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Dehydroepiandrosterone Induces Human CYP2B6 through the Constitutive Androstane Receptor

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Abstract

Dehydroepiandrosterone (DHEA), the major precursor of androgens and estrogens, has several beneficial effects on the immune system, on memory function, and in modulating the effects of diabetes, obesity, and chemical carcinogenesis. Treatment of rats with DHEA influences expression of cytochrome P450 (P450) genes, including peroxisome proliferator-activated receptor α (PPAR α)- and pregnane X receptor (PXR)-mediated induction of *CYP4As* and *CYP3A23*, and suppression of *CYP2C11*. DHEA treatment elevated the expression and activities of *CYP3A4*, *CYP2C9*, *CYP2C19*, and *CYP2B6* in primary cultures of human hepatocytes. Induction of *CYP3A4* in human hepatocytes was consistent with studies in rats, but induction of *CYP2Cs* was unexpected. The role of PXR in this response was studied in transient transfection assays. DHEA activated hPXR in a concentration-dependent manner. Because *CYP2B6* induction by DHEA in human hepatocytes might involve either PXR or constitutive androstane receptor (CAR) activation, we performed experiments in primary hepatocytes from CAR knockout mice and observed that CAR was required for maximal induction of Cyp2b10 by DHEA. Furthermore, CAR-mediated Cyp2b10 induction by DHEA was inhibited by the inverse agonist of CAR, androstanol (5 α -androstan-3 α -ol). Further evidence for CAR activation was provided by cytoplasmic/nuclear transfer of CAR upon DHEA treatment. Elucidation of CAR activation and subsequent induction of *CYP2B6* by DHEA presented an additional mechanism by which the sterol can modify the expression of P450s. The effect of DHEA on the activation of the xenosensors PPAR α , PXR, and CAR, and the consequent potential for adverse drug/toxicant interactions should be considered in humans treated with this nutraceutical agent.

Dehydroepiandrosterone (DHEA), the major secretory product of the adrenal cortex, is the most abundant steroid in humans and has multifunctional properties: it is a precursor of sex steroid hormones and a peroxisome proliferator at pharmacological dosages (Wu et al., 1989). DHEA is derived from cholesterol via a series of steps catalyzed by cytochrome P450 (P450) enzymes (Miller, 2002). Physiological concentrations of DHEA and its sulfate derivative in human plasma are in the micromolar range and increase significantly in individuals consuming high amounts of DHEA (Legrain et al., 2000). It is secreted primarily as 3 β -sulfate conjugate (DHEA/DHEA-sulfate ratio 1:250 or 1:500 in plasma), which is taken up by target tissues and hydrolyzed by sulfatases back to DHEA (Labrie et al., 1997; Webb et al., 2006). DHEA is further metabolized to androgens and estrogens in testis and ovary (Labrie et al., 2005), or to hydroxylated metabolites in the liver. DHEA is hydroxylated to 7 α / β -

hydroxy- or 16 α -hydroxymetabolites by microsomal P450 enzymes (Miller et al., 2004; Chalbot and Morfin, 2005). Some of the hydroxylated metabolites are further oxidized to oxo-derivatives (Fitzpatrick et al., 2001; Miller et al., 2004).

DHEA has been suggested to display beneficial effects in humans including reduced serum cholesterol, lower incidence of cardiovascular disease, weight loss, and improved control of plasma glucose in patients with diabetes (Kawano et al., 2003; Villareal and Holloszy, 2004). DHEA also protects against chemically induced carcinogenesis (Lubet et al., 1998) and certain immune deficiencies (Henderson et al., 1992) in animal models. Furthermore, DHEA has been documented to be a neuroactive steroid displaying neuroprotective and memory-enhancing effects (Baulieu et al., 2000). In addition to these beneficial effects, chronic administration of DHEA at high doses to rodents stimulates several pathophysiological changes such as hepatomegaly and hepatocellular carcinoma (Rao et al., 1992).

