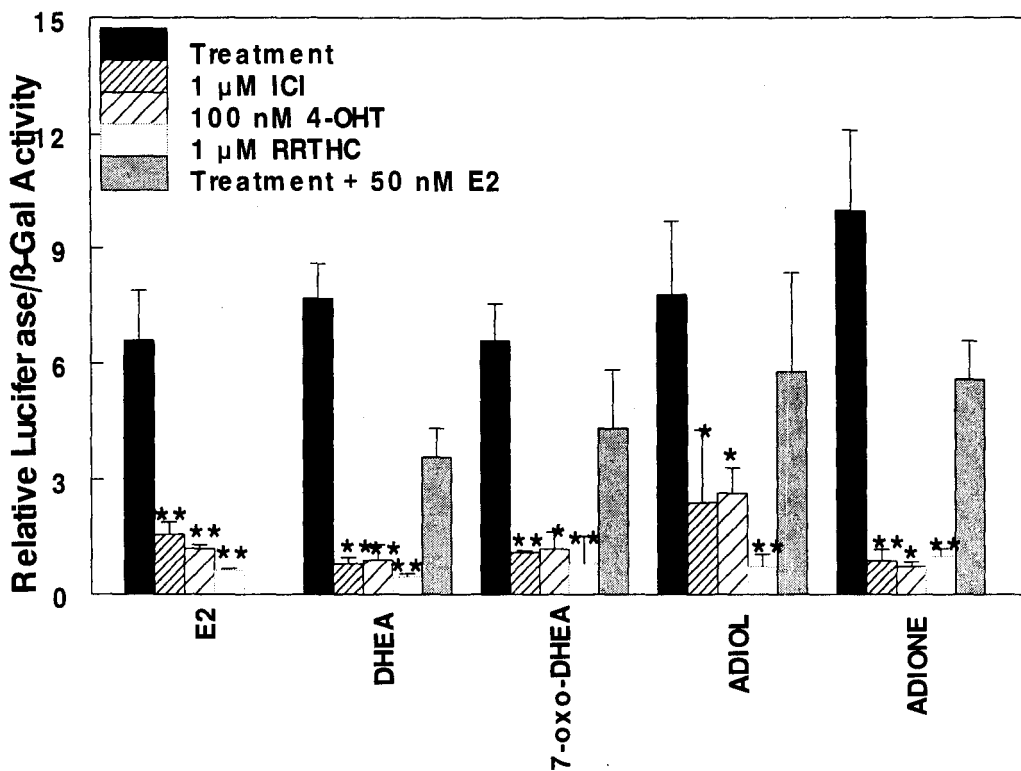


Figure 23. Inhibition of ERELUC reporter activity in the presence of cotransfected ER β in HepG2 cells. HepG2 cells were transfected with ERELUC reporter plasmid and expression vector for human ER β . Cells were treated for 24 h with 5 μ M DHEA metabolite, 1 μ M 182,780 ICI, 100 nM 4-hydroxytamoxifen (4-OHT), 1 μ M R,R,-THC, or 50 nM 17 β -estradiol (E2). Cells were harvested and lysates assayed for β -galactosidase and luciferase activities. Data represent the mean \pm S.D. of three wells. Experiments were repeated three times with similar results. Statistical significance was determined using analysis of variance followed by Student's *t* tests. *, significantly different from treated cells, $p < 0.05$ or ** $p < 0.01$.

As shown by Figure 24, cells treated with miconazole and exemestane exhibited a slight although insignificant decrease in ERELUC reporter activity in response to DHEA and metabolites in the presence of ER α . However, the same decrease was seen with E2. Figure 25 shows that neither miconazole or exemestane had a significant effect of ERELUC reporter activity in response to DHEA or its metabolites in the presence of cotransfected ER β . These results strongly suggest that DHEA and the metabolites we have added to the cells bind directly to ER α and ER β to activation of ERELUC expression and that transcriptional activation seen is not caused by metabolism of the added DHEA or DHEA metabolites to estrogen by aromatase.

In addition to the transient transfection assays, the metabolites that exhibited significant induction of ERELUC were used in a HAP ligand binding assay (Pavlik and Coulson, 1976) with recombinant human ER α or ER β to further examine ligand binding to ER α or ER β . Figure 26 shows that in our hands, the IC₅₀ value of E2 for ER α is ~10 nM which is similar to the value reported in various literature (Branham *et al.*, 2002). ADIOL bound to ER α with an IC₅₀ of ~1 μ M. DHEA and DHEA-S bound ER α with IC₅₀s of >500 μ M and 100-500 μ M respectively. Figure 27 shows that the IC₅₀ of 17 β -E₂ for ER β is ~50 nM. Again, this value is in agreement with previous studies (Branham *et al.*, 2002). Like ER α , ADIOL exhibited significant binding to ER β with an IC₅₀ of ~50 nM followed by ADIONE with an IC₅₀ of 50 μ M. DHEA and 7-oxo-DHEA exhibited IC₅₀ values for ER β of 500 μ M and did not exhibit significant binding.

