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# THE BIOLOGICAL ACTIONS OF DEHYDROEPIANDROSTERONE INVOLVES MULTIPLE RECEPTORS

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## Abstract

Dehydroepiandrosterone has been thought to have physiological functions other than as an androgen precursor. The previous studies performed have demonstrated a number of biological effects in rodents, such as amelioration of disease in diabetic, chemical carcinogenesis, and obesity models. To date, activation of the peroxisome proliferators activated receptor alpha, pregnane X receptor, and estrogen receptor by DHEA and its metabolites have been demonstrated. Several membrane-associated receptors have also been elucidated leading to additional mechanisms by which DHEA may exert its biological effects. This review will provide an overview of the receptor multiplicity involved in the biological activity of this sterol.

### Keywords

Dehydroepiandrosterone; DHEA; Hormone receptors; 11β-hydroxysteroid dehydrogenases; Cytochrome P450; Peroxisome proliveration; PPARα; PXR; Estrogen receptor; Insulin growth factor binding protein 1

## INTRODUCTION

The metabolism of sterols by cytochromes P450 and the mechanisms of hydroxylation of sterols were among the research topics of great interest to Dr. David Kupfer. On many occasions, I contacted David for his opinion about experiments I was pursuing and the discussions always led to his informing me of the current work in his laboratory. He was always free with his technical comments and encouraging about our work. David was also one person I could talk to as a colleague when I had a difficult decision to make or if I wanted to have another opinion about a critical review from a journal or study section. I last saw David at the ISSX meeting in Vancouver in September 2004 and had a conversation about his health, his concerns, and his optimism about the future. His absence will be noticed at future meetings and scientific discussions.

### Dehydroepiandrosterone: A Nutraceutical with Interesting Biological Function

Because the decline in production DHEA in the human adrenal gland is associated with some of the pathophysiological effects of aging, many people supplement their own “declining” DHEA levels with exogenous DHEA and even refer to DHEA as the “fountain of youth hormone.” Although appropriate physiological doses are not well-defined and differ in men

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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 distribution and sale 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. Thus, nutritional supplements, such as DHEA, can be sold unless the FDA proves that they are unsafe.

Because DHEA and DHEA-S have higher serum concentrations than other sterols in humans, DHEA has been viewed as a potential androgen, as a storage repository for androgens, and a precursor to sex hormones (Ebeling and Koivisto, 1994). However, other than being a precursor to sex hormones and playing a role in the development of pubic and axillary hair and the development and maintenance of immuno-competence, a physiological role for DHEA has not been clearly defined to date (Regelson et al., 1994). DHEA is produced by the adrenal gland in humans and is taken up by several tissues, including the brain, liver, kidney, and gonads, where it is metabolized to 5-androstene-3β,17β-diol, 4-androstene-3,17-dione, testosterone, estrogen, and other biologically active steroids, depending on the tissue. The work of Labrie et al. (1995) suggests 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, followed by metabolism to yield androgens and estrogens.

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 (McCormick et al., 1996; Lubet et al., 1998; Rao et al., 1999). 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 (Robinzon et al. 2003, Ben-Nathan et al. 1992, and Lapchak et al. 2001). However, the mechanism of these processes is not well-documented to date.

Because 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, it 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 only limited evidence to support its use in humans and there have been few clinical trials that clearly substantiate the efficacy and safety for use of 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.

## Biosynthesis of Dehydroepiandrosterone

Dehydroepiandrosterone (5-androsten-3β-ol-17-one) is a naturally occurring C-19 adrenal steroid derived from cholesterol by a series of cytochrome P450-dependent monooxygenase and hydroxysteroid dehydrogenase catalyzed reactions. 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 daily synthesizes DHEA from cholesterol and secretes 75−90% of the body's DHEA, with the remainder being produced by the testes and ovaries (Vermeulen, 1980; de Peretti and Forest, 1978; Nieschlag et al., 1973).

Primates produce DHEA by the $\Delta^5$-steroidogenic pathway in which the double bond at the C-5 and C-6 position is maintained during metabolism (Fig. 1). 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. Little or no DHEA is produced by the adrenal of nonprimate species, such as mice and rats. Instead, other species produce sex steroids via the $\Delta^4$-steroidogenic pathway in which cholesterol is converted to pregnenolone by $P450_{scc}$. Pregnenolone is then converted to progesterone by 3β-hydroxysteroid dehydrogenase (3β-HSD) that is taken up by peripheral steroidogenic tissues and converted to 5-androstene-3,17-dione (ADIONE) by P450C17.

Although DHEA is a primary sterol produced in these biosynthetic pathways, DHEA is largely found in circulation in its sulfated form, DHEA 3β-sulfate (DHEA-S), which can be inter-converted with DHEA by DHEA sulfotransferases and hydroxysteroid sulfatases (Kalimi and Regelson, 1990). Although DHEA-S is the hydrophilic storage form that circulates in the blood, 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 storage and DHEA further metabolism (Allolio and Arlt, 2002).

In humans, plasma DHEA concentrations are found in the range of 1−4 ng/mL (0.003−0.015 μM), but circulating DHEA-S concentrations are much higher on a molar basis, between 250 and 500 times higher (~3−10 μM) in women and men, respectively (Barrett-Connor et al., 1986; Hopper and Yen, 1975; Labrie et al., 1995). 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. The abundant circulating concentrations of DHEA-S are due in part to the clearance of DHEA from the blood at a rate of approximately 2000 L/day, whereas DHEA-S clearance is about 13 L/day (Lephart et al., 1987). Therefore, DHEA has a half-life in blood of about 1−3 hrs, while DHEA-S has a half-life of 10−20 hrs (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 compound 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 ADIONE. During fetal development, plasma DHEA-S levels are around 100−200 μg/dL (3−7 μM), but fall rapidly after birth and remain low until adrenarche. Blood DHEA levels then rise and peak at around 300 μg/dL (10 μM) during the third 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, as described by Rainey et al. (2002) with peak levels of circulating DHEA around age 25−30 of approximately 10 μM for men and 5 μM for women. The circulating levels decline throughout life in the human adult. Labrie and coworkers (1987) suggest that a decrease in 17,20-lyase activity (see Fig. 1) may be responsible for the dramatic age-related reduction in DHEA and DHEA-S secretion. Regardless, the drastic developmental changes in DHEA secretion are not observed for 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 such as cortisol, decline with age more slowly than DHEA. 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-Connor et al., 1999). This has recently been supported by the Baltimore Longitudinal Studies in men that showed a positive correlation between decreased morbidity in life and levels of DHEA-S (Roth et al., 2002); these studies have suggested that biomarkers of caloric restriction are associated with longer life in man and higher primates.

## DHEA Action Involves a Variety of Receptors

Studies to evaluate whether there are specific receptors that bind DHEA as a ligand have been a topic of interest for over 20 years. A few studies have demonstrated the existence of proteins in liver that bind this sterol, but the biochemical identity of these proteins has remained unknown (Kalimi and Regelson, 1990; Widstrom and Dillon, 2004). In the last 10 years, a series of orphan receptors, namely, peroxisome proliferators activated receptor (PPAR), pregnane X receptor (PXR), constitutive androstanol receptor (CAR), and estrogen receptor β (Goodwin et al., 2002; Kliewer et al., 2002; Moore et al., 2002; Swales and Negishi, 2004) have been elucidated and there is evidence that DHEA and some of its metabolites either bind to or activate these newly characterized receptors (Kuiper et al., 1997; Ripp et al., 2002). The biological action of DHEA and its metabolites in activation of these receptors will be described in the following sections.

## Peroxisome Proliferator Activated Receptor alpha (PPARα)

In 1989, we began a series of experiments feeding rodents with 0.45% DHEA in their diet (Wu et al., 1989; Prough et al., 1994). We noted lower rates of growth based on body weight, and the body burden of fat was decreased in rats. In addition, studies by Leighton et al. (1987) demonstrated the increase in peroxisome number and size in rats treated with dehydroepiandrosterone acetate. Our studies also showed that liver weight increased relative to body weight, suggesting hypertrophy of the liver. After surveying a series of P450-specific substrates, our results demonstrated that DHEA administration to rats induced liver microsomal metabolism of lauric acid by 17-fold (associated with P4504A), induced 6β- and 16β hydroxylation of ADIONE and testosterone by 3 to 6-fold (associated with P4503A), and suppressed 16α-hydroxylation of ADIONE and testosterone by 80% (associated with P4502C11). In addition, rat liver microsomal NADPH:cytochrome c oxidoreductase and catalase activities were both increased approximately 2-fold and fatty acyl-CoA oxidase (FACO) activity was induced 10-fold (Prough et al., 1994). These results suggested that DHEA feeding causes pronounced changes in a milieu of cytochromes P450 isoforms in rodent livers, some of which are associated with peroxisome proliferators.