DHEA administration to rats at supraphysiological doses has been reported to increase the expression of several genes including *CYP4As*, *CYP3As*, NADPH:cytochrome *c* oxidoreductase, catalase, or fatty acyl-CoA oxidase (Wu et al., 1989; Prough et al., 1994; Webb et al., 1996; Gu et al., 2003). Several drugs including clofibrate and nafenopin also induce *CYP4A* transcription by activation of peroxisome proliferator-activated receptor α (PPAR α). Although induction of *CYP4A* expression by DHEA requires activation of PPAR α , DHEA does not appear to be a ligand for PPAR α (Prough et al., 1994; Peters et al., 1996). Receptor activation is possibly the result of an effect of phosphorylation status of PPAR α (Webb et al., 2006). Furthermore, studies with primary cultures of rat hepatocytes revealed that several oxidative metabolites of DHEA produced in liver are also able to activate *CYP4A* message and protein levels (Webb et al., 1996). 7-Oxidized metabolites (7-hydroxy-DHEA and 7-oxo-DHEA) do not serve as ligands for PPAR α , but they are presumed to activate PPAR α in a way similar to that of the parent compound. Treatment of rats with pharmacological doses of DHEA results in alteration in expression of other genes responsive to peroxisome proliferators, including fatty acyl-CoA oxidase or malic enzyme. DHEA exerts regulatory effects not only on peroxisome proliferation-associated genes, but also on *CYP3A23* gene in rats (Singleton et al., 1999). This observation was due to DHEA activation of the pregnane X receptor (PXR), a member of the nuclear receptor superfamily known to mediate induction of *CYP3A* expression (Jones et al., 2000; Ripp et al., 2002). Negative regulation of *CYP2C11* expression by peroxisome proliferators has also been demonstrated in rats, although with DHEA, PPAR α coexpression does not seem to be required for negative regulation (Ripp et al., 2003). The main goal of the present study was to examine the P450-inducing capacity of DHEA and of two of its metabolites (7 α -hydroxy-DHEA and 7-oxo-DHEA) in primary culture of human hepatocytes. In addition to PXR action, we provided evidence for a role of constitutive androstane receptor (CAR) in the induction of certain P450 genes by DHEA.

Materials and Methods

Chemicals

5-Androsten-3 β -ol-17-one (DHEA), 5-androsten-3 β ,7 α -diol-17-one (7 α -hydroxy-DHEA), and 5-androsten-3 β -ol-7,17-dione (7-oxo-DHEA) were purchased from Steraloids, Inc. (Newport, RI). Dexamethasone, SR-12813 (tetra-ethyl 2-(3,5-di-*tert*-butyl-4-hydroxyphenyl) ethenyl-1,1-bisphosphonate), 5 α -androstan-3 β -ol (androstanol), and dimethyl sulfoxide (DMSO) were the products of Sigma Chemie GmbH (Deisenhofen, Germany). 3,3',5,5'-Tetrachloro-1,4-bis(pyridyloxy)benzene (TCPOBOP) was purchased from Bayer AG (Leverkusen, Germany). 6-(4-Chlorophenyl)imidazo[2,1-*b*][1,3]thiazole-5-carbaldehyde-*O*-(3,4-dichlorobenzyl)oxime (CITCO) was purchased from BIOMOL Research Laboratories (Plymouth Meeting, PA). Chemicals for hepatocyte isolation, cell culture media, and supplements were purchased from Sigma Chemie GmbH and Merck (Darmstadt, Germany).

Isolation and Culture of Human Hepatocytes

Human livers were obtained from kidney transplant donors at the Transplantation and Surgical Clinic, Semmelweis University (Budapest, Hungary). Permission of the Hungarian Regional Committee of Science and Research Ethics was obtained to use human tissues. Clinical histories of the donors are shown in Table 1. Liver cells were isolated by the method of Bayliss and Skett (1996). Hepatocytes having viability better than 90% as determined by trypan blue exclusion were used in the experiments. The cells were plated at a density of 1.7×10^5 cells/cm² in plastic dishes precoated with collagen in medium described by Ferrini et al. (1998). After overnight culture, the medium was replaced by serum-free medium. Forty-eight hours after serum deprivation, cells were cultured in the presence or absence of inducers for 48 h. Hepatocytes were treated with dexamethasone (1 and 10 μ M), DHEA (50 μ M), 7 α -hydroxy-DHEA (50 μ M), or 7-oxo-DHEA (50 μ M).