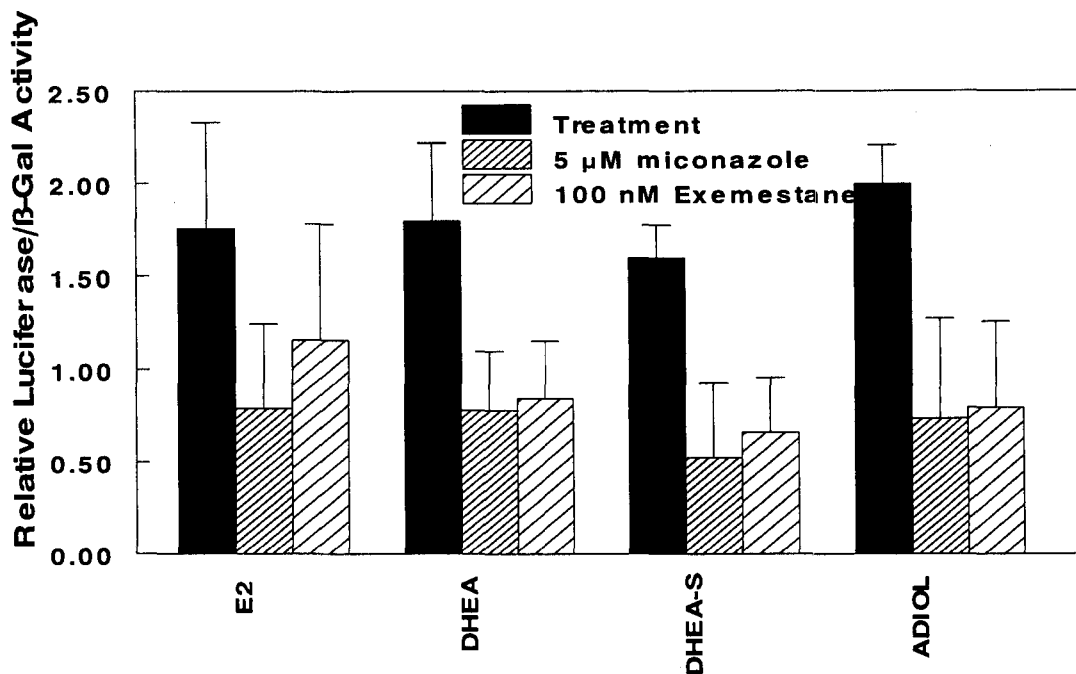


Figure 24. Effect of P450 inhibitors (non-specific inhibitor, miconazole or aromatase inhibitor, exemestane) on ERELUC reporter activity in response to DHEA and metabolites in the presence of cotransfected ER α in HEK293 cells. HEK293 cells were transfected with ERELUC reporter plasmid and expression vector for human ER α . Cells were treated for 24 h with 5 μ M DHEA metabolite and either 5 μ M P450 inhibitor, miconazole (non-specific P450 inhibitor) or 100 nM aromatase inhibitor, exemestane. Cells were harvested and lysates assayed for β -galactosidase and luciferase activities. Data represent the mean \pm S.D. of three wells. Experiments were repeated three times with similar results. None of the results were statistically different.