Subsequently, we demonstrated that ADIOL and DHEA, and to a much lesser extent, 17α-hydroxypregnenolone, at 80 mg/kg body weight, induced FACO and P4504A protein levels (Prough et al., 1994). Administration of ADIONE *per os* did not cause an increase in hepatic P4504A1 or NADPH:cytochrome P450 oxidoreductase protein or activity, nor did it alter expression of FACO activity. The time course for maximal induction of P4504A protein/activity and NADPH:cytochrome *c* reductase activity required 3−4 days of DHEA administration. Nuclear run-on assays demonstrated that these induction phenomena were regulated at the transcriptional level (Webb et al., 1996). ADIOL appeared to be slightly better as an inducer when a dose-response curve was carefully determined. Subsequent studies by Peters et al. (1996) demonstrated the requirement of the peroxisome proliferator activated receptor α for induction by DHEA sulfate. These studies clearly demonstrate that feeding of DHEA or its reduced product, ADIOL, leads to peroxisome proliferation in rats and mice. The biological action of these sterols requires PPARα, but our subsequent studies suggest that DHEA does not activate PPARα in a ligand-dependent process.

To define the mechanism of action of DHEA, we performed metabolism studies with DHEA using rat liver proteins. As expected, ADIONE and ADIOL were formed from cytosolic fractions of rat liver in the presence of $NAD^+$ and NADH, respectively (R. W. Estabrook and R. A. Prough, data not shown). In the presence of rat liver microsomes, Fitzpatrick et al. (2001) and Miller et al. (2004) demonstrated that 7α-hydroxy- and 16α-hydroxy-DHEA are major oxidative metabolites and that the affinity of DHEA ($EC_{50}$) for cytochromes P450 was approximately 5 μM (Fitzpatrick et al., 2001; X.-D. Lei and R.A. Prough, data not shown). The metabolism *in vitro* was extensive, consuming nearly all of the 50 μM DHEA substrate within 10 min. When human liver microsomal protein fractions were utilized, we observed 7α-, 7β-, and 16α-hydroxy-DHEA and at longer incubation times we also observed 7-oxo-DHEA as a product. Carbenoxolone, a potent inhibitor of 11β-hydroxysteroid dehydrogenase, prevented formation of 7-oxo-DHEA from 7α-hydroxy-DHEA in the presence of $NADP^+$ with rodent and human liver microsomal fractions and also the reduction of 7-oxo-DHEA to 7α-hydroxy-DHEA and 7β-hydroxy-DHEA by NADPH by human and pig microsomal fractions (Fitzpatrick et al., 2001; Miller et al., 2004; Robinzon et al., 2003; Robinzon and Prough, 2005). Interestingly, DHEA sulfate was not a direct substrate for the P450s and apparently must be hydrolyzed to DHEA prior to oxidative metabolism (Fitzpatrick et al. 2002; K. K. Michael Miller and R. A. Prough, unpublished data). These results demonstrate that DHEA is extensively metabolized in the liver through HSD- and P450-dependent reactions.

Subsequently, we tested whether DHEA or its known hepatic metabolites (Fitzpatrick et al. 2002; Michael Miller et al., 2004) activated PPARα using transient transfection experiments. We transfected HepG2 cells with murine PPARα and a reporter construct containing a copy of the peroxisome proliferator activated receptor responsive element (PPRE) from the 5′-flanking region of the murine fatty acyl-CoA (FACO) oxidase gene. Using the HepG2 cells, neither DHEA nor any oxidative metabolites were effective activators of PPARα in this transient transfection assay system, but the peroxisome proliferators nafenopin and Wyeth 14643 (data not shown) were excellent activators of the receptor (Fig. 2). These results with DHEA have been reported by others, but the other hepatic metabolites had not been tested in a cell-based assay. In our hands, none of the oxidative metabolites of DHEA serve as activators of PPARα. In contrast, studies with primary cultures of rat hepatocytes revealed that DHEA, ADIOL, and the 7-oxidized metabolites of DHEA do activate FACO and P4504A message and protein levels in the first 2−3 days in culture. The inductive response is not as potent as nafenopin, but 1−20 μM concentrations of DHEA and its metabolites do cause induction of these gene products in primary hepatocytes (Fig. 3). The central question remaining is how DHEA or its oxidative metabolites cause activation of PPARα in hepatocytes and what factor is missing in HepG2 cells that obviate induction of PPARα responses in reporter gene assays. This will be discussed in the section on ligand-independent activation processes, following the receptor-mediated processes regulated by DHEA.

## Pregnane X Receptor

In the last decade, another orphan receptor, the pregnane X receptor, has been elucidated which binds pregnanes, bile acids, and a number of drugs and foreign compounds (rifampacin, pregnenolone-16α-carbonitrile, etc.) as ligand activators (Moore et al., 2002; Dussault and Forman, 2002). This nuclear sterol receptor displays some interesting differences relative to other nuclear receptors. For example, there are some pronounced polymorphisms in the ligand-binding domain that exists, between the rodent and human receptor. The PXR orthologs from rabbits, rats, mice, and humans exhibit high sequence similarity in their DNA-binding domains. However, they show variation in amino acid sequence within the ligand-binding domains, and therefore exhibit significant species-specific differences in response to ligands (Jones et al., 2000). For example, the antibiotic rifampicin is a good activator of human and rabbit PXR, but a very poor activator of the rat and mouse PXR. In contrast, pregnenolone-16α-carbonitrile is

a good activator of rat and mouse PXR, but a poor activator of the human PXR. In addition, a large number of drugs and foreign compounds serve to activate the receptor, including ligands for the glucocorticoid receptor, natural products including sterols of the pregnane class, drugs like clofibrate, phthalate plasticizers, and chlorinated pesticides (Jones et al., 2000; Hurst and Waxman, 2003, 2004; LeMaire et al., 2004). The contrasting responses involved in activation of the pregnane X receptor by various compounds caused pronounced differences in induction of this receptor in rodent models and humans *in vivo*.

Because we observed induction of P4503A23 in livers of rats fed DHEA (Prough et al., 1995; Ripp et al., 2002), we suspected that PXR may also be activated by DHEA and its metabolites. We performed experiments in PPARα-null mice, treating them with DHEA. As also reported by Peters et al. (1996), we were unable to see induction of P4504A11. However, there was a 2-fold increase in the levels of enzyme activity and mRNA specific for P4503A11 in wild-type and PPARα-null mice (Ripp et al., 2002). We have also observed that P4503A11 is not induced by DHEA feeding in PXR null mice (S. J. Webb and R. A. Prough, unpublished results).

Subsequently, we utilized a luciferase reporter construct containing a functional PXR responsive element (PXRE) from the 5′-flanking region of the rat P4503A23 gene. We performed experiments using both the rodent and human PXR. DHEA did activate both receptors, but it activated the human receptor with an affinity in the 50−100 μM ($EC_{50}$ = 70 μM) compared to the rodent receptors ($EC_{50}$ = 20 μM). ADIOL and ADIONE displayed an interesting divergence in affinity between the human and rodent receptors, in that ADIOL did not activate the human PXR well. ADIONE ($EC_{50}$ = 25 μM) was better than DHEA in activating the human PXR, but was not as good as ADIOL ($EC_{50}$ = 25 for ADIOL vs. $EC_{50}$ = 70 μM for ADIONE) in activating the rodent receptor. Therefore, ADIONE, a compound used illegally in enhancing muscle mass growth, activates PXR in humans, presumably as a method of ridding the body of this sterol.

## Estrogen Receptor

In the last decade, many new and exciting functions of estrogen receptors have been elucidated. For example, Gustafsson and coworkers (Kuiper et al., 1996, 1997; Kuiper and Gustafsson, 1997) have demonstrated the existence of a second form of estrogen receptor, ERβ, leading to the intense study of the differences in gene regulation due to estrogen receptors α and β and their possible cross-talk or function as heterodimers (Klinge, 2001). Generations of null mice for each receptor has allowed researchers to define the role of each receptor in expression of critical estrogen-dependent genes (Couse et al., 1997; Dixon et al., 1997; Korach, 2000; Ogawa et al., 1999, 2000). These studies have described differences in the function of ERα and ERβ, accounting for their cell, tissue, and developmental specificity.

In addition, functions of ER by processes other than as a nuclear transcription factor have provided mechanistic details about previously unexplained unique physiological responses caused by estrogen. For example, estrogen receptors have been shown to be localized at the cell membrane and in mitochondria, as well as the nucleus. The definition of the caveolae-associated estrogen receptor apparently functioning through $G_{\alpha}$-protein coupled mechanisms, results in an additional mechanism of cell signaling other than the traditional role of ER as a nuclear receptor (Levin, 2005; Evinger and Levin, 2005).