Preparation and Culture of Primary Mouse Hepatocytes

The first breeding pair of CAR knockout C57BL/6J mice was a generous gift of D. D. Moore from the Department of Molecular and Cellular Biology, Baylor College of Medicine (Houston, Texas) (Wei et al., 2000). Further breeding of CAR knockout mice was carried out at the Biozentrum, University of Basel (Basel, Switzerland). Primary hepatocytes of control (CAR +/+) and CAR knockout (CAR -/-) mice were prepared by a two-step collagenase method (Bayliss and Skett, 1996). Liver cells were plated at a density of 3×10^5 cells/well on 12-well plates coated with collagen and maintained in medium described by Ferrini et al. (1998). Hepatocytes were exposed to TCPOBOP (10 μ M), DHEA (25 μ M), or vehicle (DMSO 0.1%) for 24 h.

P450 Enzyme Assays

Microsomal fraction from cultured human hepatocytes was prepared by differential centrifugation (van der Hoeven and Coon, 1974). Protein content of microsomes was determined by the method of Lowry et al. (1951), with bovine serum albumin as the standard. Published methods were followed to determine selective P450 enzyme activities: mephenytoin *N*-demethylation for CYP2B6 (Heyn et al., 1996), tolbutamide 4-hydroxylation for CYP2C9 (Miners and Birkett, 1996), mephenytoin 4'-hydroxylation for CYP2C19 (Srivastava et al., 1991), nifedipine oxidation (Guengerich et al., 1986), and midazolam 1'- and 4-hydroxylation (Kronbach et al., 1989) for CYP3A4/5. The incubation mixture contained NADPH-generating system (1 mM NADPH, 10 mM glucose 6-phosphate, 5 mM MgCl₂, and 2 units/ml glucose-6-phosphate dehydrogenase), microsomes, and various selective substrates for P450 forms (mephenytoin, tolbutamide, midazolam, or nifedipine). The rates of enzyme activity were linearly dependent upon the amount of microsomal protein added for the 10- to 30-min incubation period. The metabolic extraction procedures and high-performance liquid chromatography analyses were performed according to the published methods. P450 enzyme assays were performed in triplicate and means $\pm$ standard deviations were calculated. For comparison among untreated and treated groups, statistical analysis of the results was carried out using a two-tailed *t* test with *p* < 0.05 as the criterion for significance. Due to high variation in basic P450 activities of human hepatocytes, the entire experiment was repeated in hepatocytes isolated from five to six donors to confirm the results.

RNA Extraction and Quantitative RT-PCR

Total RNA was isolated from human and mouse hepatocytes using TRIzol reagent (Invitrogen, Carlsbad, CA). Ten million liver cells were homogenized in 1 ml of TRIzol reagent, and total RNA was extracted according to the manufacturer's instructions. The RNA was precipitated using ethanol and stored at -80°C for further analyses. The primers used for RT-PCR analyses are shown in Table 2. RNA (3 μ g) was reverse-transcribed into single-stranded cDNA using

Transcriptor First Strand cDNA synthesis kit (Roche Diagnostics GmbH, Mannheim, Germany), and then real-time PCR with human cDNA was performed using FastStart *Taq*DNA polymerase (LightCycler TaqMan Master, Roche Diagnostics GmbH) and UPL (Universal Probe Library) probes for *CYP2B6*, *CYP2C9*, *CYP2C19*, and *CYP3A4* (Roche Diagnostics GmbH). Mouse cDNA was analyzed with FAM-labeled *Taq* probe for *Cyp2b10* (Microsynth GmbH, Balgach, Switzerland) using AmpliTaq DNA Polymerase (TaqMan Universal PCR Master Mix, No Amperase UNG, Applied Biosystems, Foster City, CA). The quantity of target RNA relative to that of housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was determined. P450 mRNA levels were quantified by RT-PCR measurements in the same human hepatocytes in which P450 activities were measured. Statistical analysis of the results obtained by RT-PCR was carried out in a manner similar to that of P450 activities.

