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DHEA metabolites activate estrogen receptors alpha and beta

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Abstract

Dehydroepiandrosterone (DHEA) levels were reported to associate with increased breast cancer risk in postmenopausal women, but some carcinogen-induced rat mammary tumor studies question this claim. The purpose of this study was to determine how DHEA and its metabolites affect estrogen receptors α or β (ERα or ERβ) -regulated gene transcription and cell proliferation. In transiently transfected HEK-293 cells, androstenediol, DHEA, and DHEA-S activated ERα. In ERβ transfected HepG2 cells, androstenedione, DHEA, androstenediol, and 7-oxo DHEA stimulated reporter activity. ER antagonists ICI 182,780 (fulvestrant) and 4-hydroxytamoxifen, general P450 inhibitor miconazole, and aromatase inhibitor exemestane inhibited activation by DHEA or metabolites in transfected cells. ERβ-selective antagonist R,R-THC (R,R-cis-diethyl tetrahydrochrysene) inhibited DHEA and DHEA metabolite transcriptional activity in ERβ-transfected cells. Expression of endogenous estrogen-regulated genes: pS2, progesterone receptor, cathepsin D1, and nuclear respiratory factor-1 was increased by DHEA and its metabolites in an ER-subtype, gene, and cell-specific manner. DHEA metabolites, but not DHEA, competed with 17β-estradiol for ERα and ERβ binding and stimulated MCF-7 cell proliferation, demonstrating that DHEA metabolites interact directly with ERα and ERβ *in vitro*, modulating estrogen target genes *in vivo*.

Keywords

estrogen receptors; DHEA; androstendione; androstendiol; transcription

1. Introduction

In postmenopausal women, androgens are converted to estrogens by aromatase (CYP19A1) [1] and aromatase inhibitors (AI) have recently replaced the selective estrogen receptor modulator (SERM) tamoxifen (TAM) as the most efficacious endocrine therapy for treating

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Appendix A. Supplementary data
Supplementary data associated with this article can be found in the online version.

postmenopausal patients whose initial breast tumors were estrogen receptor α (ERα) positive [2]. Dehydroepiandrosterone (DHEA) and its sulfate metabolite, DHEA-S, are androgen and estrogen precursors whose role, if any, as source for ER or androgen receptor (AR) ligands in breast cancer progression has yet to be clearly defined.

DHEA is present in normal breast as well as breast tumors DHEA has been suggested to have a protective effect in premenopausal women; however, a positive correlation was reported between DHEA plasma levels and breast cancer risk in postmenopausal women [3]. Analysis of DHEA, DHEA-S, and testosterone levels in 646 postmenopausal women in the Nurses' Health Study found no significant associations between the breast cancer risk score and androgen levels [4]. However, a weak association was found between higher free testosterone and breast cancer risk [4]. The association with DHEA and breast cancer was less clear [4] More recently, circulating DHEA-S correlated with alcohol consumption, and was strongly associated with several breast cancer risk factors [5]. Circulating DHEA-S levels >90 μg/dL (> 2.4 μM) were suggested as a potential risk factor for breast cancer progression in patients treated with TAM or fulvestrant [6]. Because enzymes involved in DHEA metabolism are expressed in breast tissue [7], local production of estrogenic DHEA metabolites are of keen interest.

Plasma DHEA-S levels are higher than other sterols secreted by the adrenals [8], peak in the second decade of life, and decline thereafter [9, 10]. Circulating DHEA levels are 20–25 nM, with higher levels in peripheral tissues, *e.g.*, prostate [11]. DHEA supplementation has been suggested to have many beneficial effects during aging as endogenous DHEA declines [10]. Although oral DHEA had anti-carcinogenic effects in the mammary gland of rats after chemical, *i.e.*, NMU [12] and DMBA [13] induction of tumors, the role for DHEA as a chemopreventive agent remains uncertain. Recently, 100 μM DHEA, a supraphysiological level [9], was reported to inhibit the proliferation, cell cycle progression, and migration of ERα+ MCF-7 and triple-negative MDA-MB-231 and Hs578T breast cancer cells [14].

However, DHEA (0.5 μM) and ADIOL (2 nM) stimulated MCF-7 cell growth [15]. DHEA activated transfected ERα reporter genes in GT1-7 immortalized mouse hypothalamic neuronal cells [16] and MCF-7 cells [17]. DHEA also stimulated proliferation of MCF-7SH cells, an estrogen-independent MCF-7 variant [17]. The efficacy of DHEA as an ERα ligand was enhanced by aromatase expression, indicating conversion DHEA to estrogenic metabolites in MCF-7 cells [18]. DHEA binds endogenous androgen receptor (AR) in MDA-MB-435 breast or LNCaP prostate cancer cells with a Ki of ~ 1.2 μM and binds ERα or ERβ expressed in COS-1 cells with Ki of ~ 1.1 μM and 500 nM, respectively [11]. However, the role of ER and AR in mediating DHEA responses is uncertain, because neither fulvestrant (ICI 182,780, an ER antagonist) nor flutamide (an AR antagonist) blocked the proliferative activity of 10 nM DHEA in MCF-7 cells [19]. The finding of a lack of inhibition by ICI 182,780 may be because it is a GPR30/GPER agonist [20], and a recent report that one DHEA metabolite 7α-hydroxy-epiandrosterone (7α-OH-EpiA, 1–100 nM) inhibited MCF-7 proliferation through inhibition of GPR30/GPER and ERα and by activation of ERβ [21].

In addition to 7α-OH-EpiA, another metabolite of DHEA, 5-α-androstane-3β,17β-adiol (3β-Adiol) serves as a ligand for ERβ [22–27] and for ERα [28]. ADIOL (100 nM) showed activity similar to 3β-Adiol (100 nM) in stimulating ERα- and ERβ- mediated ERE-luciferase activity in transfected HEK-293 cells [29]. Notably, ADIOL showed greater efficacy compared to DHEA, since 10 nM ADIOL had greater ERβ-stimulatory activity compared to 10 μM DHEA. ADIOL induced transcription of *HEM45*, an estrogen-responsive gene in MCF-7 cells, presumably through ER-activation, although this was not proven [29].

Evaluating the role of DHEA and its metabolites in activating ER is critical for understanding the biological events of estrogen-mediated gene regulation in normal and diseased tissues. In this study, DHEA and several metabolites were tested for their ability to serve as ligand activators of human ERα and ERβ using *in vitro* assays.

2. Experimental

2.1. Chemicals

Androstenediol (ADIOL), androstenedione (ADIONE), 5-α-androstane-3β,17β-adiol (3β-Adiol), etiocholanolone (ETIO), DHEA, DHEA-sulfate (DHEA-S), 7α-hydroxy-DHEA (7α-OH-DHEA), 7β-hydroxy-DHEA (7β-OH-DHEA), 7-oxo-DHEA, 11β-hydroxy-DHEA (11β-OH-DHEA), 16α-hydroxy-DHEA (16α-OH-DHEA), and 17β-estradiol (E_2) were purchased from Steraloids, Inc. (Wilton, NH). ICI 182,780 was purchased from Tocris, Inc. (Ellisville, MO). Miconazole and 4-hydroxytamoxifen (4-OHT) were purchased from Sigma-Aldrich (St. Louis, MO). Exemestane was a generous gift from Pharmacia Upjohn Corp., Kalamazoo, MI. *cis*-Diethyl tetrahydrochrysene (R,R-THC, a selective ERβ antagonist/ERα agonist) was a generous gift from Dr. John A Katzenellenbogen of the University of Illinois at Urbana/Champaign [30].

2.2. Plasmids

The pCMV expression plasmid containing the cDNA for human ERα (Reese and Katzenellenbogen, 1991) was a gift from Dr. Benita Katzenellenbogen (University of Illinois at Urbana/Champaign). The pSG5 expression plasmid containing the cDNA for the long form of human ERβ (hERβ1, 530 aa) was a gift from Dr. Eva Enmark, Karolinska Hospital, Stockholm, Sweden [22]. The ERELUC reporter contains three tandem copies of a consensus estrogen response element (ERE) [31]. pCMVβgal control reporter was purchased from CLONTECH (Palo Alto, CA).

2.3. Transient transfection assays

HEK-293, HepG2, CHO-K1, MCF-7, and MDA-MB-231 cells were purchased from ATCC (Manassas, VA). Cells were plated at 1.5×10^5 cells/well in 12-well plates containing 1 ml of MEM supplemented with 5% charcoal-stripped fetal bovine serum (DCC-FBS) Twenty-four hours after plating, cells were transfected in serum free medium with hERα or hERβ expression plasmids (150 ng/ml), ERELUC reporter plasmid (250 ng/ml), and either pCMVβgal (250 ng/ml) or pRL-tk (5 ng/ml, *Renilla* luciferase control vector, Promega, Madison, WI), for normalization, using 4 μg/ml LipofectAMINE (Invitrogen, Carlsbad, CA). After overnight incubation, the transfection mixture was removed and the cells were treated with phenol red-free MEM supplemented with 5% DCC-FBS. Transfected cells were treated with DHEA and metabolites dissolved in ethanol, and harvested 24 h later with 100 μl of cell lysis buffer (Promega). β-Galactosidase and luciferase activities were determined as described [32]. Since we could not find a single cell line that displays high E_2-dependent expression, we therefore used HEK-293-transfectioned assays with ERα-dependent expression and HepG2 cells-transfection assayed with ERβ-dependent cells expression vectors were used demonstrate the independent effects of DHEA and its metabolites on ERα and ERβ transcriptional activity.