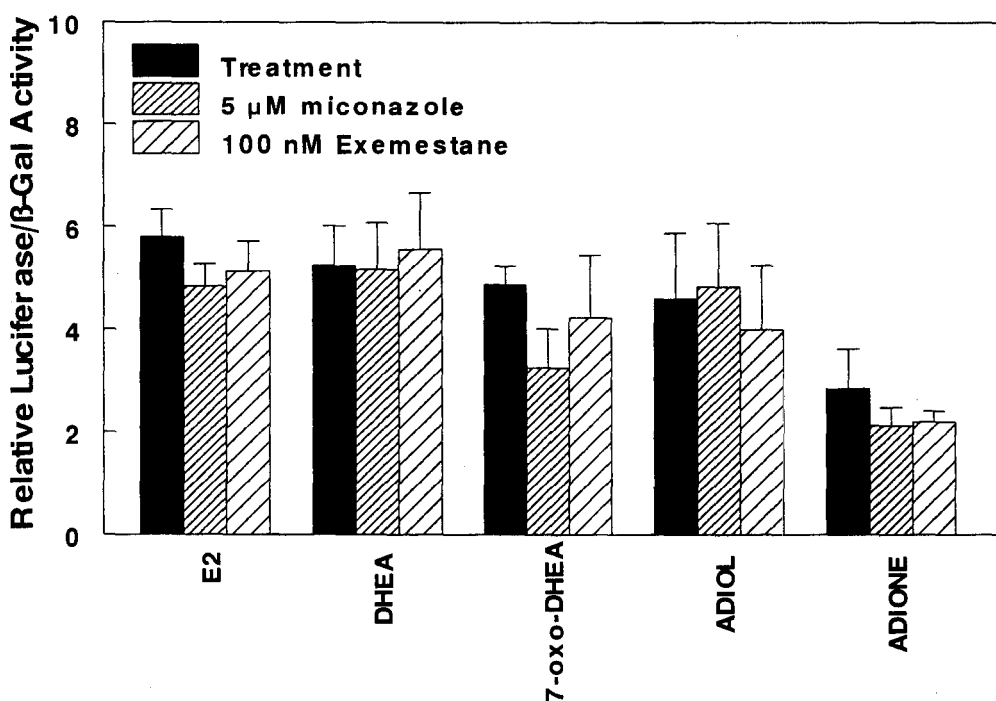


Figure 25. Lack of effect of P450 inhibitors on ERELUC reporter activity in response to DHEA and metabolites in the presence of cotransfected ER β in HepG2 cells. HepG2 cells were transfected with ERELUC reporter plasmid and expression vector for human ER β . Cells were treated for 24 h with 5 μ M DHEA metabolite and either 5 μ M P450 inhibitor, miconazole (non-specific inhibitor) or 100 nM exemestane (aromatase inhibitor). Cells were harvested and lysates assayed for β -galactosidase and luciferase activities. Data represent the mean \pm S.D. of three wells. Experiments were repeated three times with similar results.

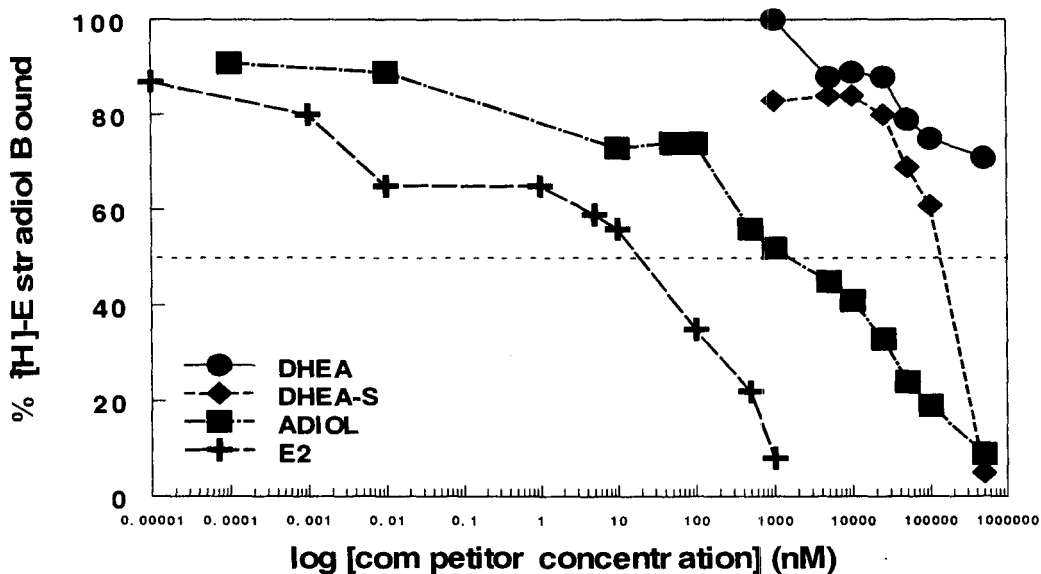


Figure 26. Competition curves for DHEA metabolite binding to ER α . [^3H]-17 β -estradiol (E2) is competing for binding to the ER with increasing concentrations of either nonradiolabeled E2 or DHEA and metabolites (DHEA-S and ADIOL). Each data point represents the mean of two independent binding assays. The competitor concentration causing 50% reduction in [^3H]-E $_2$ binding (IC_{50}) is found at the intersection of the binding curves with the 50% binding line (-----). +, E $_2$; ●, DHEA; ◆, DHEA-S; ■, ADIOL.