Cell Culture and Transfection

HuH7 cell line was obtained from the European Collection of Cell Cultures (Salisbury, UK) and maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, 1 mM glutamine, 10 mM sodium pyruvate, and 100 μ g/ml penicillin and streptomycin. The pSG5, pSG5-hPXR, pGL3(CYP3A4/XREM[−7800/−7200]/−262/+11)LUC [containing the distal xenobiotic-responsive element module (XREM, −7800/−7200) linked to the proximal CYP3A4 promoter (−262/+11)], and pSV- β -galactosidase have been described elsewhere (Pascussi et al., 2001, 2003a). For reporter assays, 5×10^5 HuH7 cells were transiently transfected with 10 ng of expression plasmid (pSG5 or pSG5-hPXR) together with 100 ng of luciferase reporter constructs pGL3(CYP3A4/XREM[−7800/−7200]/−262/+11) LUC and 50 ng of pSV- β -galactosidase expression plasmid for transfection quality control using FeGENE6 transfection reagent (Roche Applied Science, Basel, Switzerland). Approximately 20 to 30% of the cells were transfected. After 16 h, the medium was changed, and the cells were treated with DHEA (1, 10, and 50 μ M), SR-12813 (1 μ M) or solvent (DMSO 0.1%) in Dulbecco's modified Eagle's medium containing 5% delipidated and charcoal-treated calf serum. After 24 h of incubation, luciferase and β -galactosidase activities were measured as described previously (Pascussi et al., 2003a). Values are expressed as the mean $\pm$ standard deviation for four experiments measured in triplicate and represent luciferase activity divided by the β -galactosidase activity of each extract. Reporter gene activity of the DMSO-treated cells served as a control and was set to unity.

Nuclear Translocation Assay

Mouse hepatocytes were cultured on glass coverslips coated with collagen. After 4 h of attachment, serum-free medium was added to the cells. Transfection was carried out in Opti-MEM I Reduced Serum Medium (Invitrogen) using Lipofectamine 2000 transfection reagent (Invitrogen). Cells were incubated with the pEGFP-c1-hCAR plasmid kindly provided by M. Negishi (Laboratory of Reproductive and Developmental Toxicology, National Institute of Environmental Health Sciences, Research Triangle Park, NC). Eighteen hours after transfection, hepatocytes were exposed to DHEA (50 μ M) or CITCO (100 nM) for 4 h, then washed twice with phosphate-buffered saline solution and fixed in 4% (w/v) paraformaldehyde. Cells were stained with DAPI (4'-6-diamidino-2-phenylindole) and then washed again with phosphate-buffered saline. The coverslips were transferred to microscope slides and visualized in Moviol mounting medium. The intracellular localization of CAR-GFP fusion protein was determined by fluorescence microscopy using a Leica DM5000B microscope (Leica Microsystems, Wetzlar GmbH, Wetzlar, Germany) and AnalySIS Pro software (Soft Imaging System GmbH, Münster, Germany).

Statistical Analysis

For comparison among several groups, statistical analysis of the results obtained in experiments with human and mouse hepatocytes and in cell line transfection was carried out using a paired two-tailed *t* test with $p < 0.05$ as the criterion for significance (GraphPad InStat version 3.0, GraphPad Software, San Diego, CA).

Results

Previous work has clearly demonstrated the P450-inducing effect of DHEA in rats (Prough et al., 1994; Singleton et al., 1999; Gu et al., 2003). Work by Peters et al. (1996) and Ripp et al. (2002) has shown that DHEA acts on *CYP4As* and *CYP3A23* at the transcriptional level by PPAR α - and PXR-mediated events, respectively. Oxidative metabolites of DHEA have also been reported to activate PPAR α and consequently to induce *CYP4A* in rat hepatocytes (Webb et al., 2006). The present study further characterizes the actions of DHEA and of some of its metabolites, 7 α -hydroxy-DHEA and 7-oxo-DHEA (Miller et al., 2004; Chalbot and Morfin, 2005), to induce P450s in adult human hepatocytes. We also attempted to elucidate the role of nuclear receptors, PXR and CAR, in the induction of P450s by DHEA.

P450 Induction by DHEA in Human Hepatocytes

It was ascertained in preliminary experiments that 50 μ M DHEA was required for maximal induction of P450 activities in cultured human hepatocytes (data not shown). In subsequent studies, this concentration of DHEA, 7 α -hydroxy-DHEA, or 7-oxo-DHEA was used for treatment of hepatocytes. The synthetic glucocorticoid, dexamethasone, was used as a reference compound at relatively high concentrations (1 and 10 μ M). At supramicromolar concentrations, dexamethasone binds to and activates PXR, producing induction of P450s such as *CYP3A4* (Pascucci et al., 2001). P450 enzyme activities and mRNA levels were determined in primary human hepatocytes isolated from several donors (as indicated in Figs. 1 and 2) because of high individual variance in basic activities or P450 expression of the cells. To evaluate significant inducibility of human hepatocytes by DHEA and its metabolites, a paired *t* test was performed with $p < 0.05$ as the criterion for significance. Figures 1 and 2 present P450 activities and mRNA levels relative to untreated hepatocytes (0.1% DMSO-treated cells).