For the HEK-293 transient transfection experiment with coactivators SRC-2 or SRC-3, cells were plated in 24 well plates and cotransfected with 250 ng 2EREc38-LUC, 5 ng pCMV-hERα, 5 ng pRL-tk (Promega), and 250 ng of expression plasmids for SRC-2/TIF2/ GRIP1/ NCOA2 and SRC-3/AIB1/ACTR/NCOA3, generously provided by Dr. Michael Stallcup [33], as described [34]. Firefly luciferase and Renilla luciferase activities were assayed using Promega's dual luciferase reporter assay [35]. The data are expressed as luciferase activity

relative to β-galactosidase or Renilla luciferase 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.

2.4. Transfection, RNA Isolation, and Quantitative Real-Time-PCR (QRT-PCR)

HEK-293 or HepG2 cells were plated in 6 well plates at a density of 4×10^5 cells/well. HepG2 were plated in phenol red-free DMEM supplemented with 5% DCC-FBS and 1% penicillin/streptomycin. HEK293 were plated in MEM supplemented with 10% DCC-FBS, 1% penicillin/streptomycin, 2% Sodium bicarbonate, and 1% Sodium pyruvate. HEK-293 and HepG2 were transfected with expression plasmids for human ERα or ERβ1, respectively, as described previously [36] using FuGENE HD (Promega, Madison, WI). Twenty-four h post-transfection, the cells were treated with DMSO (vehicle control) or 10 nM E_2, 5 μM DHEA, or 5 μM DHEA metabolites for 24 h. RNA was isolated from the cells using QIA Shredder and the RNeasy mini kit (QIAGEN). The High Capacity cDNA Reverse Transcription kit (PE Applied Biosystems, Foster City, CA) was used to reverse transcribe total RNA from random hexamer primers. Taqman primers and probes for *TFF1* (pS2), *CTSD* (cathepsin D), *PGR* (progesterone receptor (PR)), *ESR1* (ERα), *ESR2* (ERβ), and 18S rRNA were purchased as Assays-on-Demand™ from PE Applied Biosystems. Sybr green was used for QRT-PCR to measure the expression level of *NRF1* (NRF-1) and GAPDH for normalization [35]. Sequences of the forward and reverse primers for NRF-1 were (forward) 5'-5' GCGGTG GCA TCG TTG GCA GA-3'; ((reverse): 5'-GCT GCT GCG GTT TCC CCA GA-3'. The expression of each target gene was determined in triplicate and normalized using 18S or GAPDH. QRT-PCR was performed in the ABI PRISM 7900 SDS 2.1 (PE Applied Biosystems) using relative quantification. Analysis and fold differences were determined using the comparative CT method. Fold change was calculated from the $\Delta\Delta C_T$ values with the formula $2^{-\Delta\Delta CT}$ and data are presented as relative to expression in DMSO-treated cells, *i.e.*, vehicle control.

2.5. [^{3}H] E_2 ligand binding assay

Purified recombinant human ERα or ERβ were purchased from Panvera (Madison, WI) and 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 [^{3}H] E_2 (2,3,4,7,-[^{3}H] (N)17β-estradiol, 74 Ci/mmol, NET-317, NEN) for 1 hour at 37°C prior addition of a 10% hydroxyapatite (HAP) solution in TDPK111 for the HAP ligand binding assay [37]. Eight reactions were performed for each concentration of unlabeled competitors: E_2, DHEA, DHEA-S, ADIOL, ADIONE, and 7-oxo-DHEA. An additional HAP assay was performed using miconazole (8 concentrations from 1 nM-100 μM) as a competitor for [^{3}H] E_2-ERα binding. The percent of specific competitor binding to ER was calculated by first subtracting the nonspecific binding from total [^{3}H] E_2 binding [37].

2.6. Cell proliferation BrdU assay

MCF-7 human breast cancer cells were purchased from ATCC, (Manassas, VA.) and maintained in IMEM without phenol red from BioSource International (Camarillo, CA.) supplemented with 10% FBS (Atlanta Biologicals, Norcross, GA.). For the bromodeoxyuridine (BrdU) ELISA assay (Roche Diagnostics, Indianapolis, IN), cells were plated in 96 well plates in normal growth media and allowed to attach to the plates overnight. Media was replaced with phenol red-free IMEM supplemented with 3% DCC-FBS for 24 h. Cells were treated with EtOH (vehicle control) or the indicated compound for 48 h prior to performing the BrdU ELISA assay according to the manufacturer's instructions. Within each experiment, treatments were performed in quadruplicate and values were averaged. At least 3 separate experiments were performed. Data were analyzed

by one-way ANOVA followed by Tukey and Scheffe's post-hoc testing using GraphPad Prism software(San Diego, CA)..

2.7. Statistical analysis

Experiments were conducted in triplicate or quadruplicate and means ± standard deviations were determined. Statistical comparisons among treatment groups were determined using Student's t-test or ANOVA in GraphPad Prism, with $p < 0.05$ as the criterion for significance.

3 Results

3.1. DHEA and metabolites activate ERα and ERβ in transfected cells

To determine the effect of DHEA on ERα and ERβ individually, a number of ER-null cell lines were tested to achieve the best response to DHEA for each receptor. These cell lines included HEK-293, HepG2 (human hepatoma), CHO-K1 (Chinese hamster ovary), and MDA-MB-231 (triple negative breast cancer) that were transfected with a luciferase reporter plasmid containing three tandem copies of a consensus ERE [31] (ERELUC) and expression vectors for either ERα or ERβ and treated with E_2 prior to treatment with DHEA or its metabolites. ERα displayed a high basal (ligand-independent) activity in HepG2 cells, but had a limited, ligand-dependent response to E_2 (data not shown). The highest E_2-dependent activation of ERα was detected at 50 nM in HEK-293 cells (Fig. 1A). ERβ showed E_2-concentration-dependent-activity in HepG2 cells that was similar to or greater than E_2-ERα activity in HEK-293 cells (Fig. 1A). Significantly lower levels of E_2-ERβ activity was observed in HEK-293 cells. These results for ERα and ERβ are nearly identical to those reported by Martin *et al.* [38] and therefore, we used HEK-293-transfection assays with ERα expression and HepG2 cells-transfection assays with ERβ expression vectors for subsequent experiments to demonstrate the independent effects of DHEA and its metabolites on ERα and ERβ transcriptional activity.

DHEA (5 μM) and many of its known metabolites [39] were tested for their ability to induce ERELUC expression with ERα expressed in HEK-293 cells or ERβ expressed in HepG2 cells (Fig. 1B). As a control, an empty expression vector was co-transfected with ERELUC and after treatment with DHEA or its metabolites. No additional induction of ERELUC over vehicle in either cell line, indicating that both cell types provide a viable null cell-based assay to test ERα and ERβ activation, respectively (data not shown). In HEK-293 cells, DHEA, DHEA-S, and ADIOL significantly induced ERELUC activity around 9-fold via ERα. While 11β-OH-DHEA, and 7β-OH-DHEA suppressed ERELUC activity in ERβ-expressing HepG2 cells, 7α-OH-DHEA, and 16α-OH-DHEA, increased ERELUC activity. Interestingly, DHEA and its cytosolic DHEA metabolites ADIONE and ADIOL, and 7-oxo-DHEA significantly induced ERELUC activity via ERβ in HepG2 cells (Fig. 1B). Concentration-response studies were conducted to evaluate the potency of ERα- and ERβ-mediated induction of ERELUC by DHEA and metabolites (Fig. 2A and 2B). ADIOL was the DHEA metabolite with the greatest ERα agonist activity while DHEA and DHEA-S at higher concentrations induced ERELUC expression ~2-fold with ERα. With ERβ, ADIONE was the most potent activator, inducing expression of ERELUC by ~ 10-to 12-fold. Both androst-5-ene-3,17-dione and androst-4-ene-3,17-dione (data not shown) were effective in activating ERELUC by ERβ in HepG2 cells. The conversion of the 5-ene derivative to the 4-ene form of androstenedione is believed to be an enzyme-mediated process, involving glutathione S-transferases [40]. 7-oxo-DHEA, DHEA, and ADIOL at higher concentrations also induced expression of ERELUC by ~ 6- to 8-fold with ERβ.

To establish whether the transcriptional response of DHEA and its metabolites seen with ERα and ERβ was mediated by interaction of these sterols with the receptors, cells were co-treated with the ER antagonists 4-hydroxytamoxifen (4-OHT) and ICI 182,780 (ICI) and either E_2, DHEA, DHEAS, and ADIOL (Fig. 3A and 3B) ICI and 4-OHT inhibited the ERα-mediated induction of ERELUC by E_2, DHEA, DHEA-S, and ADIOL and also the ERβ-mediated induction of ERELUC E_2, DHEA, 7-oxo-DHEA, ADIOL, and ADIONE (Fig. 3A and 3B). The ERα agonist/ERβ antagonist R,R-THC [30] significantly inhibited the ERβ-mediated induction of ERELUC activity by E_2, DHEA, 7-oxo-DHEA, ADIOL, and ADIONE, but did not inhibit ERELUC activation mediated by ERα. These data indicate that these sterols and their metabolites formed within these cells interact with the ligand binding domain of ERα and ERβ. In addition, E_2 did not act synergistically with DHEA metabolites in ERα- or ERβ-mediated induction of ERELUC activity (Fig. 3A and 3B), perhaps because it displaced a lower affinity ligand formed by metabolism. Another possibility is that these data indicate a saturation of the reporter response as often seen due to limiting amounts of coregulators or other transcriptional components.