We and others (Maggiolini et al., 1999; Chen et al., 2005) sought to evaluate the role of DHEA and its metabolites as ligand activators of ERα and ERβ. Several reports have indicated that DHEA enhances proliferation of estrogen-sensitive cells (Maggiolini et al., 1999), and recently Chen et al. (2005) have described the ability of DHEA and DHEA-S to activate the estrogen receptors and estrogen-dependent cell proliferation of MCF7 cells. Our current studies have

utilized luciferase reporter genes to measure ERα and ERβ transcriptional activation (Fig. 4). In our hands, DHEA activates ERβ nearly to the same extent as 17β-estradiol and activates ERα to a lesser extent, but requires far higher concentrations of DHEA to achieve the same level of gene expression. DHEA-S is not as good an activator of the two estrogen receptors (Fig. 4). We have noted that two oxidized metabolites activate either receptor with apparent $EC_{50}$ values two orders of magnitude below that seen for DHEA, depending upon the cell type in which the experiments are performed (data not shown). This suggests that ER-activation by DHEA may occur due to metabolism to a more active metabolite. In addition, these metabolites shown to serve as ligands for ERα and ERβ display a much higher affinity than DHEA and DHEA-S. This phenomenon is an example of pre-receptor processing of sterol ligands formed from DHEA by various hydroxysteroid dehydrogenases (Rizner et al., 2003). Studies on the function of DHEA or DHEA-S in activation of ERs must carefully consider its rapid conversion to a proximal ligand capable of binding the receptor.

## Non-ligand Dependent Receptor Activation

Several of the orphan receptors that function as metabolic receptors; i.e., constitutive androstanol receptor and Nrf2, have been shown to be activated by changes in their phosphorylation status. For example, Sidhu and Omiecinski (1997) demonstrated that the induction of P4502B in cultured rat hepatocytes is dependent upon the phosphorylation status of CAR. More recently, Negishi and coworkers have characterized this as an alternate non-ligand-dependent mechanism for activation of CAR, clearly demonstrating that this receptor can be activated either by direct ligand activation or by changes in its phosphorylation status in the liver (Honkakoski and Negishi, 1998). In the study of the basic-helix-loop-helix transcription factor Nrf2 by Kong and coworkers, this transcription factor was shown to be activated by one of two different processes, one an electrophile-dependent activation of Keap1 and the other due to a phosphorylation event (Kong et al., 2001; Shen et al., 2004).

Related to our work on PPARα activation by DHEA, it is clear that DHEA or any of its metabolites do not serve as ligands for this receptor. Although ligand activation and changes in receptor protein levels have been shown to clearly alter PPARα function, phosphorylation of PPAR can also affect its activity (see review by Diradourian et al., 2005). Shalev and Meier (1996) were the first to demonstrate PPARα phosporylation in primary rat adipocytes. Using immuno-precipitation procedures with polyclonal antibodies to PPARα and phospho-PPARα, PPARα phosphorylation was clearly evident when cells were treated with phosphatase inhibitors, okadaic acid, and vanadate. These authors also showed that insulin treatment increased PPARα phosphorylation in a time-dependent manner, reaching a 4-fold higher level of receptor phosphorylation over untreated cells in 15 minutes. Subsequent studies have demonstrated that insulin stimulates phosphorylation at serines 12 and 21 in the N terminal A/B domain of the receptor, and that this phosphorylation is necessary for insulin-dependent activation of PPARα in transient transfection assays (Juge-Aubry et al., 1999). Passilly et al. (1999) also demonstrated PPARα phosphorylation in rat hepatic Fao cells. Phosphorylation was stimulated by PPARα activators, ciprofibrate, or the Wyeth compound, in contrast to the studies of Shalev and Meier (1996) and Passilly et al. (1999) also demonstrated that treatment with the phosphatase inhibitor okadaic acid resulted in a 25% decrease in induction of FACO, a PPARα- responsive gene. Barger et al. (2000) and Barger and Kelly (2000) have also reported a phosphorylation-dependent decrease in PPARα activity in cardiac myocytes in response to an α adrenergic receptor agonist. Using selective inhibitors of p38-dependent mitogen-activated protein kinases (p38MAPK) or extracellular signal regulated kinases (ERK), they demonstrated that inhibiting the ERK-MAPK pathway eliminated agonist mediated repression of PPARα activity. Using *in vitro* kinase assays, they went on to show that ERK-MAPK phosphorylation was predominantly in the A/B domain of PPARα at S6, 12, and/or 21.

The relationship between phosphorylation and PPARα activity is complex. Early studies by Shalev et al. (1996) showed that insulin stimulates both PPARα phosphorylation and activity. Similarly, Blanquart et al. (2004) showed that treating cells with okadaic acid, that would presumably cause receptor hyper-phosphorylation, stimulated ligand-dependent PPARα activity in Cos 7 cells by stabilizing the receptor leading to increased receptor protein. On the other hand, statin drugs decreased PPARα phosphorylation in HepG2 cells, but resulted in an increased transcriptional activity (Martin et al., 2001). Further, Barger et al. (2001) demonstrated that PPARα phosphorylation in primary rat neonatal cardiomyocytes stimulated its transcriptional activity, while in an earlier report the same authors using the same cells, showed in that PPARα phosphorylation by ERK-MAPK decreased PPARα activity. In both cases, phosphorylation was localized to serines 6, 12, and 21 in the N-terminal AF1 domain. To add to the complexity, protein kinase A (PKA) can also phosphorylate PPARα, although in this case the site of phosphorylation is in the DNA binding domain rather than the N terminal A/B domain. Calcium-dependent protein kinases (PKCα and βII) have also been shown to phosphorylate PPARα in human liver cells.

Which phosphatases are capable of removing phosphate residues from PPARα is also unclear. Studies with general phosphatase inhibitors like okadaic acid and vanadate have been widely used to demonstrate the importance of phosphorylation status on PPARα activity. However, these agents do not distinguish between various phosphorylation sites and are not sufficiently specific to identify the relevant phosphatases in the cell. We have also observed inhibition of transcriptional activity of a rat cytochrome P4502C11 reporter construct by okadaic acid in transient transfection experiments in HepG2 cells and hepatocytes (Fig. 5). In this experiment, we noted that both DHEA and napenopin, a PPARα ligand, suppressed expression of this reporter gene. However, nafenopin suppression required the presence of PPARα, while DHEA suppression was PPARα-independent. Upon addition of up to 40 nM okadaic acid, the suppression by either compound was reversed to control values.

Similar experiments were performed with a FACO reporter construct using nafenopin to activate the reporter (data not shown); okadaic acid potently inhibited the transactivation in a PPARα-dependent manner. While the relationship between PPARα phosphorylation and activity seems complex, it is clear that phosphorylation, in addition to ligand activation and changes in receptor protein, contributes to PPARα transcriptional activity. Presumably, the balance between these multiple pathways determines the ultimate transcriptional activity of the receptor. Interestingly, DHEA decreases PPARα phosphorylation status at S12 and 21 in rat hepatocytes, in addition to stimulating PPARα responsive genes. While there is not necessarily a cause and effect relationship between DHEA-mediated activation and PPARα phosphorylation status, the correlation between these two events is intriguing.

## Membrane-Associated DHEA Receptors

Most of the research to date identifying the mechanism(s) responsible for DHEA action has focused on cytosolic/nuclear hormone receptors, as this is the principal mechanism for steroid action, as described previously for the estrogen receptor (Levin, 2005; Evinger and Levin, 2005). While no steroid nuclear hormone receptor with high affinity for DHEA, or its sulfated conjugate, has been identified to date, DHEA binding and modulation of membrane-associated proteins has been demonstrated. Research focused on membrane-initiated DHEA action has recently been extensively reviewed (Widstrom and Dillon, 2004). The most convincing reports to date are those characterizing high affinity binding of DHEA to a plasma membrane receptor in bovine aortic endothelial cells (BAEC) and primary human umbilical vein endothelial cells (HUVEC). DHEA binding to isolated plasma membranes (BAEC) was of high affinity ($K_d$ = 48.7 pM; within the physiological range of DHEA concentration), saturable, and specific, since neither 17β-estrogen, testosterone, androstenedione, 17α-hydroxypregnenalone, nor DHEA-S

inhibited DHEA binding. DHEA binding was inhibited by non-hydrolyzable GTPγ S and pertussis toxin, indicating involvement of a G-coupled protein. Moreover, DHEA-stimulated binding of GTPγ S to $G\alpha_{i2}$ and $G\alpha_{i3}$, but not $G\alpha_{i1}$ or $G\alpha_{i0}$, and DHEA binding was inhibited by antibodies to $G\alpha_{i2}$ and $G\alpha_{i3}$. High-affinity binding of DHEA to the G-coupled protein was functionally linked to increased synthesis of NO (Liu and Dillon, 2002; Liu and Dillon, 2004), a key modulator of vascular function. DHEA stimulated NO synthesis in BAEC in a time- and concentration-dependent manner. The $EC_{50}$ of DHEA stimulation of NO synthesis was 87 pM with maximal stimulation occurring within 5 min.