Nifedipine oxidation, and midazolam 1'- and 4-hydroxylation activities of *CYP3A4* increased by 3- to 4-fold in dexamethasone-treated cells as expected from the work of others (Ledirac et al., 2000). Treatments with DHEA or its metabolites resulted in a more than 2-fold induction of *CYP3A4* activities (Fig. 1a). The elevation in enzyme activities was nearly concomitant with the -fold changes in levels of *CYP3A4* mRNA (Fig. 1b). Treatments of hepatocytes with DHEA or its two oxidative metabolites produced nearly identical increases in enzyme activities or mRNA levels of *CYP3A4*. These results suggested that DHEA and its metabolites caused transcriptional activation of *CYP3A4* gene involving the action of nuclear receptors, most likely of PXR.

Tolbutamide hydroxylation activity and expression of *CYP2C9* were also significantly induced by DHEA and two of its metabolites (Fig. 2a). The degree of the elevation of *CYP2C9* activity and mRNA levels relative to the control cell population (2- to 3-fold) was identical with that of dexamethasone-treated cells. Similar results were observed for *CYP2C19*. DHEA treatment caused an appreciable 2- to 3-fold increase in mephenytoin 4-hydroxylation activity and mRNA levels of *CYP2C19* (Fig. 2b). The two oxidative metabolites of DHEA also exerted the same *CYP2C19*-inducing effect as the parent compound. Furthermore, mephenytoin *N*-demethylation activity of *CYP2B6* was potently induced by DHEA and its metabolites (Fig. 2c). Nearly identical increases in *CYP2B6* activity were obtained in liver cells treated with DHEA, 7 α -hydroxy-DHEA, and 7-oxo-DHEA. The 48-h treatment resulted in an

approximately 2- or 2.5-fold increase similar to that of dexamethasone. Similar effects were observed at the level of CYP2B6 mRNA (Fig. 2c).

PXR Activation by DHEA

DHEA is known to induce expression of *CYP4As* through activation of PPAR α . It is also an activator of PXR that mediates induction of *CYP3A23* expression in rats (Ripp et al., 2002). It has been demonstrated that PXR is involved in regulation of the expression of several P450s (CYP2B6, CYP2C9, CYP2C19, and CYP3A4) in human (Pascussi et al., 2001, 2003b). Our results in human hepatocytes also suggested that DHEA transcriptionally activated P450 genes involving the action of PXR. To assess whether DHEA was able to activate human PXR, transient cotransfection assay was performed in the HuH7 cell line. The effect of DHEA on transcriptional activation of hPXR was investigated using reporter plasmid (pGL3(CYP3A4/XREM[−7800/−7200]/−262/+11)LUC). The reporter plasmid was cotransfected with hPXR. DHEA treatment resulted in a concentration-dependent increase in ligand-induced transactivation of reporter construct by hPXR (Fig. 3). However, the 3-fold induction observed at 50 μ M DHEA was lower than the increase in luciferase activity caused by the well known PXR agonist SR-12813 (Jones et al., 2000). It should be noted that DHEA was able to slightly activate reporter plasmid without coexpression of hPXR to some extent (1.8-fold).

The Role of CAR in CYP2B Induction

The finding that DHEA was active in CYP2B6 induction in human hepatocytes raised the question of whether CAR activation may occur as a result of DHEA treatment or PXR activation by DHEA is responsible for CYP2B6 induction. Hepatocytes isolated from CAR knockout and wild-type mice were tested for their responsiveness to DHEA. RT-PCR analysis of RNA prepared from hepatocytes of wild-type mice revealed a substantial increase (approximately 8-fold) in mRNA of the mouse ortholog *Cyp2b10* as a result of DHEA treatment compared with vehicle-treated controls (Fig. 4). Mouse hepatocytes from wild-type animals exhibited strong induction of *Cyp2b10* (approximately 20-fold) after exposure to the mouse CAR activator TCPOBOP (10 μ M). Lack of expression of CAR in hepatocytes from CAR-ablated mice resulted in abolition of *Cyp2b10* induction by TCPOBOP. Although the induction of *Cyp2b10* expression by DHEA was also reduced in primary hepatocytes of CAR knockout mice, it was not completely inhibited. Approximately 4-fold induction of *Cyp2b10* by DHEA was still present in liver cells of CAR $^{-/-}$ mice (Fig. 4). These observations clearly established that CAR was involved in maximal induction of *Cyp2b10* by DHEA, suggesting that both CAR and PXR may be required for the maximal inductive effect of DHEA on *Cyp2b10* gene expression.