Aromatase inhibitors (AI), by preventing the conversion of androgen precursors into active estrogens, have significantly greater activity against breast cancer in postmenopausal women with estrogen-sensitive tumors compared to TAM [41]. Since HepG2 cells have been reported to have high aromatase activity [42], the effect of DHEA and its metabolites seen in the ERELUC assay may be due to their metabolism to estrogenic compounds. To examine whether the activation of ERELUC in ERα and ERβ transfected cells is mediated by conversion of DHEA metabolites to estrogens, ERα-transfected HEK-293 and ERβ-transfected HepG2 cells were pretreated with 5 μM miconazole, a general P450 inhibitor, or 100 nM exemestane, an AI. Cells treated with miconazole showed reduced ERα- and ERβ-activation of ERELUC activity in response to E_2 and ADIOL (Fig. 4A and 4B) and to DHEA, ADIONE, and 7-oxo-DHEA for ERβ (Fig. 4B). Miconazole did not inhibit DHEA-stimulated ERα transcriptional activity (Fig. 4B). Exemestane did not significantly inhibit E_2, DHEA, or ADIOL induced ERELUC activity in ERα-transfected cells (Fig. 4A). In ERβ-transfected cells, exemestane significantly inhibited DHEA-, ADIOL-, and 7-oxo-DHEA- induced ERELUC reporter activity ~ 20–30% (Fig 4B). In contrast, exemestane had no effect on ADIONE activation of ERβ (Fig 4B). The inhibition of the transcriptional activity of the tested ligands, except DHEA for ERα, by miconazole may be related to the ability miconazole to inhibit the interaction of coactivators with ERα and ERβ, as was reported for pregnane X receptor (PXR) [43]. To test this idea, HEK-293 cells were transfected with an ERE-LUC reporter plasmid and pCMV-hERα in combination with either an empty expression plasmid or expression plasmids for the SRC-2/TIF2/ GRIP1/ NCOA2 or SRC3/AIB1/NCOA3 coactivators and treated with 10 nM E_2 in the presence or absence of pretreatment with miconazole. Miconazole increased basal ERE-luciferase reporter activity ~ 0.8-fold, but did not affect *Renilla* luciferase activity (Supplemental Fig.1 and data not shown). Although the mechanism for this increase is unknown, there is only one report on a direct effect of miconazole on gene expression: miconazole increased the expression of rat hepatic microsomal epoxide hydrolase gene transcription *in vivo* starting 12 h after oral administration [44]. Miconazole inhibited E_2-induced ERα- ERE-LUC activity (Supplemental Fig. 1). Coactivators SRC-2 and SRC-3 relieved the repression by miconazole in (Supplemental Fig. 1). These data are consistent with the idea that miconazole may inhibit ERα-coactivator interaction as reported for PXR [43], but complete confirmation requires additional experiments.

Alternatively, miconazole may destabilize ER-ligand interaction since miconazole inhibits dexamethasone-glucocorticoid receptor (GR)-binding [45]. We examined whether miconazole inhibits E_2-ER binding in a ligand binding competition assay and detected only a 15% inhibition of [^{3}H] E_2 binding to recombinant human ERα at 100 μM miconazole

which is 20 times higher than the 5 μM concentration used in cell treatments (Supplemental Fig. 2). Taken together, these results suggest that DHEA and ADIOL are not converted into E_2 or estrone to activate ERα in HEK-293 cells, but DHEA, ADIOL, and 7-oxo-DHEA are at least partially converted to estrogens in HepG2 cells to activate ERβ. In contrast, ADIONE activates ERβ without conversion to an estrogen in HepG2 cells.

3.2. DHEA and metabolites enhance endogenous ER-target gene transcription

As an initial test to examine how DHEA and its metabolites affect endogenous ER-target gene transcription, HEK-293 cells were transfected with ERα and HepG2 cells were transfected with ERβ. Increased expression of *ESR1* and *ESR2* were detected in HEK-293 and HepG2, respectively (Supplementary Fig. 3). Twenty-four h after transfection, the cells were treated for 24 h with 10 nM E_2 or 5 μM DHEA, DHEA-S, or other DHEA metabolites that had activated ERE-luciferase activity (Fig. 1B). Fig. 5A shows that the positive control E_2 increased *TFF1* (pS2), *PGR* (progesterone receptor (PR)), and *CTSD* (cathepsin D) transcription. The relative induction of pS2 mRNA was higher than that for PR or cathepsin D with 24 h treatment. Similar to the induction of ERE-reporter activity (Fig. 1B), DHEA, DHEA-S, 7-oxo-DHEA, and ADIOL increased *TFF1* expression. Likewise, DHEA and DHEA-S increased *PGR* and *CTSD* expression. In agreement with the lack of ERα activity with 7α-OH-DHEA in the ERE-luciferase assay (Fig. 1B), no induction of *TFF1, PGR*, or *CTSD* was detected (Fig. 5A). While 16α-OH-DHEA and 7-oxo-DHEA showed no activation of *PGR* and *CTSD*, *TFF1* expression was increased, in contrast to the lack of ERα-induced ERE-luciferase activity with these DHEA metabolites. DIOL increased *TFF1*, but not *PGR* or *CTSD*. We conclude that endogenous ERα target genes show similar, although not identical, responses to DHEA ligands in a gene-specific manner in ERα-transfected HEK-293 cells.

For ERβ-transfected HepG2 cells, we did not detect induction of *TFF1, PGR*, or *CTSD* (data not shown). ERβ regulates *NRF1* (nuclear respiratory factor-1, NRF-1) expression in response to E_2 and 4-OHT in breast and lung cancer cells [35, 36]. In agreement with the ERELUC activation data for ERβ (Fig. 1B), a statistically significant increase in NRF-1 mRNA expression was seen with E_2, DHEA, α-OH-DHEA, 16α-OH-DHEA, and ADIOL (Fig. 5B). In contrast to the ERELUC for ERβ (Fig. 1B), ADIONE did not increase endogenous NRF-1 expression. On the other hand, 3β-Adiol, identified as an ERβ-selective agonist in prostate [23, 46], increased NRF-1 expression in HepG2 cells. We conclude that some DHEA ligands show ERβ agonist activity on NRF-1 expression, but ADIONE does not.

3.3 DHEA metabolites compete with E_2 for binding ERα and ERβ

To determine if DHEA and its metabolites serve as ligands for ERα and ERβ, *in vitro* ligand binding competition assays [37]. Fig. 6A shows that in our hands, the IC_{50} value of E_2 for ERα is ~1–10 nM, similar to the value reported in the literature [22, 47]. ADIOL bound to ERα with an IC_{50} of ~0.1 μM. DHEA and DHEA-S bound ERα with IC_{50}s of >500 μM and 100–500 μM, respectively. Fig. 6B shows that the IC_{50} of E_2 for ERβ is ~1–5 nM, in agreement with previous studies [22, 47]. Like ERα, ADIOL exhibited significant binding to ERβ with an IC_{50} of ~50 nM followed by ADIONE with an IC_{50} of 50 μM. DHEA and 7-oxo-DHEA exhibited IC_{50} values for ERβ of 500 μM and did not exhibit significant ERα binding. Previous work by Gustafsson's group demonstrated that 4-androstene-3,17-dione did not compete with E_2 for ERα or ERβ [22]. To our knowledge, no one has evaluated the ER binding of 5-androstene-3,17-dione that would be the anticipated product of oxidation of DHEA and, this intermediate may have appreciable rates of formation in mammalian tissues. The DHEA concentrations at which E_2 is displaced are not physiologically relevant

[9], and thus we conclude that DHEA and DHEA-S do not compete with E_2 for binding ERα or ERβ.

3.4 DHEA and its metabolites stimulate MCF-7 cell proliferation

To examine the biological activity of the DHEA metabolites in a cell line expressing endogenous ERα and low levels of ERβ [36], a 48 h BrdU assay was performed in MCF-7 breast cancer cells (Fig. 7). As a positive control, the E_2-induced stimulation of MCF-7 cell proliferation was inhibited by the selective estrogen receptor disrupter (SERD) ICI 182,780 (fulvestrant), demonstrating that ER was responsible for E_2-induced cell proliferation (Fig. 7A). Similarly, DHEA, DHEA-S, 7-oxo-DHEA, ADIOL and ADIONE, all at 5 μM, increased MCF-7 proliferation (Fig. 7A). or The aromatase inhibitor (AI) exemestane inhibited the proliferative activity of DHEA by ~ 50% while showing no inhibitory activity with ADIOL or ADIONE. The general P450 inhibitor miconazole inhibited MCF-7 cell proliferation induced by E_2, DHEA, ADIOL, and ADIONE, again indicating that miconazole appears to have off target effects. To address the potential involvement of endogenous AR in MCF-7 cells in the proliferative effects of DHEA and its metabolites, cells were pretreated with bicalutamide, an AR antagonist, and also treated with dihydrotestosterone (DHT). Bicalutamide inhibited DHEA-, 7-oxo-DHEA-, ADIOL-, and ADIONE- induced proliferation in MCF-7 cells. DHT induced a concentration-dependent increase in MCF-7 cell proliferation that was blocked by bicalutamide. These later results are in agreement with a report showing that testosterone stimulated MCF-7 cell proliferation [17] and AR protein expression increases ~ 2–3 fold with 48 h treatment of MCF-7 cells with 10 nM DHT [48]. Together these data suggest that DHEA is metabolized to compounds that stimulate MCF-7 cell proliferation through both ER- and AR- mediated pathways.