While estrogen activates NO synthesis through a membrane-directed mechanism, similar to that observed for DHEA, the ER antagonist tamoxifen had no effect on DHEA-induced NO synthesis. This finding, together with the fact that estrogen did not compete for DHEA-specific binding to plasma membranes of BAEC, suggests that DHEA functions through a membrane-associated G-coupled protein receptor distinct from estrogen receptor action. Endothelial NO synthase (eNOS) can be activated by increased cytosolic calcium or direct phosphorylation. DHEA did not affect cytosolic calcium concentrations in BAEC, suggesting a mechanism independent of calcium modulation. A mechanism for DHEA activation of eNOS by direct phosphorylation is supported by Lui and Dillon's finding that DHEA-stimulated cGMP accumulation and NO synthesis were inhibited by the tyrosine kinase inhibitor, herbimycin A, and the MEK inhibitor, PD098059. The identification of a high affinity, G-protein coupled membrane-associated “receptor” specific for DHEA that signals an increase in NO synthesis in vascular endothelial cells is consistent with the anti-atherosclerotic properties attributed to DHEA

This is the first report which extensively describes a direct physiologically relevant cell signaling cascade for DHEA action. However, definitive evidence for a DHEA-specific membrane receptor must include isolation and sequencing of the gene for the purported receptor. Identification and characterization of this receptor will allow generation of DHEA analogs that specifically activate the endothelial membrane receptor and improve vascular function, but lack other potentially undesirable androgenic effects of DHEA. High-affinity DHEA binding was also observed in the heart and liver, but only 39.8 and 23.5%, respectively, of that observed in the plasma membranes of BAEC.

DHEA and DHEA-S have also been shown to modulate membrane-associated signaling cascades in neuronal cells (Maurice et al., 1999; Dubrovsky, 2005). DHEA is a neuroactive steroid; that is, it is synthesized *de novo* in neuronal tissues (neurosteroid) and activates/inhibits several neurotransmitter systems through non-genomic mechanisms. DHEA and DHEA-S act as excitatory neurosteroids in that they antagonize γ-aminobutyric acid type A ($GABA_A$) receptors, and stimulate *N*-methyl-D-aspartate (NMDA) receptors. DHEA-S and DHEA are endogenous activators of the neuromodulator $\sigma_1$-receptor. The $\sigma_1$-receptors are a class of membrane-associated proteins that potently modulate excitatory neurotransmitter systems, including the glutamate and cholinergic systems. DHEA modulation of NMDA receptors may be indirect through stimulation of the $\sigma_1$-receptors. DHEA and DHEA-S have common psychopharmaco-logical actions with $\sigma_1$-receptor agonists, such as anti-amnesic and anxiolytic properties, and may be involved in neuroendocrine control of cocaine and morphine addiction.

DHEA-S and DHEA both stimulate [$^{35}$S]GTPγ S binding to synaptic membranes of prefrontal cortex in a dose-dependent manner (10 nM to 10 μM) that was blocked by NE-100, a sigma-receptor antagonist. Furthermore, the DHEA-S-induced stimulation was blocked by pertussis toxin and reconstituted with recombinant $G_{i1}$, suggesting a G-coupled protein mechanism. Ueda et al. (2001) demonstrated DHEA-S stimulation of the nociceptive flexor test, which measures responses to pain, at both low (<1 fmol intraplantarly) and high (>1 pmol intraplantarly) concentrations when injected into the planta of the hind paw. This biphasic

response was proposed to occur through two different receptor mechanisms: DHEA stimulation at higher concentrations resulting from stimulation of $\sigma_1$-receptors on nociceptor endings and at lower concentrations resulting from histamine release from mast cells, respectively (Ueda et al., 2001). The high dose DHEA response was blocked by the $\sigma_1$-receptor antagonists NE-100 and BD1063 and is attributed to substance P release upon $G\alpha_{i1}$ and phospholipase C activation. The low dose DHEA-S response was not blocked by pertussis toxin, but completely inhibited by equimolar concentrations of progesterone, by either PLC inhibitors or thapsigargin, and by diphenhydramine, an H1 antagonist. In a follow-up report, this group demonstrated low dose DHEA-S induced mast cell degranulation and histamine release that was coupled through $G_{q/11}$ protein-coupled membrane receptor (Mizota et al., 2005). Interestingly, this receptor activation by DHEA was inhibited by preaddition of progesterone, suggesting that the process must be unrelated to the receptor described by Dillon and coworkers (Widstrom and Dillon, 2004). While intriguing, these studies raise many questions about the physiological function of these processes.

These recent advances in elucidating the presence of unique receptors that bind DHEA on the surface of specific cells raises the possibility that a multiplicity of such receptors may exist for DHEA and other neurosteroid products (Fig. 6). This exciting area of research will provide better understanding of DHEA's action as a neurosteroid and its role in cognition. The biochemical nature of the receptors involved and its cell biological action, whether as a caveolin-associated receptor or some other cell membrane receptor type, remains to be elucidated. However, the non-genomic activation of biochemical signaling systems by DHEA and other sterols that allow regulation at physiological concentrations of these sterols or their metabolites will be the focus of considerable study in the future.

## Other Target Genes Accounting for the Biological Effects of DHEA

Several years ago we applied expression assay analysis to liver mRNA samples from rats treated with DHEA in a synthetic diet (AIN76A diet, Purina, Richmond, IN) to AIN76A diet alone (Gu et al., 2003). We found a number of gene products up- or down-regulated by DHEA feeding (0.45% in diet); several of these were gene products we had previously observed regulated by DHEA feeding (e.g., CYP4A1, CYP4A3, fatty acyl-CoA oxidase). We have characterized several of these genes and attempted to describe the mode of regulation by DHEA. The following section will describe our studies on three of these genes; namely, CYP2C11, 11β-hydroxysteroid dehydrogenase, and insulin-like growth factor binding protein 1.

**P4502C11—**Cytochrome P4502C11 (CYP2C11) message expression was shown to be suppressed in rat liver by chemicals that are peroxisome proliferators in our gene array studies. CYP2C11 is expressed in male rat liver and codes for a protein involved in 16α-hydroxylation of steroids, epoxygenation of arachidonic acid, and drug metabolism. Waxman and coworkers (Janeczko et al., 1990; Sundseth et al., 1992) described the mechanism of sex-specific and growth hormone-dependent regulation of transcription of cytochrome P450 2C11 (male) and 2C12 (female) genes. Ström et al. (1994, 1995) described the responsive elements in the 5′-flanking region of this rat gene that results in gender-specific regulation of this gene. There are related genes in humans that code for proteins involved in steroid hydroxylation reactions, *CYP2C9* and *CYP2C19*. Whether peroxisome proliferator chemicals suppress expression of human CYP2C genes is not known. However, expression of *CYP2C11* is markedly suppressed by peroxisome proliferator chemicals (Corton et al., 1998) and by DHEA (Prough et al., 1995). Its regulation is complex and involves factors like growth hormone acting via a STAT5-dependent pathway as described by Delesque-Touchard et al. (2000).

In our first publication in this area (Wu et al., 1989), we noted that ADIONE and testosterone 16α-hydroxylation activities are suppressed by feeding rats with 0.45% DHEA by 70−80%. We subsequently showed that message levels for P4502C11 are decreased 70% in livers of animals fed DHEA (Ripp et al., 2003), suggesting that this most likely was due to the decrease in P4502C11 expression in liver. We studied this effect using a P4502C11 reporter construct containing 1.5 kb of the flanking region of the rat P4502C11 gene (Ripp et al., 2003). In HepG2 cells, both DHEA and nafenopin down-regulated this reporter, but nafenopin negative regulation required co-expression of PPARα, while DHEA regulation did not. The loci of regulation was found to be −348 and −60 bp near the transcription start site. Mutation of a hexanucleotide sequence at −70 bp ablated regulation by either DHEA alone or nafenopin in the presence of co-expressed PPARα. HepG2 cell nuclear extract from cells without co-transfected PPARα did not form PPARα-RXR heterodimer binding complexes with the DNA containing a PPRE from the 5′-flanking region of the CYP2C11 gene. When nuclear extract from cells transfected with a murine PPARα expression vector were used in electrophoretic mobility shift assays, we could observe PPAR-RXR complexes with the PPRE from the rat FACO gene. These results suggest that receptors other than PPARα must allow regulation by DHEA, but negative regulation by peroxisome proliferators requires PPARα. These studies have verified the results of our array studies (Gu et al., 2003). This suggests that there may be an additional transcription factor in HepG2 cells capable of binding DHEA and activating the CYP2C11 gene, but it does not appear to be PPARα.