To evaluate the role of CAR in DHEA action, the effect of androstanol on *Cyp2b10* induction was also investigated in mouse hepatocytes. Androstanol significantly reduced the induction of *Cyp2b10* by the CAR ligand, TCPOBOP. *Cyp2b10* mRNA levels of the cells treated with TCPOBOP decreased by approximately 50 to 60% in the presence of androstanol (Fig. 5). Androstanol was also observed to repress the induction of *Cyp2b10* by DHEA. Androstanol treatment decreased *Cyp2b10* mRNA levels by 40 to 50% in DHEA-induced hepatocytes. Thus, androstanol, the inverse agonist of CAR (Forman et al., 1998), reduced the expression of *Cyp2b10* both in DHEA- and in TCPOBOP-induced cells.

The capability of DHEA to trigger CAR translocation from cytoplasm to nucleus was also tested in mouse primary hepatocytes expressing human CAR. The subcellular localization of hCAR was determined by fluorescence microscopy in hepatocytes infected with plasmid coding for hCAR in fusion with enhanced green fluorescent protein (pEGFP-c1-hCAR). In control hepatocytes, hCAR-GFP was clearly located in the cytoplasm, whereas it was undetectable in the nuclei in the absence of stimulation (Fig. 6). After 4 h exposure to the

selective human CAR agonist CITCO (Maglich et al., 2003), cytoplasmic/nuclear transfer of CAR was observed primarily in nuclei of the cells, as expected. In response to DHEA treatment, the pattern of CAR-GFP localization was similar to that seen after CITCO treatment (Fig. 6). The corresponding DAPI-stained and merged images provided further visual evidence indicating that DHEA promoted efficient CAR translocation into the nucleus of hepatocytes.

Discussion

DHEA administration to rats affects the expression of several P450 genes. DHEA has been shown to increase the levels of CYP4As (Prough et al., 1994) and CYP3A23 (Singleton et al., 1999), and to suppress the expression of *CYP2C11* (Ripp et al., 2003). Nuclear receptors such as PPAR α and PXR appear to be involved in DHEA action in rats (Peters et al., 1996; Ripp et al., 2002). In addition, 7-oxidized metabolites of DHEA also induce CYP4A1 message and protein levels in rat hepatocytes contributing to the effect of the parent compound (Webb et al., 2006). In the present study, we therefore investigated the induction of P450s by DHEA and two of its oxidative metabolites in primary cultures of human hepatocytes and, in particular, the role of nuclear receptors PXR and CAR in DHEA action.

Human hepatocytes in culture as induction system were validated by testing the classical inducer compound, the steroid dexamethasone, on P450 gene expression. Concentrations of dexamethasone equal to or above 1 μ M potently increased both the activities and mRNA levels of CYP3A4 and, to a lesser extent, of CYP2C9, CYP2C19, and CYP2B6. The present study then revealed that DHEA treatment also elevated the expression and activities of CYP3A4, CYP2C9, CYP2C19, and CYP2B6 similar to those of dexamethasone. In addition, the DHEA metabolite 7 α -hydroxy-DHEA or 7-oxo-DHEA resulted in the same inducing action as did the parent compound. This means that biotransformation does not lead to inactivation of DHEA as P450 inducer.

The finding that DHEA potently induced *CYP3A4* in human hepatocytes was consistent with previous studies in rats (Singleton et al., 1999). PXR activation has been proposed to mediate the increase in *CYP3A* expression (Ripp et al., 2002). But in contrast to studies in rats (Ripp et al., 2003), DHEA did not mediate suppression of *CYP2Cs* in human hepatocytes. Moreover, DHEA treatment led to up-regulation of both *CYP2C9* and *CYP2C19*, suggesting the involvement of PXR. DHEA was able to activate hPXR in a concentration-dependent manner, although it appeared to be a weak PXR activator compared with the well known PXR agonist SR-12813.