4. Discussion

DHEA has been suggested to share some of the same beneficial properties as estrogens in diseases of aging including atherosclerosis, osteoporosis, depression, and Alzheimer's disease, but lack their carcinogenic effects in breast [8, 10, 49]. Here we demonstrate that DHEA metabolite ADIOL activated ERα transcriptional activity in HEK-293 cells; whereas, ADIONE, ADIOL, DHEA, 7-oxo-DHEA, 7α-OH-DHEA, and 16α-OH-DHEA activated ERβ-mediated transcription in HepG2 cells. One caveat of our studies is the use of aromatase null HEK-293 [50–53] and aromatase positive HepG2 [42, 54]. Thus, it is possible that some of the DHEA metabolites were converted to estrogens in HepG2, but not in HEK-293 cells. Figure 8 presents a scheme of the DHEA metabolites examined in the present study and their role in activation of ERα and ERβ. In support of these findings, a recent paper reported that the androgenic DHEA metabolite 7β-Hydroxy-epiandrosterone (7β-OH-EpiA) activated ERβ in transfected MDA-MB-231 cells and GPER/GPR30 in SKBR3 cells [21]. Alone at 1–100 nM concentrations 7β-OH-EpiA did not affect cell proliferation, but inhibited E_2-induced proliferation of MCF-7 and ERβ-overexpressing MDA-MB-231 cells with no dose-response detected [21]. The observation that different sterols (ADIOL or ADIONE) stimulated activation of ERα and ERβ to levels similar to those by E_2, respectively, is reminiscent of the differences seen between the rodent versus human PXR, for which we reported that ADIOL was the preferred ligand for the murine and ADIONE for human PXR [55]. Human and murine PXR are also regulated differentially by other compounds, such as rifampicin (hPXR) and pregnenolone-16α-carbonitrile [56, 57]. The finding that DHEA and its metabolites showed greater ERβ agonist activity agrees with their higher affinity ERβ interaction, as shown here and reported previously [22], but is the first systematic study of DHEA and its metabolites as activators of ERα and ERβ transcriptional responses.

ERs plays an important role in the physiology of many tissues [58, 59] by direct binding to EREs, by interaction with other transcription factors; *e.g.*, AP-1 and Sp1, binding to their responsive elements [60], and by activating membrane ER or GPR30/GPER [61, 62]. Here we focused on DHEA and metabolite action on genomic/transcriptional ER activity. The ER antagonists ICI 182,780 and 4-OHT blocked the agonist activity of DHEA and its activating metabolites with both ERs. Likewise, the P450 inhibitor, miconazole, blocked the transcriptional activity of E_2, DHEA, except for ERα, and all DHEA metabolites, thus suggesting an 'off target' effect, *e.g.*, inhibition of coactivator-ER interaction as indicated by the ability of transfected SRC-2/GRIP1/NCOA3 and SRC-3/AIB1/NCOA to alleviate the repression of E_2-induced luciferase reporter activity by miconazole in ERα-transfected HEK-293 cells (Supplemental Fig. 1) as reported previously for PXR [43]. The reason for lack of inhibition of DHEA-induced ERα transcriptional activity by miconazole in HEK-293 cells is unclear. It is possible that DHEA stimulates ERα transcription indirectly through DHEA activation of its GPR plasma membrane receptor [63], thus possibly activating ERα phosphorylation. Alternatively, DHEA may interact with a second site in ERα other than the LBD, as has been reported for 4-hydroxytamoxifen [64]. Both of these ideas would require further testing beyond the scope of the present study. In contrast to a report that miconazole inhibited DEX-GR binding [45], miconazole did not compete with E_2 for ERα binding, indicating that miconazole is not inhibiting transcriptional activity by reducing ligand-ER interaction. The aromatase inhibitor, exemestane inhibited DHEA-, ADIOL-, and 7-oxo-DHEA-activativation of ERβ-induced reporter expression in HepG2 cells, but did not affect ERα transcriptional activity. This suggests conversion of DHEA, ADIOL, and 7-oxo-DHEA to active ERβ ligands in HepG2 cells, but not HEK-293 cells, a result congruent with steroid metabolic activity in HepG2 cells [39, 65]. Since HEK-293 cells are routinely used for transfection studies, knowledge of their endogenous sterol metabolism appears not to have been extensively studied. Indeed, exemestane inhibited DHEA-induced MCF-7 cell proliferation, indicating that metabolism is involved. Others reported that DHEA is metabolized to E_2 in MCF-7 cells [66] which agrees with our MCF-7 cell proliferation and ERβ-transcriptional responses in HepG2 cells. In contrast, ADIONE activated ERβ transcriptional activity was not inhibited by exemestane. We noted that ADIONE competed with E_2 for binding ERβ with an IC_{50} of ~ 50 μM (Fig. 6). This observation accounts for the direct transactivation of ERβ by ADIONE. We also note that DHEA and its metabolites stimulate MCF-7 cell proliferation by activating AR, results in agreement with previous reports [17, 67].

Our data are concordant with and extend previous reports on the estrogenic activity of DHEA metabolites. DHEA was reported to bind ERα and ERβ expressed in COS-1 cells with Kd ~ 1.2 and 0.5 μM, respectively [11]. We did not detect DHEA binding as a ligand to baculovirus-expressed rh ERα and ERβ at physiologically relevant DHEA concentrations in our studies. DHEA, its metabolites ADIOL, 3β-Adiol, testosterone, and DHT all stimulated proliferation and ERE-driven luciferase activity in MCF-7 cells, albeit at significantly higher (1–10 μM) concentrations than E_2 [17]. DHEA is metabolized by CYP7B to 7α-hydroxy-DHEA in human prostate tissues and CYP3A4 in human liver [38, 39]. 7α-Hydroxy-DHEA (50 μM) stimulated activity of an ERE-TK-luciferase reporter in COS cells transactivated with ERα or ERβ [38]. Similarly, 0.1 μM 3β-Adiol and 5-androstene-3β,17β-diol (ADIOL) activated ERE-luciferase activity in HEK-293 cells transfected with ERα or ERβ [29].

Reports on the activities of DHEA and its metabolites in cancer cells have been contradictory. Early studies with diethylnitrosamine-induced hepatic tumors in rats demonstrated that DHEA (0.6% in the diet) inhibited tumor cell growth, perhaps by the inhibition of glucose-6-phosphate dehydrogenase, a key enzyme in the biosynthesis of both NADPH and ribose phosphate derivatives [68]. Likewise, estrone-stimulated growth of ZR-75-1 breast cancer xenografts in nude mice was suppressed by twice daily s.c. injections

of 0.3 mg DHEA [69]. Conversely, studies with MCF-7 breast cancer cells demonstrated that ADIOL, DHEA, and DHEA-S increased cell proliferation, albeit less than E_2 [17] [70]. Over-expression of aromatase stimulated DHEA-dependent MCF-7 cell proliferation, implicating conversion to estrogens [18]. Recently, interest in DHEA metabolites has been revived with the observation that DHT and its metabolite 3β-Adiol stimulated MCF-7 and T47D breast cancer cell proliferation through ERα [28, 71]. These and related studies suggested that DHEA and its metabolites may serve as activators of ERα upon metabolism by aromatase to form estrogens.

We detected gene-specific and DHEA metabolite-specific differences in induction of endogenous gene transcription in HEK-293 transfected with ERα and HepG2 transfected with ERβ. There are many reasons that could account for the different responses of ERα detected with 16α-OH-DHEA, 7-oxo-DHEA, or ADIONE treatment in HEK-293 cells between the ERE-luciferase assay and induction of endogenous *TFF1* expression. First, the ERE-luciferase reporter is an artificial construct with three tandem perfect, consensus EREs, which binds ERs with high affinity [31, 72, 73], compared to the endogenous *TFF1* which is regulated by imperfect ERE and an AP-1 element [74]. Second, endogenous genes have chromatin structure and epigenetic modifications that have profound effects on transcription. In agreement with previous reports of low ERβ activity in transfected HepG2 cells [75], we detected low induction of endogenous NRF-1 expression in response to E_2, DHEA, 7α-OH-DHEA, 16α-OH-DHEA, 7-oxo-DHEA, and 3β-Adiol. ADIONE gave the highest activation of ERELUC by ERβ in transiently transfected HepG2, the reason for the lack of ADIONE-induction of NRF-1 expression in HepG2-ERβ cells is unknown. Certainly, the relative deficiency of specific coregulators or differences in the epigenome may explain the lack of effect of ADIONE on NRF-1 expression. To our knowledge, only one report on NRF-1 regulation in HepG2 has been published and that showed that an 8 h combined treatment of proinflammatory LPS and TNFα increased NRF-1 by activating NFκB [76]. It is clear from genome-wide studies identifying ERβ-regulated genes in U2OS [77], HEK293 [78], T47D [79], and MCF-7 [80] cells overexpressing ERβ, that there are cell-type differences in gene regulation. Thus, the selection of another ERβ target gene in HepG2 cells would necessitate a separate study and would be of limited physiological relevance since ERβ is not expressed in human liver [81].

There are significant differences in the transactivation of ERs based on relative AF-1 activation differences between cell lines [82–85]. Since AF-1 is stimulated by phosphorylation of ser 118 in ERα by MAPK [86, 87] and DHEA activates a membrane-initiated G-protein-coupled receptor (GPCR)- MAPK pathway in human endothelial cells [88, 89], we examined if the MEK1/2 inhibitor, PD98059 would block transcriptional activation of ERα and ER by DHEA and its metabolites. However, PD998059 did not block DHEA or metabolite-induced ERELUC activity, suggesting that ERα- and ERβ-dependent activation of transcription by DHEA and its metabolites is independent of MAPK-mediated phosphorylation (data not shown).