**11β-HSD1—**11β-hydroxysteroid dehydrogenase enzymes maintain appropriate concentrations of active glucocorticoids in cells and tissues. The oxidation state at the 11 position of the sterol ring is the key determinant of glucocorticoid activity, with the hydroxyl being active (as in cortisol and corticosterone) and the keto inactive (as in cortisone and deoxycorticosterone). There are two enzymes that mediate this oxidation-reduction, 11βHSD type 1 and type 2. The enzymes are expressed in different tissues depending on specific requirements for active or inactive glucocorticoid. For example, 11β-HSD2, which functions as an NAD-dependent dehydrogenase to inactivate cortisol to cortisone (or corticosterone to deoxycorticosterone in rodents), is highly active in mineralocorticoid target tissues, especially the kidney, where low levels of active glucocorticoid are required to prevent fortuitous ligand activation of the mineralocorticoid receptor. In contrast, 11β-HSD1 is highly expressed in glucocorticoid targeted tissues such as liver, adrenal, ovary, and adipose (Ricketts et al., 1998). While originally described as a dehydrogenase, it is now known that this enzyme functions as an NADPH-dependent keto-reductase, regenerating active glucocorticoids from inactive 11-keto metabolites. As such, 11β-HSD1 is a local pre-receptor amplifier of glucocorticoid action and an important factor in the physiological response to glucocorticoids (Tomlinson et al., 2004; Bujalska et al., 2002; Jamieson et al., 1995). Interestingly, the 11-keto reductase activity of the enzyme is dependent on local availability of NADPH. 11β-HSD1 is found in the endoplasmic reticulum coupled to hexose-6-phosphate dehydrogenase, an enzyme capable of generating NADPH locally to drive reduction of the glucocorticoid's 11-keto group to the alcohol (Jamieson et al., 1995; Edwards et al., 1988).

Altering levels of 11β-HSD1 gene expression can affect the local concentration of active glucocorticoid and have important physiological and pathophysiological consequences. Transgenic mice over-expressing 11β-HSD1 in adipocytes show signs of central obesity, hyperlipidemia, hyperglycemia, and insulin resistance, similar to the symptoms of metabolic syndrome in humans (Masuzaki et al., 2001). Liver specific over-expression of 11β-HSD1 also results in anomalies, including hypertension, abnormal lipoprotein profiles, and an attenuated metabolic syndrome-like state without overt obesity (Patterson et al., 2004; Carr and Brunzell, 2004). In contrast, the 11β-HSD1 knockout mice are resistant to hyperglycemia induced by a high fat diet (Kotelevtsev et al., 1997). They show increased insulin sensitivity in liver, increased serum HDL levels (Morton et al., 2001), and are also protected against age-related

declines in hippocampal function (Yau et al., 2001). These effects may not be surprising given the putative role of DHEA as a neurosteroid and the physiological role of glucocorticoids in fat and carbohydrate metabolism.

Metabolic syndrome is a condition, which if left untreated, leads to type II diabetes (Deen, 2004). It is currently estimated that 20−24% of the US population and 40−44% of the population over 60 suffer from this disorder (Abuissa et al., 2005). While the clinical progression of metabolic syndrome is variable, it is clearly associated with increased risk of atherosclerosis and cardiovascular heart disease. The apparent link between levels of 11β-HSD1 expression and metabolic syndrome make an understanding of 11β-HSD1 gene expression vital. Like many "TATAless" promoters, HSD1 transcription is strongly influenced by GC rich and CCAAT boxes. Williams et al. (2000) showed in transfection experiments that the CCAAT enhancer binding protein (C/EBP) is absolutely required to achieve high level expression of 11β-HSD1 promoter-driven reporter constructs. We have also observed an absolute requirement for C/EBP in transient transfection studies (Fig. 7).

Other factors in addition to C/EBP modulate 11β-HSD1 transcription. Hermanowski-Vosatka et al. (2000) have shown that PPARα negatively regulates 11β-HSD1 transcription. Liver X receptor (LXR) has also been shown to affect transcription in both animal and cell culture models (Stulnig et al., 2002). We have also seen transcriptional down-regulation of 11β-HSD1 by PPARα (Fig. 7), although regulation by LXR was not apparent in our transient transfections (data not shown). Hermanowski-Vosatka et al. (2000) suggested a feedback loop for regulating of 11β-HSD1 gene expression. In their model, glucocorticoids induce PPARα expression, which subsequently down-regulates 11β-HSD1, thus decreasing production of active glucocorticoid. Our data support the observation that increased levels of PPARα down regulate 11β-HSD1 transcription. However, we also found that the 11β-HSD1 promoter is induced by glucocorticoids in a glucocorticoid-dependent manner (data not shown). In our model, glucocorticoids would initially increase 11β-HSD1 expression, but subsequently down-regulate expression through GR-dependent induction of PPARα. This feed-forward regulation would ensure a rapid and high level initial response to increased circulating glucocorticoids, which would later be attenuated by induction of PPARα and its suppression of 11β-HSD1 expression.

An overlap between the anti-glucocorticoid effects of DHEA and effects of the 11β-HSD1 knockout on insulin sensitivity, lowered serum triglycerides, and increased serum HDL, suggests a possible relationship between changes in 11β-HSD1 expression and DHEA-mediated alterations in lipid and carbohydrate metabolism. We previously showed that 11β-HSD1 expression is decreased in livers of animals treated with DHEA (Gu et al., 2003). Apostolova et al. (2005) have seen similar results in 3T3-L1 preadipocytes and mouse adipose tissue. These results suggest that 11β-HSD expression is regulated by several nuclear receptors providing other mechanisms for regulation of this gene critical in altering the diabetic state.

**Insulin growth factor binding protein 1 (IGFBP-1)—**IGFBP-1 is one of six proteins that bind with high affinity to the insulin-like growth factors (Firth and Baxter, 2002). IGFBP-1 is synthesized predominantly in the liver and secreted into the circulation where it binds to IGF-1. The majority of IGF-1 is complexed with IGFBP-3 and an acid-labile protein, and as this ternary complex does not cross the endothelial barrier, is thought to principally be responsible for IGF-1 transport in the circulation. The IGFBP-1/IGF-1 binary complex, however, is believed capable of permeating the endothelial barrier and, thereby, IGFBP-1 can alter local IGF-1 action at the cellular level. The primary function of IGFBP-1 is thought to be negative regulation of IGF-1 action through regulating the amount of bioavailable IGF-1. The IGF-1/IGFBP-1 complex is involved in glucose and lipid metabolism, cellular proliferation and differentiation, apoptosis, organism growth, and aging. Dysregulation of this axis has been

reported in cancer, metabolic X syndrome, atherosclerosis, diabetes, obesity, intra-uterine growth retardation, and aging. Thus, DHEA regulation of IGFBP-1 expression and subsequent modulation of IGF-1 bioavailability and signaling may account for some of DHEA's beneficial effects on diabetes, obesity, atherosclerosis, and cancer.

IGFBP-1 expression is tightly regulated by nutritional status. It is one of only a few genes that are negatively regulated by insulin, through a mechanism similar to insulin regulation of the gluconeogenic enzymes phosphoenolpyruvate carboxykinase and glucose-6-phosphatase (Suwanichkul et al., 1993; Lee et al., 1997). The negative regulation of IGFBP-1 by insulin suggests that IGFBP-1 has glucose counter-regulatory effects. Serum IGFBP-1 levels follow a diurnal pattern of expression correlating to fluctuations in insulin levels. Interestingly, DHEA and DHEA-S levels are decreased by insulin, resulting from increased clearance rates and decreased synthesis (Nestler and Kahwash, 1994; Yamaguchi et al., 1998; Ueshiba et al., 2002). Indeed, transgenic mice over-expressing rat IGFBP-1, under control of mouse phosphoglycerate kinase promoter, demonstrate hyperinsulinemia in the first week of life and go on to develop fasting hyperglycemia (Rajkumar et al., 1999). In another transgenic model, over-expression of human IGFBP-1, is under control of its native promoter to ensure physiological response to hormonal stimuli, its expression led to an attenuated hypoglycemic response to IGFBP-1, but not to insulin; these animals demonstrated fasting hyperglycemia and hyperinsulinemia and glucose intolerance in later life (Crossey et al., 2000). These two reports demonstrate that dysregulation of the insulin/IGF-1/IGFBI-1 axis has profound effects on glucose homeostatis.

The anti-obesity effects of DHEA may involve modulation of the IGFBP-1/IGF-1 axis. The insulin-like growth factor-1 (IGF-1) is important in adipocyte proliferation and differentiation, and IGFBP-1 counteracts IGF-1 action in adipocytes. IGFBP-1 inhibits IGF-1 stimulated clonal expansion of 3T3-L1 preadipocyte cells (Siddals et al., 2002). Over-expression of IGFBP-1 in transgenic mice results in impaired adipogenesis, and primary preadipocytes from the transgenic animals were resistant to IGF-1 stimulated clonal expansion and differentiation (Rajkumar et al., 1999). Thus, DHEA regulation of adipogensis/adiposity may be mediated in part through modulation of the IGF-1/IGFBP-1 axis.