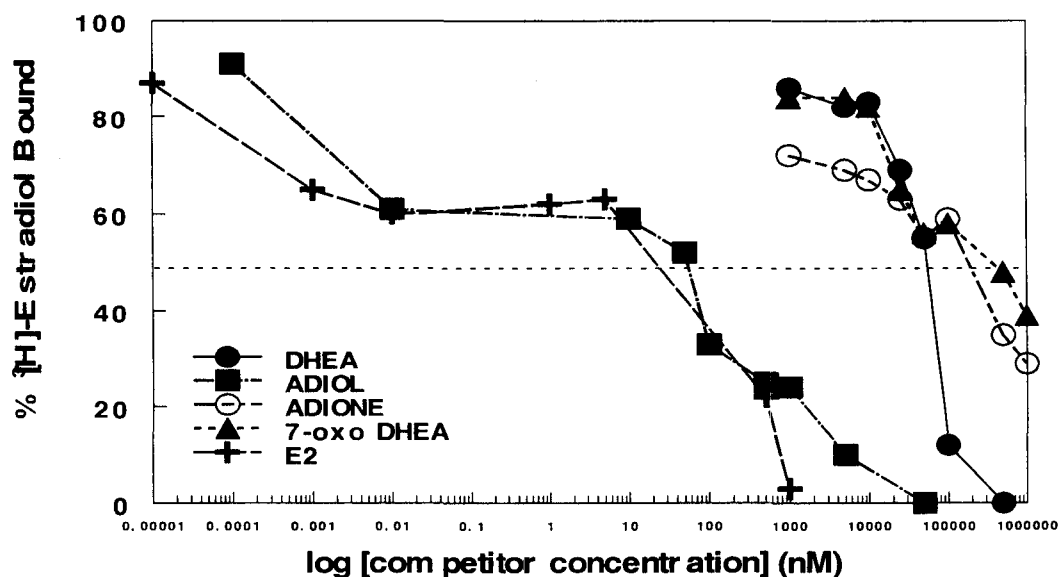


Figure 27. Competition curves for DHEA metabolite binding to ER β . [^3H]-17 β -estradiol (E2) is competing for binding to the ER with increasing concentrations of either nonradiolabeled E2 or DHEA and metabolites (ADIOL, ADIONE, and 7-oxo-DHEA). Each data represents the mean of two independent binding assays. The competitor concentration causing 50% reduction in [^3H]-E $_2$ binding (IC $_{50}$) is found at the intersection of the binding curves with the 50% binding line (-----).+, E2; ●, DHEA; ▲, 7-oxo-DHEA; ■, ADIOL; ○, ADIONE

DISCUSSION

ER plays an important role in the physiology of many tissues (Couse and Korach, 2001). Upon binding estrogen or estrogen-like ligands, ER regulates the expression of certain genes by binding estrogen responsive elements (EREs), promoters within the 5'-flanking region of these genes. Estrogens are known to have profound effects on both female and male reproductive systems, as well as, important roles in cardiovascular system and maintenance of bone tissue (Nilsson and Gustafsson, 2002). Although estrogens are purported to play a protective role in certain diseases such as atherosclerosis, osteoporosis, and Alzheimer's disease, estrogens can also promote carcinomas in other tissues (Hoskins and Weber, 1994). DHEA has been purported to share some of the same beneficial properties as estrogens without the carcinogenic effects of estrogen.

The activation of an ERE-driven luciferase reporter by hER α and hER β was examined in response to E2, DHEA and DHEA metabolites. DHEA, DHEA-S and ADIOL activated ER α -mediated ERE reporter expression in HEK293 cells, whereas DHEA, 7-oxo-DHEA and the cytosolic metabolites, ADIOL and ADIONE activated ER β -mediated ERE reporter expression in HepG2 cells. The ER antagonists ICI 182,780 and 4-OHT blocked the agonist activity of DHEA and metabolites, whereas the P450 inhibitor, miconazole, and the aromatase inhibitor, exemestane, failed to inhibit the induction of ERELUC. Taken together, these results indicate that agonist activity on ERELUC is mediated by a direct interaction of DHEA and selected metabolites with the ligand-binding domain of ER α and ER β . The direct binding of ADIOL to both ER α and ER β was confirmed by ligand binding assay (Figure 26 and Figure 27). However,

according to the ligand binding assay, DHEA-S binds ER α weakly and DHEA bound even more weakly. DHEA, ADIONE, and 7-oxo-DHEA also bind weakly to ER β . This suggests that ADIOL binds to both receptors with relatively high affinity whereas the other metabolites activate the ER α and ER β mediated ERELUC activity by weakly binding the receptors. However, it should be noted that since DHEA-S is present in physiological concentrations of a log higher than DHEA, exogenous DHEA supplementation could possibly increase physiological DHEA-S levels to a point that activates ER α . As a result, the possibility for DHEA-S to be a potent ligand for ER (Le Bail *et al.*, 1998) as well as other receptors should be considered.

Interestingly, in addition to E2, the most potent inducer of ER α -mediated ERELUC transcriptional activation is ADIOL, while the potent inducer of ER β -mediated ERELUC transcriptional activation is ADIONE. Since the ligand-mediated activation of ERELUC is more robust with ER β in HepG2 cells than ER α in HEK293 cells, CHO-K1 (Chinese hamster ovary) and MDA-MB-231 (breast cancer) cells were transfected with both ERs. None of the cell lines examined exhibited both ER α and ER β mediated activation of ERELUC transcriptional activity by E2 and the DHEA metabolites. In HepG2 cells, the action of the AF-1 domain is greater than that of the AF-2 domain, while in HEK293 cells, the activity of the AF-1 domain is similar to that of the AF-2 domain (Metivier *et al.*, 2001). Since AF-1 is known has been found to be stimulated by phosphorylation by mitogen-activated protein kinase (MAPK) (Kato *et al.*, 1995), the ER β mediated activation of ERELUC transcriptional activity by DHEA and metabolites were examined in the presence of the MAPK inhibitor, PD98059. There was no significant difference in the ERELUC transcriptional activity in the presence or absence

of the inhibitor, suggesting that DHEA and metabolite activation of ER α and ER β transcription is not dependent on phosphorylation (data not shown).

In summary, these studies demonstrate that DHEA and metabolites are able to directly activate ER α and ER β in cell-based assays suggesting that DHEA and metabolites mediate the activation of the classic estrogen receptor. These results provide insight into the mechanism of action of DHEA as well as its role in the progression of breast cancer.