In conclusion, these studies demonstrate that DHEA and its metabolites activate ERα and ERβ in an ER-subtype and cell-selective manner. The most potent ligands for ERα and ERβ are ADIOL and ADIONE, respectively, in an ERELUC assay, but potencies differ for endogenous estrogen target genes. DHEA, ADIOL, and 7-oxo-DHEA are converted by aromatase to estrogens to activate ERβ in HepG2 cells, but ADIONE appears to directly activate ERβ, albeit with low affinity (IC_{50} ~50 μM). Since circulating levels of DHEA and DHEA-S are high and variable in humans and DHEA can be metabolized in peripheral and tumor tissues, our results provide new insight into the mechanism of action of DHEA and its metabolites as possible contributors in the progression of hormone-sensitive cancer in

patients expressing high circulating and tissue levels of these sterols and their metabolizing enzymes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

ADIOL	androstenediol (androst-5-ene-3β,17β-diol)
3β-Adiol	(5α-androstane-3β,17β-adiol)
ADIONE	androstenedione (androst-5-ene-3,17-dione)
CYP	cytochrome P450
DHEA	dehydroepiandrosterone (3β-hydroxy-androst-5-ene-17-one)
DHEA-S	DHEA 3β-sulfate
DMSO	dimethyl sulfoxide
E_2	17β-estradiol
ER	estrogen receptor
ERα	estrogen receptor α
ERβ	estrogen receptor β
ERE	estrogen response element
ETIO	etiocholanolone (3α-hydroxy-5β-androstan-17-one)
HAP	hydroxyapatite
NRF-1	nuclear respiratory factor-1
SD	standard deviation
4-OHT	4-hydroxytamoxifen
7α-OH-DHEA	7α-hydroxy-DHEA
7β-OH-DHEA	7β-hydroxy-DHEA
11β-OH-DHEA	11β-hydroxy-DHEA
16α-OH-DHEA	16α-hydroxy-DHEA
7-oxo-DHEA	3β-hydroxyandrost-5-ene-7,17-dione
ICI 182,780	(ICI, Fulvestrant)
R,R-THC	*cis*-diethyl tetrahydrochrysene

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Highlights

- DHEA and its metabolites increased ERα and ERβ transcriptional activity
- Androstenediol binds ERα with an IC_{50} ~0.1 μM
- Androstenedione binds ERβ with an IC_{50} ~5 nM
- DHEA metabolites stimulate MCF-7 cell proliferation
- Miconazole inhibits ERα-coactivator interaction

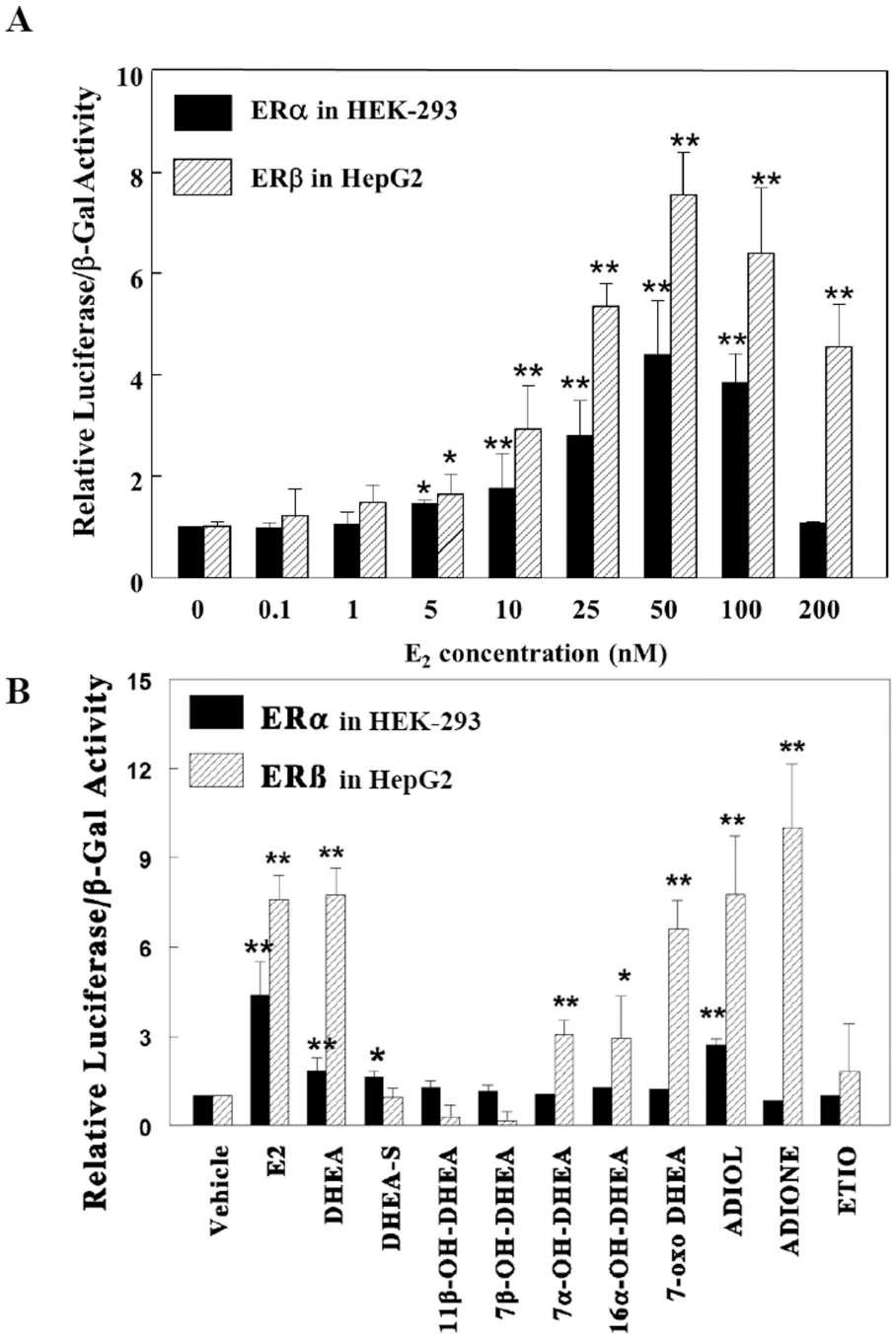

Figure 1. E_2 activates ERE-luciferase activity in transiently transfected cells

HEK-293 cells were transfected with ERα and HepG2 were transfected with ERβ expression plasmids Both cell lines were cotransfected with ERELUC (estrogen-response element luciferase) and pCMV-β-gal reporters and were treated for 24 h with EtOH (vehicle) or A) the indicated concentrations of E_2 or B) 10 nM E_2, 5 μM DHEA or 5 μM of the indicated DHEA metabolites. The cells were harvested and the lysates were assayed for β-galactosidase and luciferase activities. Data represent the mean ± S.D. of three separate experiments. Statistical significance is indicated *versus* vehicle (EtOH control), * $p<0.05$, ** $p<0.01$.