DHEA is atheroprotective in rodent models. Early studies on DHEA action demonstrated a 50% reduction in atherosclerotic plaque formation, and a 30−40% reduction in aortic fatty streak formation in two separate hypercholesterolemic rabbit studies (Gordon et al., 1988; Arad et al., 1989). Epidemiological studies demonstrated an inverse relationship with DHEA-S levels and cardiovascular disease in men over 50 years of age (Barrett-Connor et al., 1986). The IGF-1/IGFBP-1 axis has also been implicated in the development of atherosclerotic plaques. Atherosclerotic plaque formation involves vascular smooth muscle cell (VSMC) proliferation, migration, and apoptosis. IGF-I stimulates VSMC proliferation and migration, requiring the IGF-IR and $\alpha_5\beta_1$ integrin (fibronectin receptor). IGFBP-1 has an RGD $\alpha_5\beta_1$ integrin binding site in its carboxyl-terminus and has been shown to also affect VSMC migration. It both inhibits IGF-I-dependent VSMC migration, and, in the absence of IGF-I, induces VSMC migration (Gockerman et al., 1995).

The relative contribution of locally synthesized versus systemically circulating IGF-I/IGFBP-1 on VSMC function is not known. However, IGF-1, IGF-IR, and several IGFBPs, including IGFBP-1, are synthesized locally in damaged aortic vascular smooth muscle cells, suggesting a paracrine/autocrine action (Bayes-Genis et al., 2000). Targeted over-expression of IGF-I in smooth muscle cells resulted in aortic VSMC hyperplasia, confirming paracrine action of IGF-I (Wang et al., 1997). Thus, altered levels of expression of IGFBP-1/2, IGF-I, and IGF-IR, both locally and systemically, may have profound effects on the development of atherosclerosis. In addition, DHEA modulation of IGF-1/IGFBP-1 axis resulting from either

local or systemic (hepatic expression) effects may be the basis of positive effects of DHEA on the development of atherosclerotic plaques.

Dysregulation of IGF-1 signaling is implicated in certain cancers of the colon, liver, prostate, and breast (LeRoith and Roberts, 2003). IGF-1R and IGF-1 expression levels are increased in tumors of the colon; and patients with acromegaly, a condition characterized by increased growth hormone and IGF-1 production, are at an increased risk of developing colon cancer (Jenkins, 2004). IGF-1 stimulation of the colorectal cancer C10 cell line resulted in stabilization of β-catenin, an oncogene overexpressed in colonic tumors (Playford et al., 2000). While IGF-1 stimulates proliferation and oncogenesis, several studies have demonstrated that IGFBP-1 has anti-cancer properties. For example, in a prospective nested case-control study of 32,826 women of the Nurses' Health Study, IGFBP-1 levels were found to be inversely proportional to the incidence of colorectal cancer (multivariable relative risk, 0.28; 95% CI, 0.11−0.75; Wei et al., 2005). Transgenic mice over-expressing IGFBP-1 developed considerably fewer hepatic pre-neoplastic lesions and lesions of smaller size upon administration of *N, N*-diethynitrosamine than wild-type animals (Lu and Archer, 2003). All of these lesions had increased expression of IGF-1 and IGF-2. IGFBP-1 induces cell detachment and apoptosis through integrin interaction via the RGD domain in human breast cancer cells (Perks et al., 1999). IGFBP-1 inhibits growth and induces apoptosis in cultured LNCaP cells, whereas IGF-1 stimulates cell growth (Ngo et al., 2003).

Gu et al. (2003) demonstrated a 4.9- and 3.1-fold increase in hepatic expression of the insulin-like growth factor binding protein–1 (IGFBP-1) in rats administered DHEA *per os* or the peroxisome proliferators nafenopin by *i.p*. injection, respectively, by gene array analysis. We subsequently confirmed induction of IGFBP-1 by DHEA and nafenopin in the liver of the rat (Fig. 8) using quantitative RT PCR. Similar results were shown in studies with mice treated with DHEA (data not shown). In mildly fasted mice, nafenopin induced IGFBP-1 expression by 2.8-fold, and dexamethasone, a known inducer of IGFBP-1, induced its expression by 3.4-fold (data not shown). The nutritional state of the animal significantly affected DHEA induction of hepatic IGFBP-1 expression, as DHEA-induction was abolished in animals sacrificed in the early morning, presumably in a fed state when insulin levels are high. Insulin is a potent negative regulator of IGFBP-1 expression in the fed state, while in the mildly fasted state, insulin levels are decreased, allowing the regulation by other signaling systems. The role of other receptors that bind DHEA and its metabolites in regulation of IGFBP-1 may provide other mechanisms of regulation of IGFBP-1 and is under study in our laboratory. However, the utility of DHEA and other peroxisome proliferators in regulating IGFBP-1 as a molecular target against cardiovascular disease or cancer appears obvious.

## SUMMARY AND CONCLUSIONS

There are many studies evaluating the role of DHEA in ameliorating age-related diseases. When we began our studies in 1989, no receptors had been identified for DHEA or its metabolites. In addition, due to is status as a nutraceutical, there had been no human studies to elucidate the safety of its use in high dosage and the studies on hepatocarcinogenesis by DHEA had not been performed by Reddy and coworkers (Rao et al., 1999) prior to that time. Ongoing research has shown now that pharmacological levels of DHEA and most of its metabolites lead to activation *in vivo* of a number of nuclear (PPARα, PXR, ER) and membrane-associated receptors. These observations identify some of the molecular targets of DHEA actions, such as 11β-HSD, IGFBP-1, and some cytochromes P450.

A more complex problem is whether there is an absolute requirement for DHEA or a metabolite in important physiological processes. Studies to understand the membrane-bound receptors appear to be a fruitful area of research to elucidate additional physiological functions for DHEA

or its metabolites, such as 7-oxo-DHEA. This seems particularly important, since there are correlations between the levels of circulating DHEA-S and longevity in monkeys and man.

Clearly, this compound is carcinogenic in rodents that display peroxisome proliferation when administered at pharmacological dosages; however, mechanisms exist to allow the liver and biliary system to eliminate these compounds. On the other hand, administration of supra-physiological levels of the sterols would appear unwise, because of the implication that it is carcinogenic and is easily converted into the sex steroids, testosterone and estrogen. While DHEA supplementation may have beneficial effects in humans, its possible role in increasing risk for cancer requires careful consideration. Additional studies are required to understand the far-reaching effects of administration of pharmacological levels of DHEA in humans.

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## ABBREVIATIONS

ADIOL, 5-androsten-3β,17β-diol; ADIONE, 4-androstene-3,17-dione; DHEA, 5-androstene-3β-ol-17-one; ERα, estrogen receptor alpha; ERβ, estrogen receptor beta; PPARα, peroxisome proliferators activated receptor alpha; PXR, pregnane X receptor.

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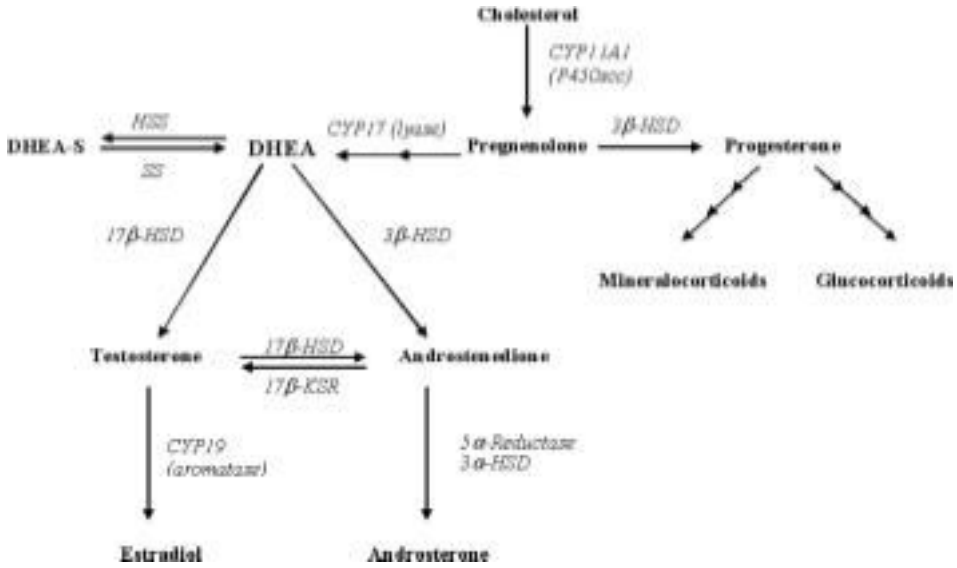


**Figure 1.**

Metabolic pathway for DHEA synthesis and utilization. The biosynthetic pathway from cholesterol to various sterol metabolites. The enzymes involved are: CYP11A1 (P450scc or cholesterol side-chain cleavage P450 enzyme); CYP17 (lyase or steroid 17 alpha-hydroxylase/ 17,20 lyase); CYP 19 (aromatase); 3α or β-HSD, 3α or β-hydroxysteroid dehydrogenase; 17β-HSD, 17β-hydroxysteroid dehydrogenase; 17β-KSR, 17β-ketosteroid reductase; HSS, hydroxysteroid sulfotransferase; SS, sterol sulfatase.