CHAPTER IV

DISCUSSION

Dehydroepiandrosterone (DHEA) is C-19 steroid produced by the adrenal gland of humans. DHEA is synthesized from cholesterol by a series of cytochrome P450 mono-oxygenase and hydroxysteroid dehydrogenase catalyzed reactions. DHEA can also be metabolized to androgens and estrogens in steroidogenic tissues where it is then taken up by target tissues, such as testes and ovaries, and converted to sex steroids.

In its sulfated form, DHEA-S, DHEA is the most abundant circulating steroid in humans. Plasma DHEA levels are highest in the second or third decade of life, but decline significantly in late adulthood. The decline in DHEA levels has been purported to be associated with some of the deleterious affects of aging such as memory loss, arteriosclerosis, obesity and cancer (Barret-Conner *et al.*, 1999). As a result, DHEA is marketed as a “fountain of youth hormone” and sold over-the-counter as a dietary supplement.

Treatment of humans with exogenous DHEA has been suggested to have beneficial effects including anti-atherosclerotic properties, enhancement of immune function and memory as well as amelioration of diabetes, systemic lupus erythematosus and obesity (Robinzon *et al.* 1999, Ben-Nathan *et al.*, 1992, and Lapchak *et al.*, 2001). In addition,

DHEA has been reported to have anti-carcinogenic effects in various organs in rodents especially the mammary gland after chemical cancer induction (Mayer, 1998 and Feo *et al.*, 2000). In contrast, Maggiolini *et al.* (1999) reported that DHEA and androstendiol (ADIOL) directly activate transfected ER α reporter genes as well as stimulate proliferation of breast cancer cell lines. Additionally, Calhoun and coworkers (2003) suggest that high DHEA-S levels pose a risk factor for breast cancer patients treated with tamoxifen.

Although DHEA is sold as an over-the-counter supplement and therefore is not regulated by the Food and Drug Administration, a physiological role and the mechanism of action of DHEA have not been defined to date. Therefore, the mechanism of action of this sterol becomes important to study.

It has been suggested that the mechanism by which DHEA exerts its pleotropic effects in humans may involve the metabolism of DHEA in target tissues to biologically active species that are thereby responsible for the various physiological and pharmacological effects of DHEA. In fact, 7-hydroxylated and 7-oxygenated metabolites of DHEA have been reported to have effects in the brain and immune system (Lathe, 2002). Additionally, 16-hydroxylated metabolite of DHEA has been reported to be the main phenolic steroid during pregnancy (Hampl and Starka, 2000)

In addition to being metabolized to active metabolites, DHEA has also been reported to mediate gene expression by serving as a ligand for nuclear receptors. For instance, Ripp *et al.* (2002) demonstrated that in *in vitro* cell-based assays DHEA and its metabolites ADIOL and ADIONE were able to activate the human pregnane X receptor (PXR) which is a nuclear receptor involved in the regulation of the CYP3A subfamily of enzymes that catalyze the oxidation of endogenous steroids as well as the metabolism of a wider array of drugs. DHEA

and ADIOL are known to activate another nuclear receptor, peroxisome proliferator activated receptor alpha (PPAR α) *in vivo* (Peters *et al.*, 1996). Recent studies suggest that in addition to nuclear receptors, DHEA can activate membrane receptors followed by activation of intracellular cell signaling cascades (Liu and Dillon, 2002).

The overall goal of this project was to investigate the mechanism of action of DHEA as it pertains gene regulation mediated by selected nuclear receptors. Our hypothesis was that DHEA is metabolized to biologically active metabolites that exert their action through various receptors and pathways. To begin to address our hypothesis and to investigate the metabolism of DHEA, the liver microsomal metabolism of DHEA by various species was quantified and the P450s responsible for DHEA metabolism were elucidated. A gas chromatography-mass spectrometry method was developed for identification and quantification of DHEA and metabolites. The DHEA metabolites produced by liver microsomal cytochrome P450s exhibited stereo- and regio-selectivity.

7 α -OH-DHEA was the major metabolite formed by rat, hamster and pig followed by 16 α -OH-DHEA. Several unidentified metabolites were formed by hamster liver microsomes, and androstenedione was produced only by pig microsomes. Liver microsomal fractions from one human demonstrated that DHEA was oxidatively metabolized to 7 α -OH-DHEA, 16 α -OH-DHEA, and a previously unidentified metabolite, 7 β -OH-DHEA. Other human microsomal fractions exhibited much lower rates of metabolism but with similar metabolic profiles.

Using expressed cytochrome P450 preparations, CYP3A4 and CYP3A5 were shown to be the cytochromes P450s responsible for production of 7 α -OH-DHEA, 7 β -OH-DHEA and 16 α -OH-DHEA in adult liver microsomes, whereas the fetal form CYP3A7 produced

16 α -OH and 7 β -OH-DHEA. CYP3A23 uniquely formed 7 α -OH-DHEA, whereas other P450s, CYP2B1, CYP2C11 and CYP2D1 were responsible for 16 α -OH-DHEA metabolite production in rat liver microsomal fractions. These metabolites could potentially serve as activators of nuclear receptors or be utilized in following the developmental pattern of CYP3A isoforms.