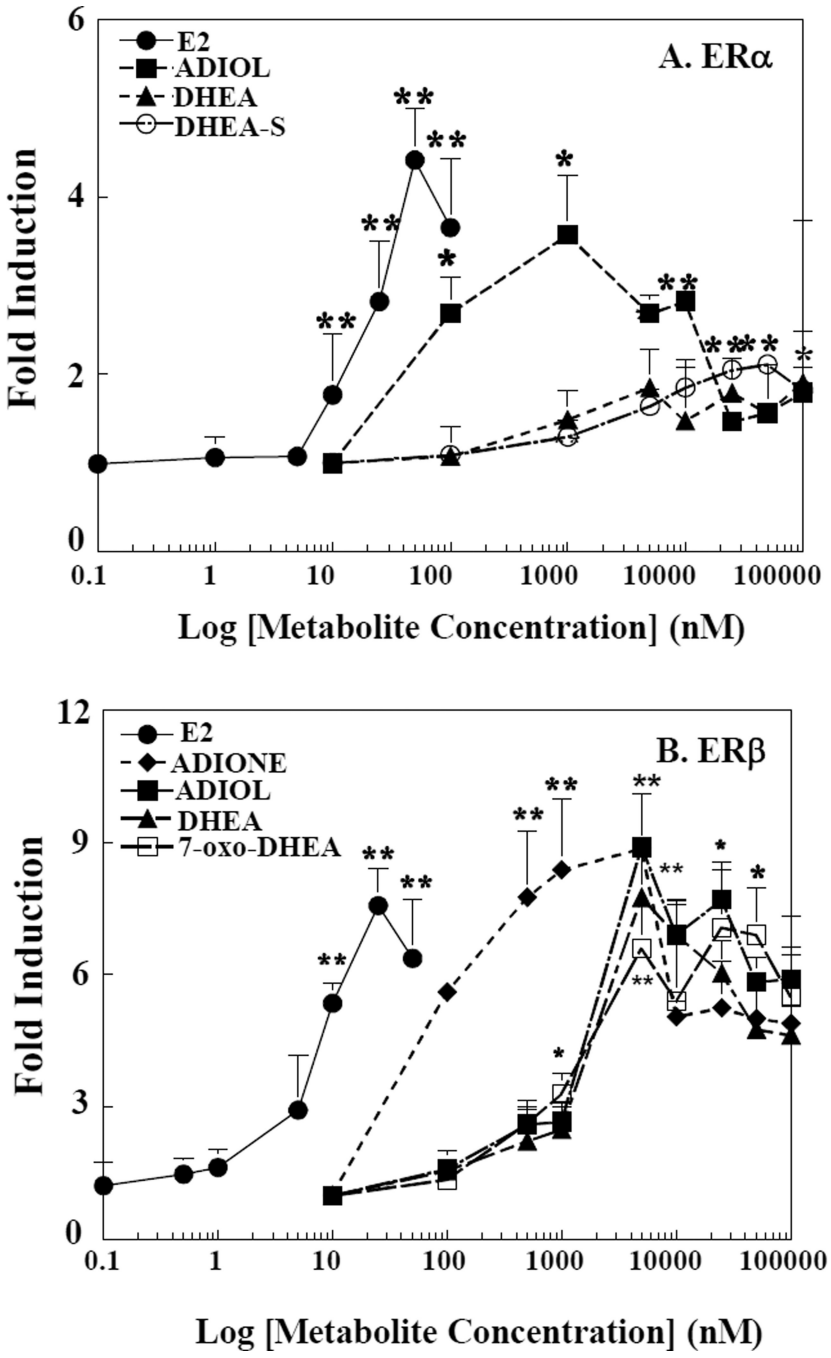

Figure 2. Concentration-dependent activation of ERα by DHEA, DHEA-S, and ADIOL in ER transfected cells

HEK-293 and HepG2 cells were transfected with ERELUC reporter plasmid and expression vector for either human ERα (A) or ERβ (B), respectively. Cells were treated for 24 h with varying concentrations of E2, DHEA, DHEA-S, 7-oxo-DHEA, ADIONE and ADIOL. Cells were harvested and lysates were assayed for β-galactosidase and luciferase activities. Data represent the mean ± S.D. of three separate experiments. *, significantly different from vehicle-treated cells, $p < 0.05$, **,$p<0.01$. E2, ●; DHEA, ▲; DHEA-S, ○; ADIOL, ■; ADIONE, ♦; and 7-oxo-DHEA, □.

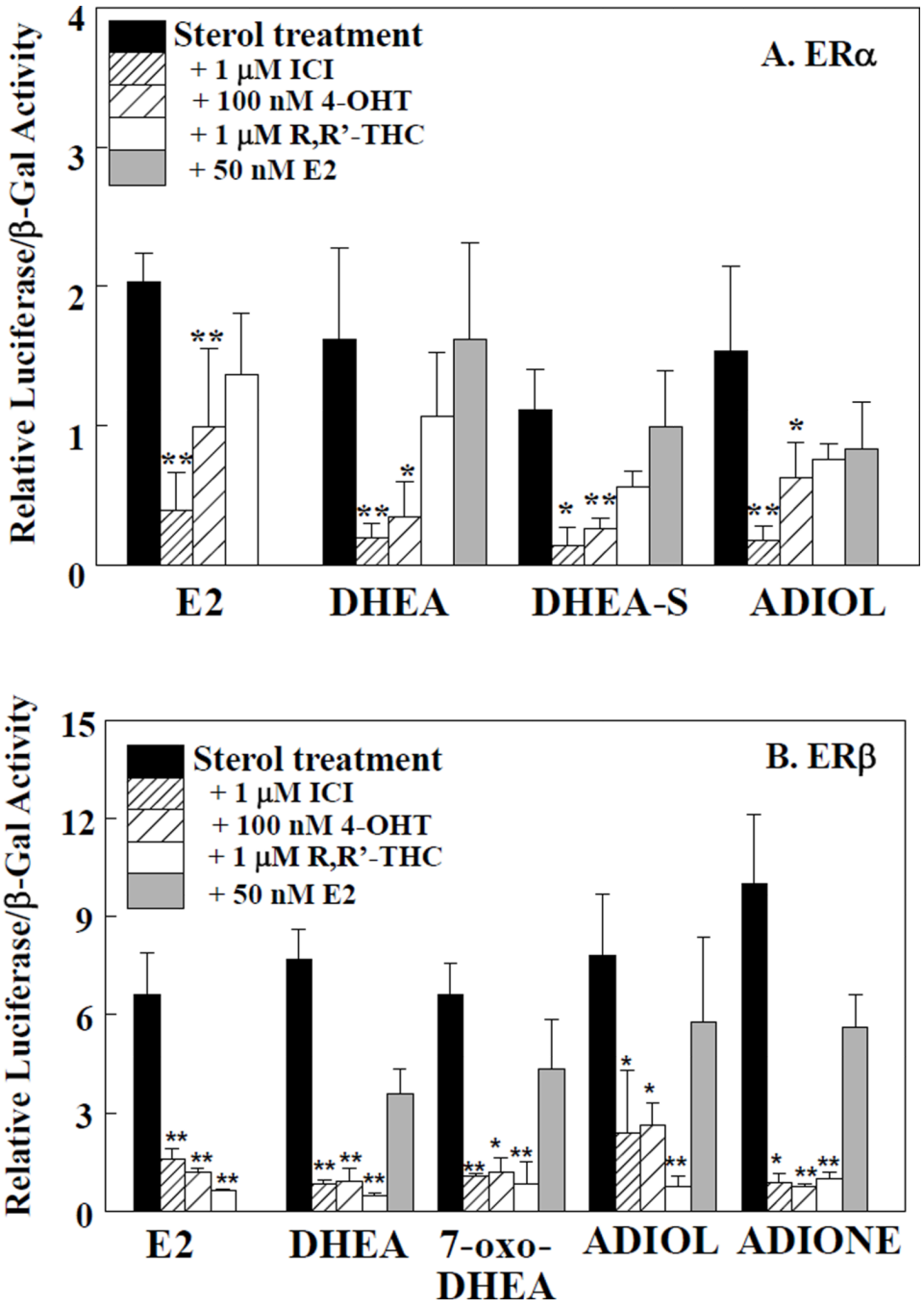

Figure 3. Inhibition of ERELUC reporter activity in the presence of cotransfected ERα in ER transfected cells

HEK-293 and HepG2 cells were transfected with an ERELUC reporter plasmid and an expression vector for either human ERα or ERβ, respectively. Cells were treated for 24 h with 5 μM DHEA metabolite in the absence or presence of either 1 μM 182,780 ICI, 100 nM 4-hydroxytamoxifen (4-OHT), 1 μM R,R-THC, or 50 nM 17β-estradiol (E2). Cells were then harvested and lysates assayed for β-galactosidase and luciferase activities. Data represent the mean ± S.D. of three wells. Experiments were repeated three times with similar results. A. ERα-mediated transactivation; B. ERβ-mediated transactivation. 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.

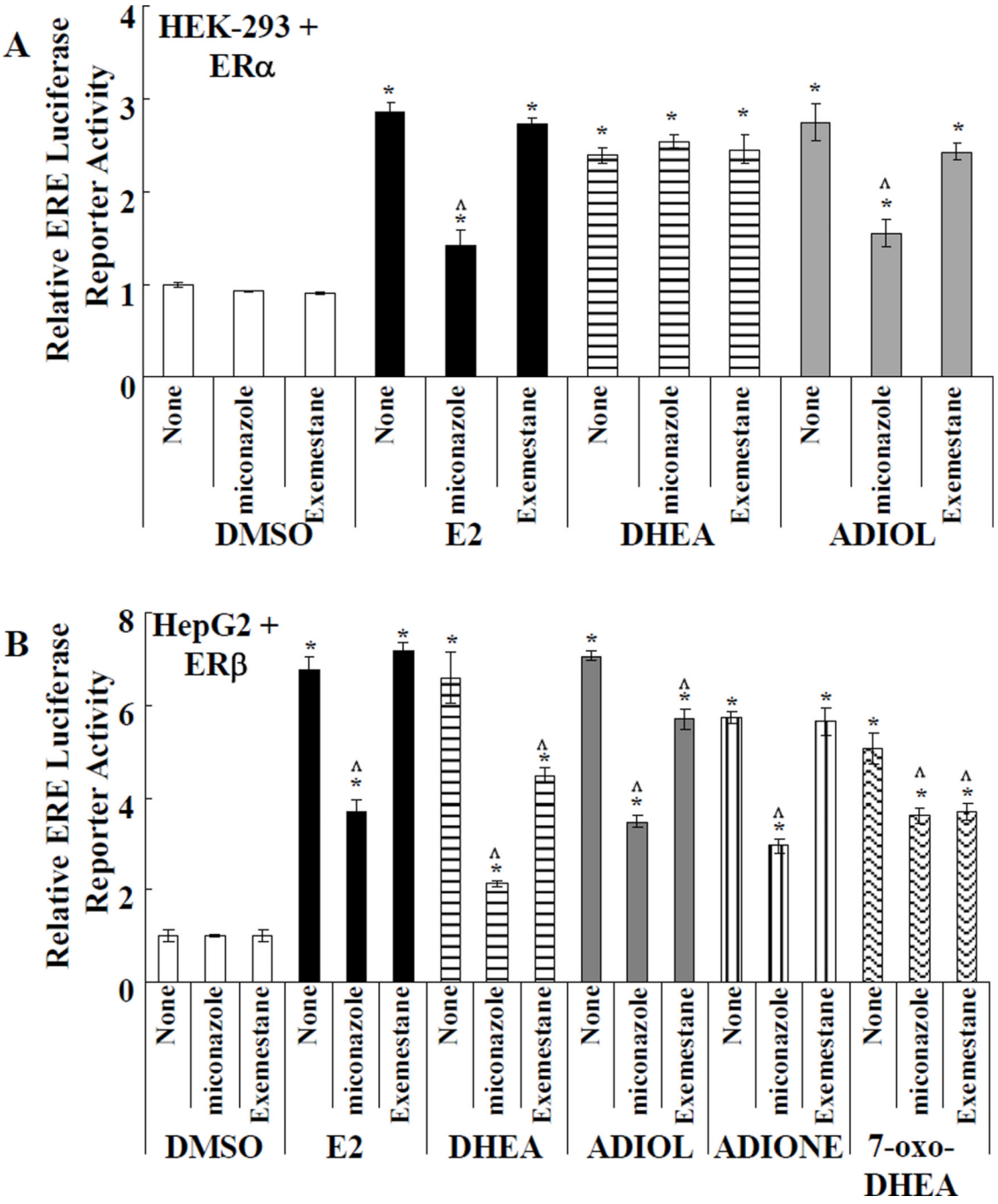

Figure 4. Effect of non-selective P450 inhibitor miconazole or aromatase inhibitor (AI) exemestane on ER-mediated transcriptional activation by DHEA and metabolites