DHEA-mediated transcription of *CYP2B* has not been unequivocally shown in previous studies. The current studies demonstrated an efficient *CYP2B6* induction exerted by DHEA in primary cultures of human hepatocytes. The activation of human CAR by compounds such as phenobarbital and CITCO or mouse CAR by TCPOBOP has been well documented (Maglich et al., 2003). Experiments carried out in hepatocytes of CAR knockout mice demonstrated that ablated levels of CAR reduced, but did not abolish, response to DHEA. Partial inhibition of DHEA-mediated *Cyp2b10* induction by the inverse agonist androstanol presented further evidence for the contribution of CAR to the enhancement of *Cyp2b10* expression. The inactive nuclear receptor CAR, which is cytoplasmic in hepatocytes, undergoes rapid nuclear translocation upon treatment with CAR activators, phenobarbital, or CITCO. Although binding of CAR activators to the receptor may not occur, CAR translocation to the nucleus is the limiting step in the CAR activation process, followed by CAR/RXR heterodimer formation and binding of the proteins to the DNA of target genes (Waxman, 1999). The activation of CAR by DHEA was thus demonstrated in in vitro nuclear translocation assays. Using primary cultures of mouse hepatocytes expressing hCAR, the effect of DHEA on cytoplasmic/nuclear shuttling of CAR was compared with that of CITCO. In agreement with Maglich et al.

(2003), the occurrence of translocation of hCAR from cytoplasm into the nucleus after CITCO treatment was well recognized. DHEA treatment also induced CAR translocation into the nucleus, presenting further evidence for CAR activation by DHEA. These findings suggested that CAR was required for maximal induction of *Cyp2b10* by DHEA. However, in the absence of CAR, there still is an apparent receptor-mediated induction of P450 mRNA, protein, and activity that could be due to PXR (Ripp et al., 2003) or yet unidentified transcription factors.

In conclusion, we provide evidence for the induction of several P450s (*CYP3A4*, *CYP2C9*, *CYP2C19*, and *CYP2B6*) by DHEA and by two of its oxidative metabolites, 7 α -hydroxy-DHEA and 7-oxo-DHEA, in human hepatocytes. Our results also provide convincing evidence for the activation of hPXR and hCAR by DHEA as prerequisite for the transcriptional activation of these P450 genes. In other studies, the activation of several nuclear receptors including PPAR α , PXR, and estrogen receptor by DHEA and its metabolites has been demonstrated. CAR activation and subsequent induction of *CYP2B6* by DHEA present an additional mechanism by which DHEA, a widely used nutraceutical, can modify the expression of P450s. Because the inductive response of the PXR and CAR system is associated with changes in the kinetics of numerous drugs and steroids and includes drug-drug interactions and adverse drug effects, the unrestricted use of DHEA as a panacea for various health problems should be carefully considered.

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ABBREVIATIONS

DHEA, dehydroepiandrosterone or 5-androsten-3 β -ol-17-one
 androstanol, 5 α -androstan-3 α -ol
 CAR, constitutive androstane receptor
 CITCO, 6-(4-chlorophenyl)imidazo[2,1-*b*][1,3]thiazole-5-carbaldehyde-*O*-(3,4-dichlorobenzyl)oxime
 P450, cytochrome P450
 DAPI, 4'-6-diamidino-2-phenylindole
 7 α -hydroxy-DHEA, 5-androsten-3 β ,7 α -diol-17-one
 7-oxo-DHEA, 5-androsten-3 β -ol-7,17-dione
 DMSO, dimethyl sulfoxide
 GAPDH, glyceraldehyde 3-phosphate dehydrogenase
 GFP, green fluorescent protein
 PPAR α , peroxisome proliferator-activated receptor α
 PXR, pregnane X receptor
 TCPOBOP, 3,3',5,5'-tetrachloro-1,4-bis(pyridyloxy)benzene
 SR-12813, tetra-ethyl 2-(3,5-di-*tert*-butyl-4-hydroxyphenyl)ethenyl-1,1-bisphosphonate
 RT-PCR, reverse transcriptase-polymerase chain reaction
 FAM, 5-carboxyfluorescein
 XREM, xenobiotic-responsive element module
 h, human.