In order to examine the DHEA-mediated activation of ER α and ER β , DHEA and its metabolites were tested for their ability to activate ER α and ER β in transient transfection assays of HepG2 and HEK293 cells. Two cell types were used because ER α displayed high basal rate in HepG2 cells, whereas ER β was not well activated by 17 β -estradiol in cells other than HepG2 cells. DHEA, DHEA-S and ADIOL activated ER α -mediated ERE reporter expression in HEK293 cells and DHEA, 7-oxo-DHEA, ADIOL, and ADIONE activated ER β -mediated ERE reporter expression in HepG2 cells. The antiestrogens ICI 182,780 and 4-hydroxytamoxifen blocked the agonist activity of DHEA and metabolites suggesting that the agonist activity of DHEA and metabolites is mediated by a direct interaction between ligand and ER. The general P450 inhibitor, miconazole and exemestane, an aromatase inhibitor, decreased E2 and DHEA-mediated ERELUC activities mimicked by ER α to the same extent. Neither miconazole nor exemestane inhibited ER β activated ERELUC transcription with E2, DHEA or DHEA metabolites. Taken together, these results suggest that DHEA and metabolites do not exert their activity by being metabolized to estrogens in this cell-based assay, but serve as direct ligands for ER α and ER β . This was demonstrated directly in competitive binding assays with ER α and ER β .

DHEA and ADIOL were inducers of both ER α - and ER β -mediated ERELUC transactivation. However, DHEA-S was an inducer of ER α , while ADIONE and 7-oxo-

DHEA were inducers of ER β . A ligand-binding assay confirmed the direct binding of ADIOL to both ER α and ER β . However, the other metabolites had a higher IC₅₀ values for their respective receptors, suggesting a weaker binding of the other metabolites to ER α and ER β . Since DHEA-S has relatively higher circulating concentrations in the human, its potential role as a potent ligand for nuclear receptors should be considered.

In summary, this study demonstrates that DHEA is extensively metabolized by liver microsomal fractions and the human produces a unique metabolite, 7 β -OH-DHEA. These data support the hypothesis that the metabolism of DHEA to various metabolites may play a role in the biological action of DHEA. Additionally, DHEA and metabolites were also shown to directly activate ER α and ER β in *in vitro* cell-based assays, suggesting DHEA mediates the activation of the classic estrogen receptor. Ultimately, the results of these studies provide new insights into the many mechanisms of action described for DHEA and demonstrate that DHEA exerts its biological effects through metabolism to multiple active metabolites that mediate the action of nuclear receptors via several mechanisms (Figure 28).