A) HEK-293 cells were transfected with ERα (A) and HepG2 cells were transfected with ERβ (B). All cells were co-transfected with ERELUC reporter plasmid and pRL-tk. After 24 h, cells were incubated for 24 h in phenol red-free MEM supplemented with 5% DCC-FBS plus DMSO, 10 nM E2, 5 μM DHEA, 5 μM ADIOL, 5 μM ADIONE, or 5 μM 7-oxo-DHEA, as indicated in each panel. Where indicated, cells were preincubated with 5 μM miconazole or 100 nM Exemestane (AI) for 6 h prior to addition of the hormone treatment. Values are the average of 3 separate determinations +/− SEM * Significantly different from DMSO (vehicle control), $p < 0.05$. ^ Significantly different from DHEA alone, $p < 0.05$.

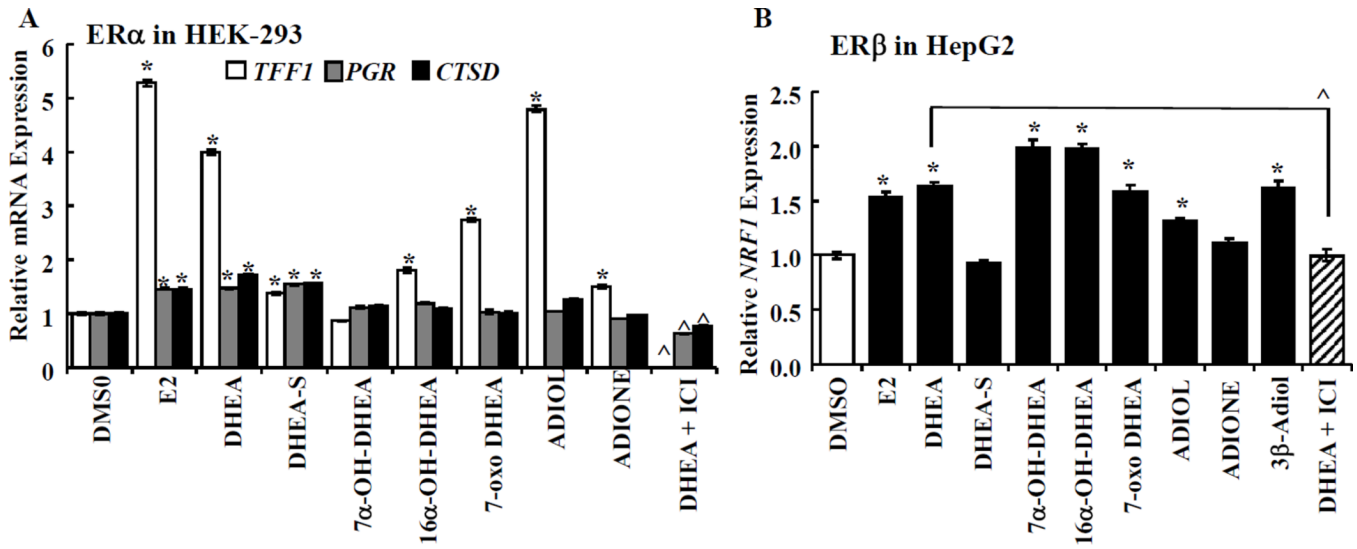

Figure 5. DHEA and metabolites increase endogenous gene transcription

HEK-293 cells were transfected with ERα (A) and HepG2 were transfected with ERβ (B) expression plasmid for 24 h and then treated with the indicated hormones (10 nM E_2 or 5 μM sterols) or 100 nM ICI 182,780 (ER antagonist) for 24 h. QPCR for A) TFF1 (pS2), PGR (PR), CTSD (cathepsin D) mRNA was normalized to 18S. B) NRF1 (NRF-1) was normalized to GAPDH. Values are the avg. ± SEM of triplicate determinations. * Significantly different from DMSO (vehicle control), $p < 0.05$. ^ Significantly different from DHEA alone, $p < 0.05$.

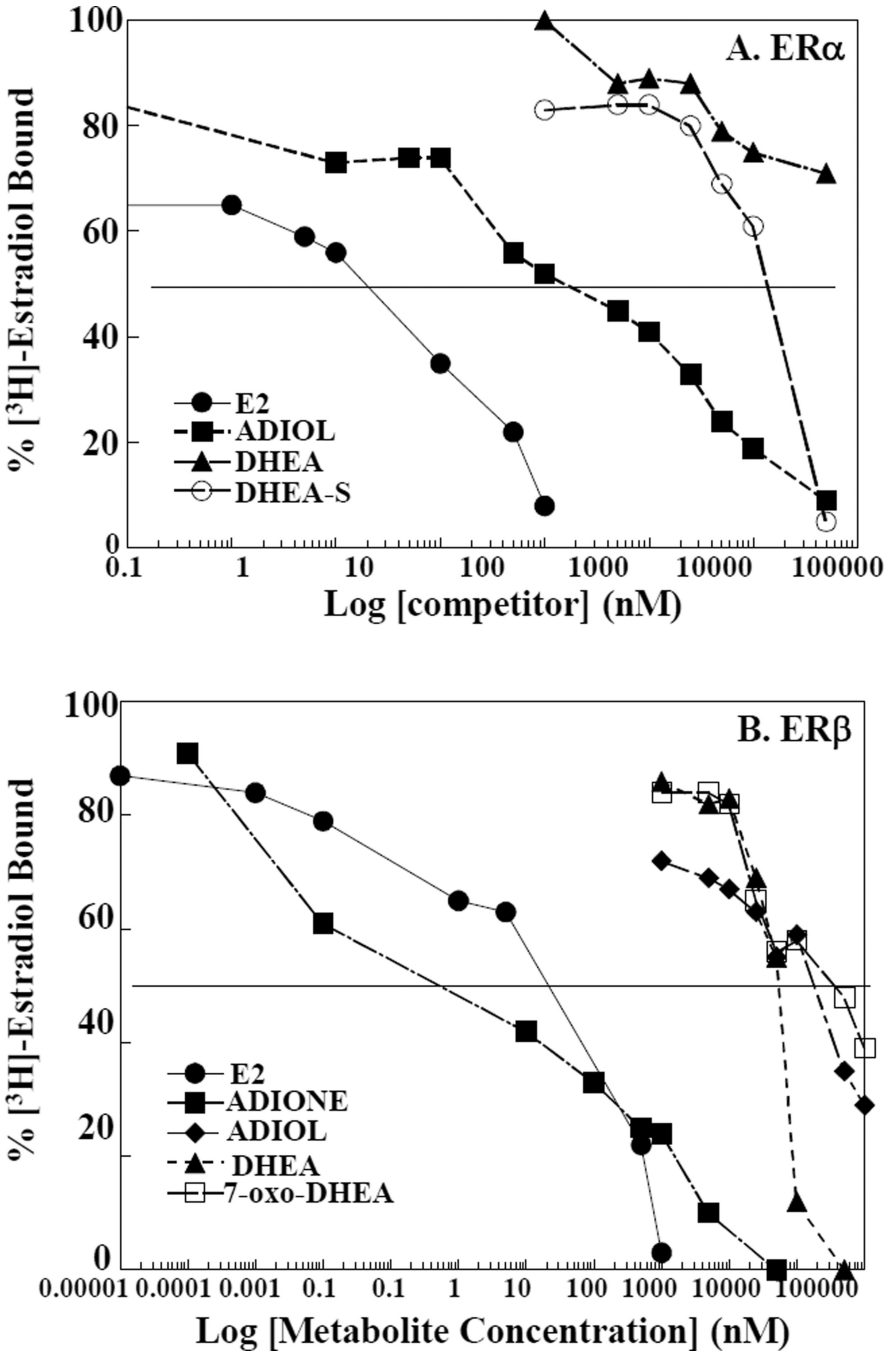

Figure 6. Competitive binding of DHEA metabolite to ERα and ERβ

An ligand binding competition assay using [^{3}H] E_2 was performed using baculovirus expressed ERα (A) or ERβ (B) with increasing concentrations of nonradiolabeled E_2, DHEA or DHEA metabolites (DHEA-S, 7-oxo-DHEA, ADIONE and ADIOL). The values on the Y-axes are expressed as the percentage of [^{3}H] E_2 bound and each data point represents the mean of two independent binding assays. The competitor concentration causing 50% reduction in [^{3}H] E_2 binding (IC_{50}) is found at the intersection of the binding curves with the 50% binding line (---------). E2, ●; DHEA, ▲; DHEA-S, ♦; ADIOL, ■; ADIONE, ♦; and 7-oxo-DHEA, □.