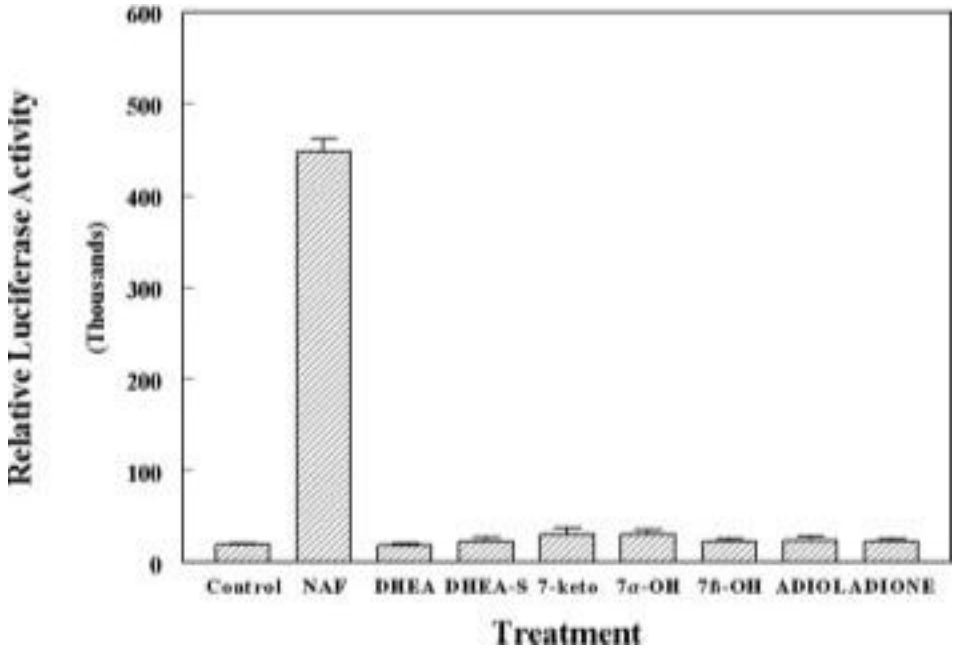


**Figure 2.**

DHEA and its metabolites do not activate PPARα in cell-based assays. HepG2 cells were transfected with a luciferase construct containing the peroxisome proliferators responsive element from murine fatty acyl CoA oxidase, an expression plasmid for murine PPARα, and an expression plasmid for β-galactosidase. The transfected cells were treated with 50 μM concentrations of nafenopin, a known peroxisome proliferating chemical, DHEA sulfate, DHEA, or a series of its oxidized metabolites (7-keto-DHEA, 7α-hydroxy-DHEA, 7β-hydroxy-DHEA, ADIOL, or ADIONE.) The cells were harvested 24 hours later and assayed for β-galactosidase and luciferase activity. The values are the average of three samples ± standard deviation.

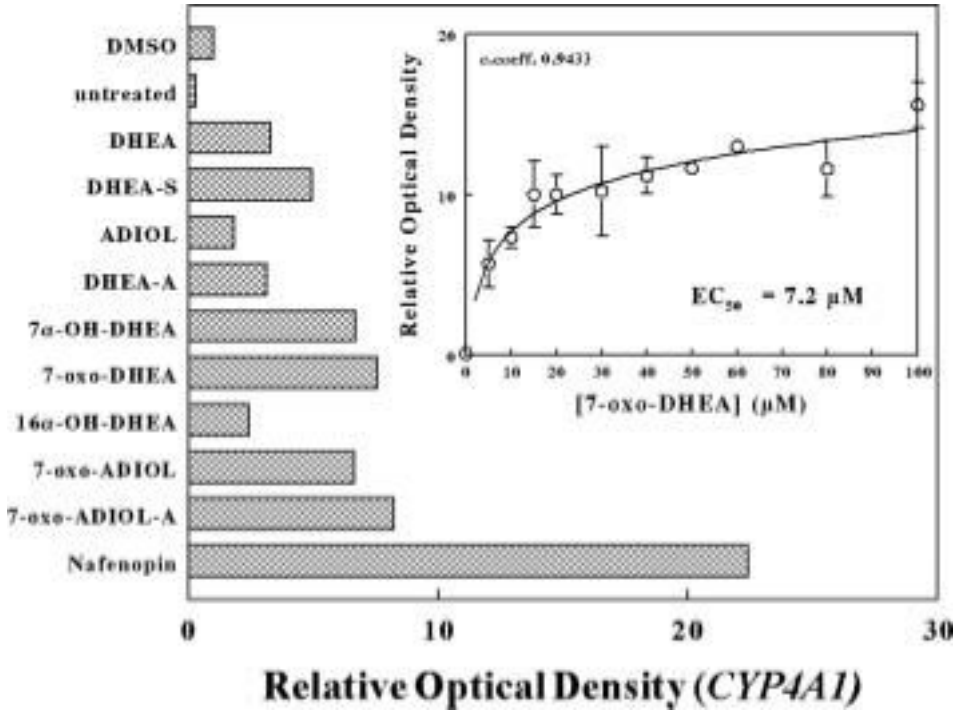


**Figure 3.**

DHEA and its metabolites induce P4504A1 message in primary cultures of rat hepatocytes. Rat hepatocytes were isolated as described previously (Prough et al., 1995) and treated with 50 μM concentrations of nafenopin, DHEA sulfate, DHEA, or a series of its oxidized metabolites (ADIOL, DHEA acetate, 7α-hydroxy-DHEA, 7-oxo-DHEA, 16α-hydroxy-DHEA, 7-oxo-ADIOL, or 7-oxo-ADIOL acetate). The mRNA was isolated and analyzed by Northern analysis; the density of each band was measured using a densitometer. Inset: The concentration-response obtained for increasing concentrations of 7-oxo-DHEA. The $EC_{50}$ value of 7.2 μM was observed for 7-oxo-DHEA. The values are the average of three samples ± standard deviation.

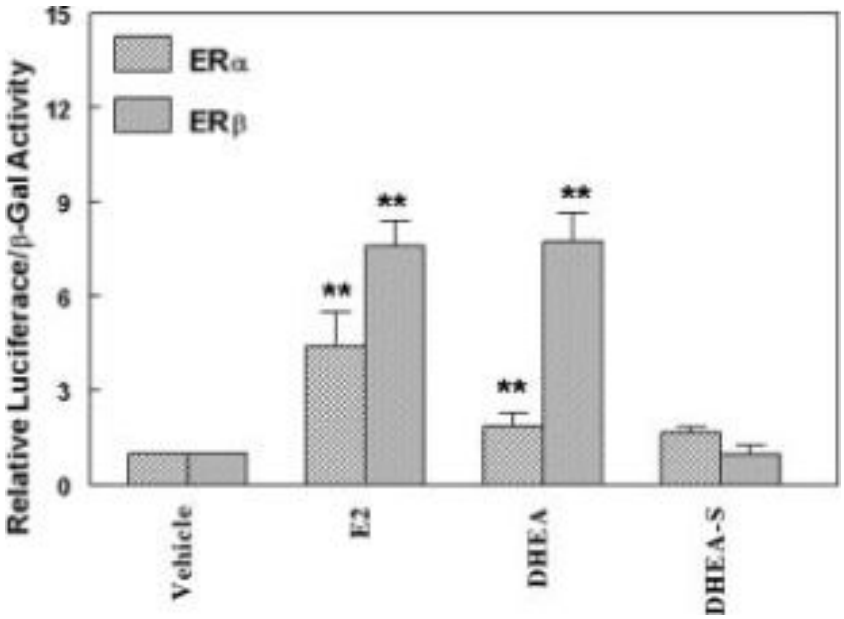


**Figure 4.**

DHEA activates estrogen receptor α and β in HepG2 cells. HepG2 cells were transfected with a luciferase construct containing a canonical estrogen responsive element, expression plasmids for either human ERα or ERβ, and an expression plasmid for β-galactosidase. The transfected cells were treated with 50 μM concentrations of DHEA or DHEA sulfate. For an estrogen standard, 17β-estradiol was added at a concentration of 50 nM. The cells were harvested 24 hours later and assayed for β-galactosidase and luciferase activity. The values are the average of three samples ± standard deviation.