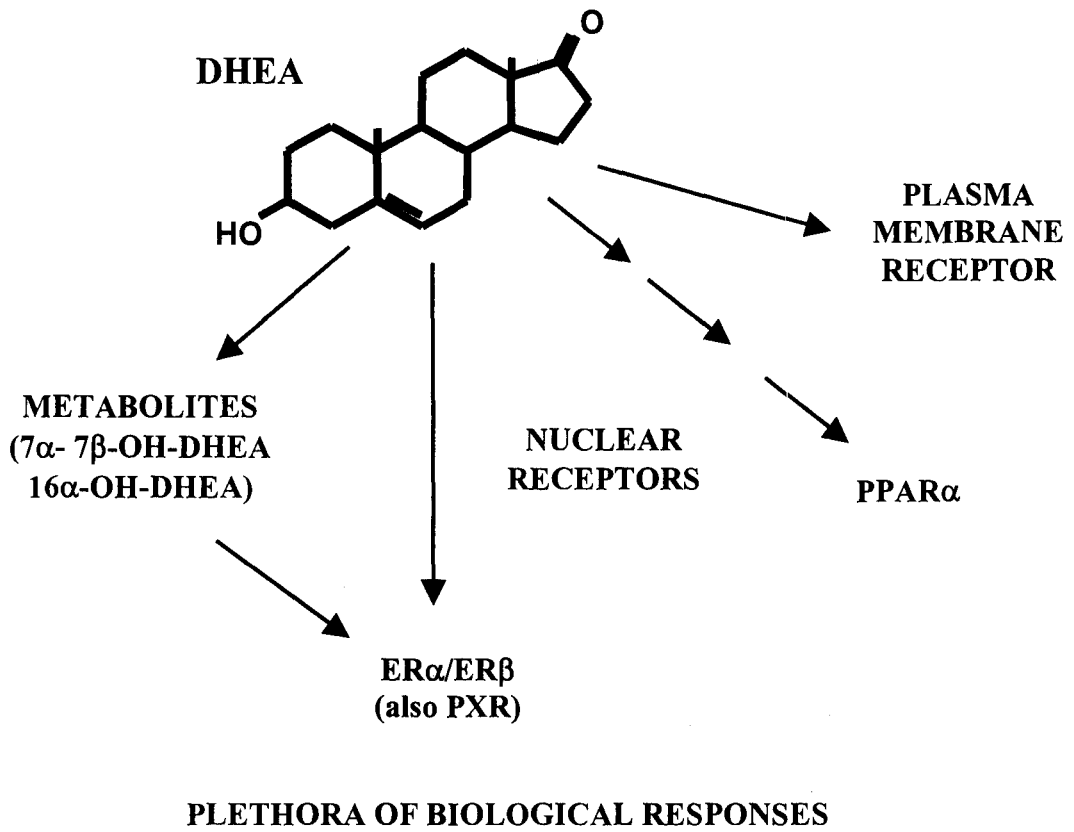


Figure 28. DHEA action is mediated by multiple receptors and metabolites.

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APPENDICES

LIST OF ABBREVIATIONS

3 β -HSD: 3 β -hydroxy steroid dehydrogenase

4-OHT: 4-hydroxytamoxifen

7 α -OH-DHEA: 7 α -hydroxy-DHEA

7 β -OH-DHEA: 7 β -hydroxy-DHEA

16 α -OH-DHEA: 16 α -hydroxy-DHEA

7-oxo-DHEA: 3 β -hydroxy-androst-5-ene-7,17-dione

ACTH: adrenocorticotrophin

ADIOL: androstendiol (androst-5-ene-3,17-diol)

ADIONE: androstenedione (androst-5-ene-3,17-dione)

AF-1: activation function-1

AF-2: activation function-2

BSTFA-TMS: N,O-bis(trimethylsilyl)trifluoroacetamide

CAR: constitutive androstane receptor

CYP: cytochrome P450

DBD: DNA binding domain

DHEA: dehydroepiandrosterone (3 β -hydroxy-androst-5-ene-17-one)

DHEA-S: DHEA 3 β -sulfate

DMSO: dimethyl sulfoxide

DTT: dithiothreitol

E2: 17 β -estradiol

EET: epoxyeicosatrienoid acid

EI: electron ionization

ER: endoplasmic reticulum

ER: estrogen receptor

ERE: estrogen response element

ETIO: etiocholanolone

FAD: flavin adenine dinucleotide

FDA: Food and Drug Administration

FMN: flavin mononucleotide

FXR: farnesoid X receptor

GC/MS: gas chromatography-mass spectrometry

HAP: hydroxyapatite

HETE: hydroxyeicosatetraenoic acid

HPETE: hydroperoxyeicosatetraenoic acid

LC-MS: liquid chromatography-mass spectrometry

LBD: ligand binding domain

LXR: liver X receptor

MAPK: mitogen activated protein kinase

MOX: methoxyamine · HCl

NADPH: nicotinamide adenine dinucleotide phosphate

NAD: nicotinamide adenine dinucleotide

P450: cytochrome P450

P450 oxidoreductase: NADPH/cytochrome P450 oxidoreductase

PKA: protein kinase A

PKC: protein kinase C

PMSF: phenyl methyl sulfonyl fluoride

PPAR: peroxisome proliferator activated receptor

PPRE: PPAR α response element

RXR: retinoic acid receptor

SD: standard deviation

TR: thyroid hormone receptor

VDR: vitamin D receptor

CURRICULUM VITA

KRISTY K. MICHAEL MILLER

Business Address:

Department of Chemistry
University of Evansville
1800 Lincoln Avenue
Evansville, IN 47722
Phone: (812) 479-2035 Fax: (812) 475-6429

Home Address:

713 Northeast First Street
Washington, IN 47501
Phone: (812) 254-5735
Email: kristykmiller@yahoo.com

EDUCATION

University of Louisville**Department of Biochemistry and Molecular Biology**

- Ph.D. – April 2004

Indiana University - Bloomington

- B.S. December 1999 - Chemistry
- Secondary Teacher Certification December, 1999 - Chemistry and Mathematics

TEACHING EXPERIENCE

Assistant Professor of Chemistry

- University of Evansville Department of Chemistry – August 2004
 - Biochemistry
 - Biochemistry Laboratory
 - General Chemistry
 - *Honors* General Chemistry Laboratory

Adjunct Instructor at Vincennes University

- Anatomy and Physiology – June 1, 2004 – July 7, 2004

Adjunct Instructor at Oakland City University

- Introduction to Physical Science - May 31, 2002 - June 28, 2002

Graduate Teaching Assistant

- Graduate Biochemistry II – University of Louisville – 2002
 - Graded homework
 - Conducted weekly review sessions

Guest Lecturer

- “Steroid Hormones” – Transylvania University - November 28, 2001

Future Professors Program

- University of Louisville – 2001-2002

Secondary Teaching Certification

- Chemistry
- Mathematics

RESEARCH EXPERIENCE

University of Louisville

Department of Biochemistry and Molecular Biology

- Dissertation Title: "DHEA Action is Mediated By Multiple Receptors and Metabolites"
- Under the supervision of Professor Russell A. Prough Ph.D. - January 2000-April 2004
- Research involves elucidation of mechanism of gene regulation by dehydroepiandrosterone (DHEA)
 - Cell culture
 - Transient transfection of plasmid DNA
 - β -galactosidase and luciferase assays
 - Cell proliferation assays
 - Enzyme assays and kinetic determinations
 - Gas chromatography/Mass Spectrometry
 - Animal dosing and tissue isolation
 -