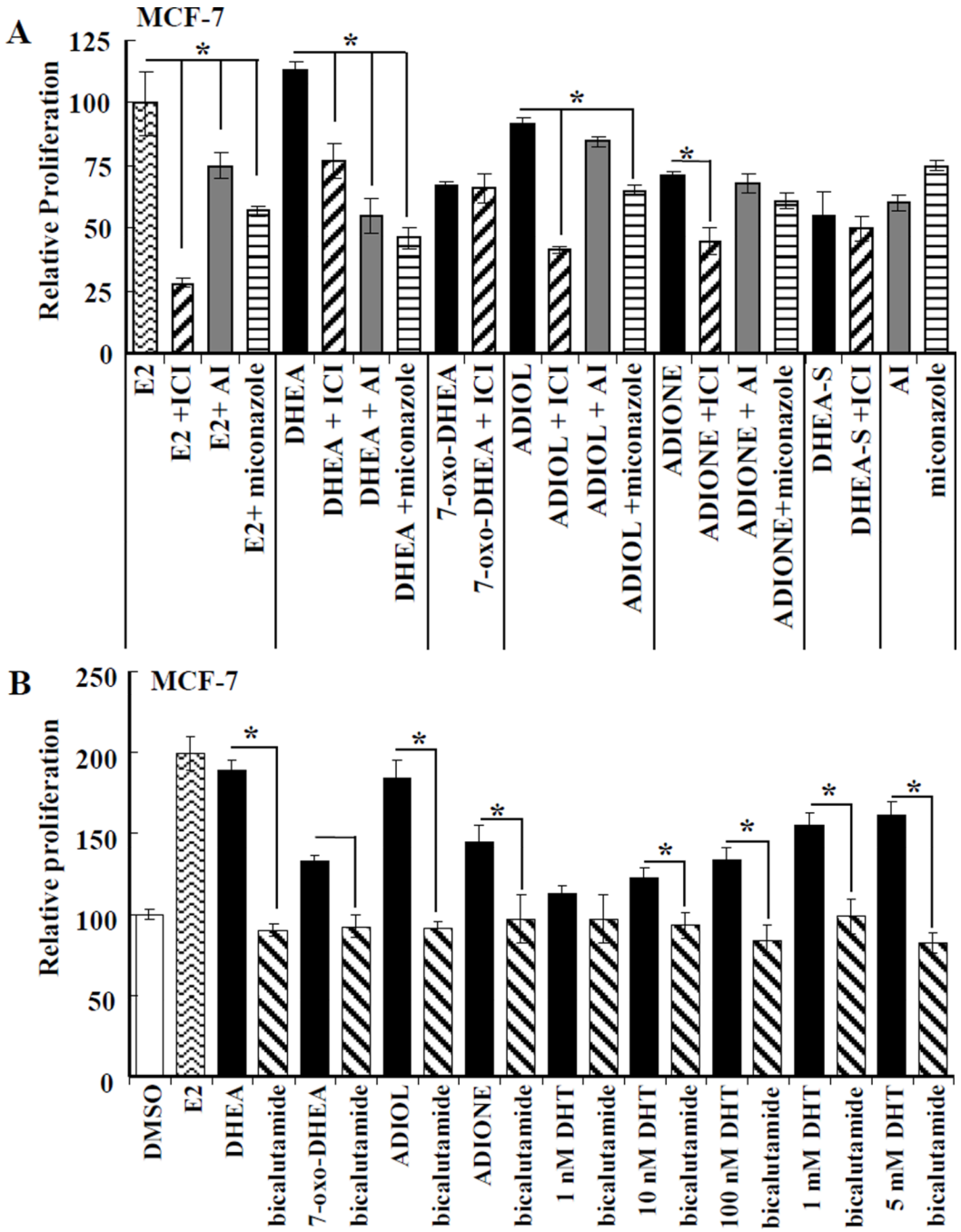

Figure 7. DHEA metabolites stimulate the proliferation of MCF-7 breast cancer cells

The effect of E_2 (10 nM), and 5 μM of either DHEA, DHEA-S, 7-oxo-DHEA, ADIOL or ADIONE alone or in combination with 100 nM ICI 182,780 (fulvestrant, a pure ER antagonist, pretreatment for 6 h), 100 nM Exemestane (AI), or 5 μM miconzaole (A) or in the presence of 10 μM bicalutamide (Casodex, pretreatment for 4 h) on the proliferation of MCF-7 cells was measured by BrdU assay after 48 h treatment as described in Experimental. A) The absorbance values were converted to percent of E_2 values for relative proliferation values. B) Values were normalized to DMSO vehicle control. The data are the mean ± S.E.M. of 3–4 independent experiments in which each treatment was performed in

quadruplicate. *, Statistically different from that particular indicated ligand treatment alone; $p < 0.05$ (one-way ANOVA followed by Student, Newman, Keuls *post-hoc* testing).

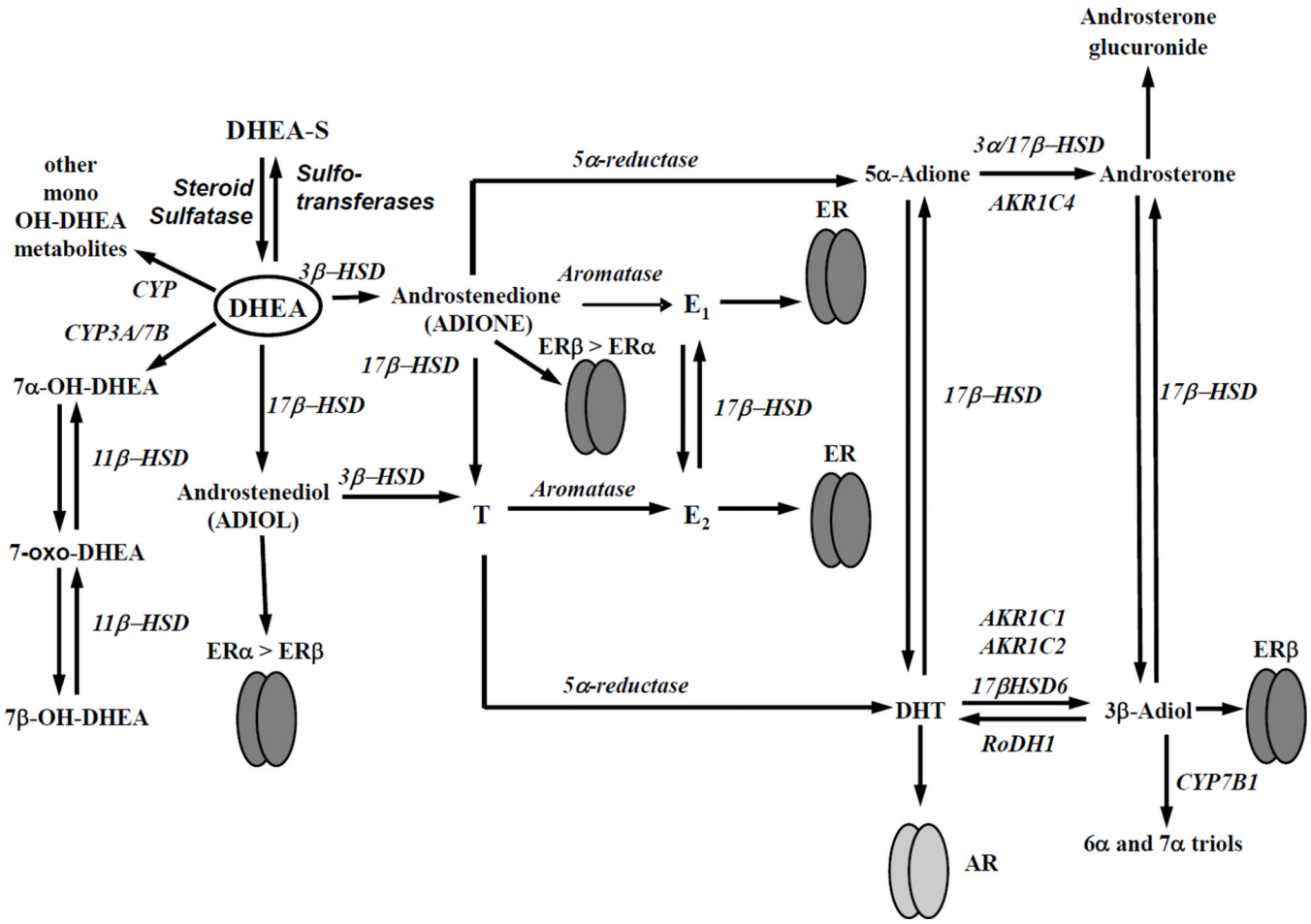

Figure 8. Model of how DHEA metabolites activate ERα and ERβ

DHEA is converted to ADIOL, ADIONE, and mono-hydroxylated metabolites capable of activating the estrogen receptors. ADIOL and ADIONE are high affinity ligand activators of ER similar to the 3β-diol derived from DHT as demonstrated by Muthusamy et al [46]. Other DHEA metabolites, 7-hydroxy- and 7-keto-DHEA are low affinity ligand activators of ER, relative to ADIOL or ADIONE. Further information on the synthesis and metabolism of DHEA is reviewed in [39, 90–92].