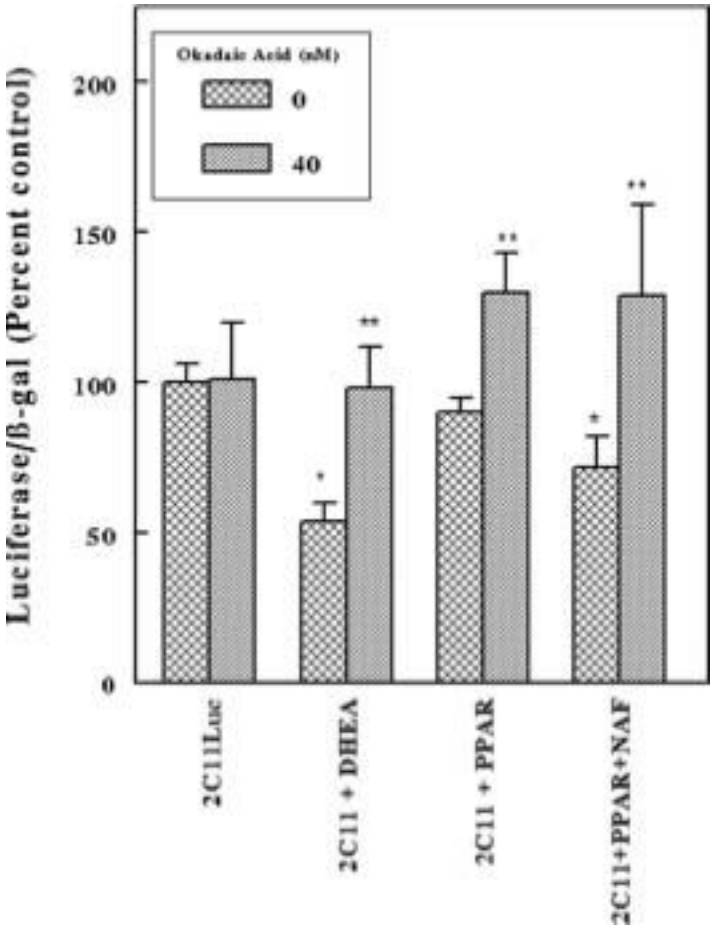


**Figure 5.**

Effect of okadaic acid on activation of a P4502C11 reporter construct by DHEA and Nafenopin. HepG2 cells were transfected with a luciferase construct containing the 5′-flanking region of rat P4502C11 gene, expression plasmids for murine PPARα, and an expression plasmid for β-galactosidase. The transfected cells were treated with either 50 μM DHEA or nafenopin. The cells were harvested 24 hours later and assayed for β-galactosidase and luciferase activity. The values are the average of three samples ± standard deviation.

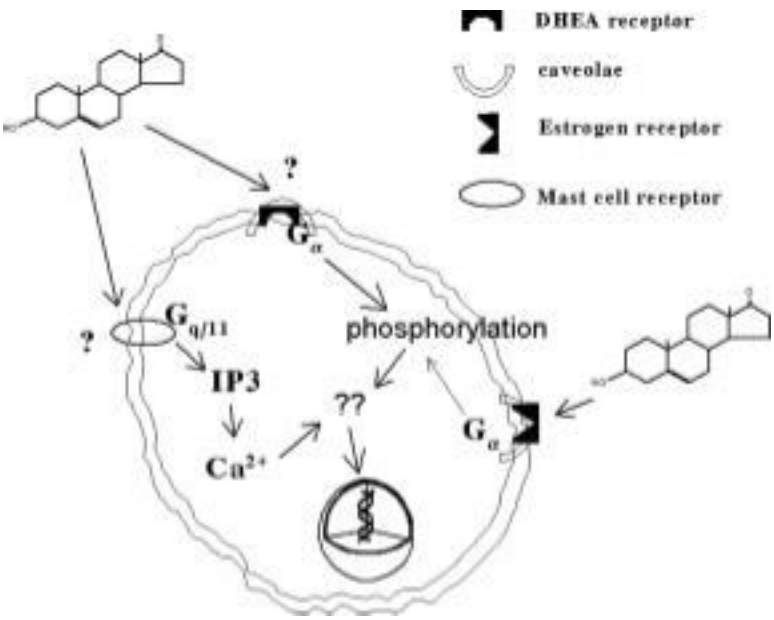


**Figure 6.**
Membrane-associated receptors for DHEA. The scheme shows several mechanisms of membrane-associated receptor activation by DHEA. One involves a caveole-associated receptor for DHEA acting through a Giα receptor system (Widstrom and Dillon, 2004), one involves membrane-associate estrogen receptor α or β, and one involves a $G_{q/11}$protein-coupled membrane receptor found in mast cells (Ueda et al., 2001).

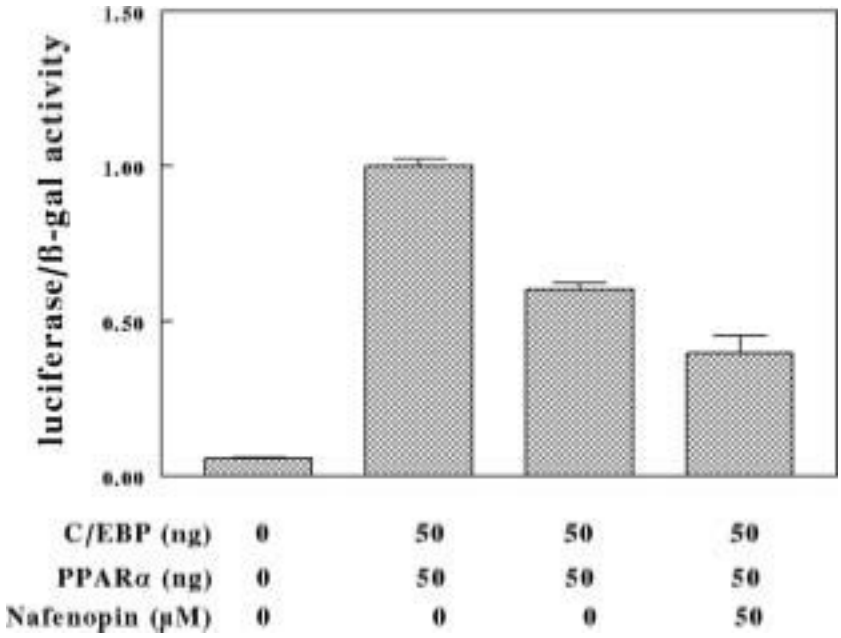


**Figure 7.**

Expression of 11β-hydroxysteroid dehydrogenase is regulated by peroxisome proliferators. HepG2 cells were transfected with a luciferase construct containing the 5′-flanking region of 11β-HSD, expression plasmids for murine PPARα, and an expression plasmid for β-galactosidase. In addition, an expression plasmid for C/EBP was transfected in certain experiments. The transfected cells were treated with 50 μM nafenopin. The cells were harvested 24 hours later and assayed for β-galactosidase and luciferase activity. The values are the average of three samples ± standard deviation.

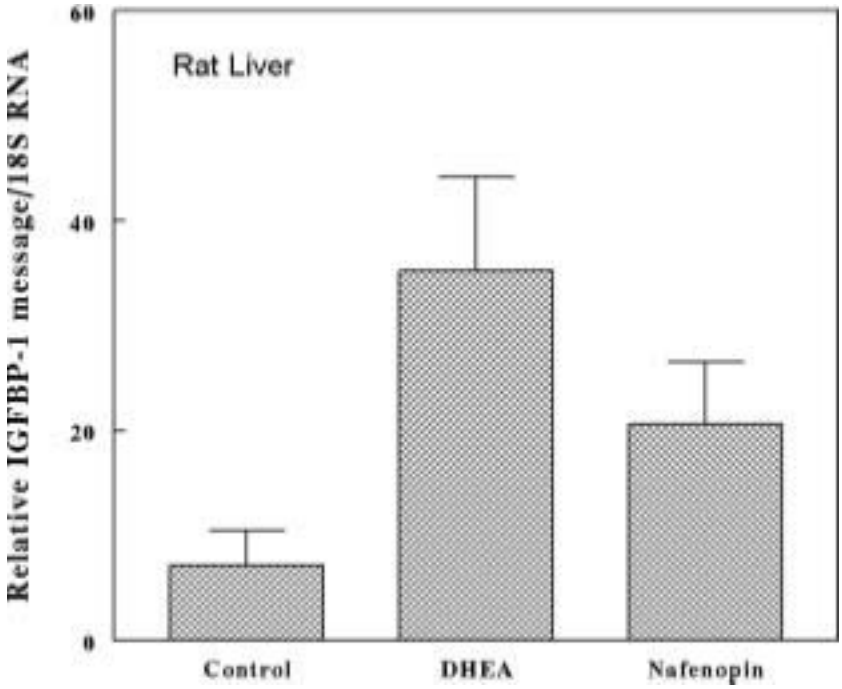


**Figure 8.**

DHEA and the peroxisome proliferators nafenopin induce expression of IGFBP-1 in rat liver. Male Sprague-Dawley rats were maintained on an *ad libitum* diet of AIN76A chow and administered either DHEA *per os* (0.45% DHEA in AIN76A chow) for 5 days, or nafenopin by daily *i.p.* injection for four days (80 mg/kg). First-strand reverse transcription was performed on 1 μg of total hepatic RNA and used for measuring changes in IGFBP-1 expression by quantitative RT-PCR using gene-specific primers. Values represent the average and standard deviation of the absolute mRNA quantities normalized to 18S rRNA from three independent animals per treatment group.