ACTIVITIES/AFFILIATIONS

Graduate Executive Committee (GEC)

- University of Louisville Department of Biochemistry - 2002-2003
 - Student representative to faculty within the department at the University of Louisville
 - Responsible for welcoming prospective students

International Society for the Study of Xenobiotics (ISSX) – 2002 - present

- Attend and present at annual national meetings

American Society for Pharmacology and Experimental Therapeutics (ASPET) – 2002 - present

PUBLICATIONS

Peer Reviewed Journal Articles:

1. Robnson B, **Michael KK**, Ripp SL, Winters SJ, and Prough RA (2003). Glucocorticoids inhibit interconversion of 7- hydroxy- and 7- oxo metabolites of dehydroepiandrosterone (DHEA): A role for 11 β -hydroxysteroid dehydrogenases? *Arch. Biochem. Biophys.* **412**:251-258.
2. **Michael Miller KK**, Cai J, Ripp SL, Pierce WM, Rushmore TH and Prough RA (2004). Regioselectivity accounts for the diversity of dehydroepiandrosterone metabolites produced by liver microsomal cytochrome P450s. *Drug Metab. Dispos.* **32**:305-313
3. Robnson B, **Michael Miller KK** and Prough RA (2003). Synthesis of [3 H]-7 α -hydroxy-, 7 β -hydroxy-and 7-oxo-dehydroepiandrosterone using pig liver microsomal fractions. *Analytical Biochem.* **SUBMITTED.**

Manuscripts in preparation:

1. **Michael Miller KK**, Klinge CM, Ripp SL and Prough RA (2004). DHEA and Metabolites Activate Estrogen Receptor β . **in preparation**.

Abstracts:

1. **Michael KK**, Cai J, and Prough RA. "Species Differences in the Metabolism of DHEA." Midwest Regional Molecular Endocrinology Conference – Bloomington, IN – oral presentation.
2. **Michael KK**, Cai J, and Prough RA. "Species Differences in the Metabolism of DHEA." *Drug Metabolism Reviews* 34:107 (2002). International Society for the Study of Xenobiotics – Orlando, FL – poster.
3. Ripp SL, **Michael Miller KK**, and Prough RA (2003). "DHEA Suppresses CYP2C11 Expression through a PPAR-Independent Mechanism." *American Society of Pharmacology & Experimental Therapeutics* (2003). Experimental Biology - San Diego, CA.
4. **Michael Miller KK**, Klinge CM, Ripp SL and Prough RA (2003). "DHEA and Metabolites Activate Estrogen Receptor β ." *Drug Metabolism Reviews* (2003). International Society for the Study of Xenobiotics – Providence, RI – poster, **FINALIST**.
5. Prough RA, Geoghegan TE, Gu S, and **Michael Miller KK** (2004). "Novel Receptors for Dehydroepiandrosterone and Metabolites." *Drug Metabolism Reviews* (2004). International Society for the Study of Xenobiotics – Vancouver, BC – presentation.
6. **Michael Miller KK**, Tamasi V, Ripp SL, and Prough RA (2004). "DHEA Regulates PPAR α by Phosphorylation." *Drug Metabolism Reviews* (2004). International Society for the Study of Xenobiotics – Vancouver, BC – poster.

AWARDS/HONORS

- Who's Who Among Students in American Universities and Colleges - 2004
- American Heart Association Predoctoral Fellowship - July, 2001- July, 2003
- Reynolds Scholarship – Indiana University – 1995-1999
- Indiana Higher Education Award – Indiana University – 1995-1999
- Honors Program - Indiana University - 1995–1997
- Honors Division Scholarship – Indiana University – 1995-1997

REFERENCES

Russell A. Prough Ph.D. - Professor
Department of Biochemistry and Molecular Biology
University of Louisville School of Medicine
Health Sciences Center, Bldg. A
Louisville, KY 40202
Phone: (502) 852-5217 Fax: (502) 852-6222
e-mail: russ.prough@louisville.edu

Kenneth Coy - Chemistry Teacher
Bedford North Lawrence High School
R.R. 13 Box 410
Bedford, Indiana 47421
Phone: (812) 279-9756 Fax: (812) 279-9304
e-mail: COYK@nlcs.k12.in.us

Sharon L. Ripp Ph.D. – Senior Research Scientist
Pfizer Global Research and Development
Groton Laboratories
Eastern Point Road 4009
Groton, CT 06340
Phone: (860) 715-6492 Fax: (860) 441-4109
e-mail: sharon_l_ripp@groton.pfizer.com

Jill V. Lyles Ph.D. – Indiana University
3313 Rolling Oak Drive
Bloomington, IN 47401
Phone: (812) 330-9119
e-mail: Rlyles@cs.com