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Fig. 1.

Induction of CYP3A4 activities (a) and mRNA levels (b) in primary human hepatocytes. Human liver cells were treated for 48 h with dexamethasone (DXM, 1 and 10 μ M), DHEA (50 μ M), and its metabolites: 7 α -hydroxy-DHEA (OH-DHEA, 50 μ M) and 7-oxo-DHEA (oxo-DHEA, 50 μ M). CYP3A4 activities were determined in microsomes prepared from hepatocytes isolated from five donors. Levels of CYP3A4 mRNA in human hepatocytes ($n = 5$ donors) were quantified and normalized to GAPDH as described under *Materials and Methods*. Statistical analysis of the results obtained in untreated and treated cells indicated significant increase in both the activities and mRNA levels of CYP3A4 as a consequence of various treatments. Controls for each independent experiment were assigned values of 1 and results of

treatments are expressed relative to the controls. Error bars represent standard deviations from the mean of five donors.

Fig. 2.

Induction of CYP2C9 (a), CYP2C19 (b) and CYP2B6 (c) activities and mRNA levels in primary human hepatocytes. Cells were treated for 48 h with dexamethasone (DXM, 1 and 10 μ M), DHEA (50 μ M) and its metabolites: 7 α -hydroxy-DHEA (OH-DHEA, 50 μ M) and 7-oxo-DHEA (oxo-DHEA, 50 μ M). P450 activities were determined in microsomes prepared from hepatocytes isolated from six donors. Levels of CYP2C9, CYP2C19, and CYP2B6 mRNAs were quantified and normalized to GAPDH as described under *Materials and Methods*. Statistical analysis of the results obtained in untreated and treated cells indicated significant increase in both the activities and mRNA levels as a consequence of various treatments. Controls for each independent experiment were assigned values of 1 and results of treatments

are expressed relative to the controls. Error bars represent standard deviations from the mean of six donors.

Fig. 3. hPXR activation in response to DHEA and the PXR agonist SR-12813. HuH7 cells were transiently transfected with expression plasmid (pSG5 or pSG5-hPXR) together with luciferase reporter constructs pGL3(CYP3A4/XREM[−7800/−7200]/−262/+11)LUC and pSV-β-galactosidase expression plasmid for transfection quality control. The cells were treated for 24 h with DHEA (1, 10, and 50 μ M), SR-12813 (1 μ M), and normalized luciferase/β-galactosidase activities were determined as described under *Materials and Methods*; the activities are the mean $\pm$ S.D. ($n = 4$).

Fig. 4.

Cyp2b10 induction by DHEA and TCPOBOP in primary hepatocytes of wild-type and CAR knockout mice. Hepatocytes from wild-type (wt) and CAR knockout mice were treated with DHEA (25 μ M) or TCPOBOP (10 μ M). Levels of Cyp2b10 mRNA were quantified and normalized to GAPDH as described under *Materials and Methods*. The normalized levels of Cyp2b10 mRNA are expressed as the average $\pm$ S.D. *, significantly different from the controls, $p < 0.05$.

Fig. 5.

The effect of androstanol on Cyp2b10 expression in DHEA- and TCPOBOP-induced mouse hepatocytes. Cyp2b10 of mouse hepatocytes was induced by DHEA (25 μ M) or TCPOBOP (10 μ M) in the presence or absence of the inverse agonist of CAR, androstanol. Levels of Cyp2b10 mRNA were quantified and normalized to GAPDH as described under *Materials and Methods*. The normalized levels of Cyp2b10 mRNA are expressed as the average $\pm$ S.D.

Fig. 6. Subcellular localization of hCAR. Primary mouse hepatocytes were transfected with an expression vector containing GFP fused to human CAR. After transfection, cells were treated with 0.1% DMSO (control), CITCO (100 nM), or DHEA (50 μ M). DAPI staining was used for visualization of nuclei as described under *Materials and Methods*.

TABLE 1

Clinical histories of human donors

Donor	Age	Sex	Race	Cause of Death	Medication
HH-075	yr				
HH-075	63	Male	Caucasian	Subarachnoidal hemorrhage	Noradrenaline
HH-078	47	Female	Caucasian	Subarachnoidal hemorrhage	Dopamine, noradrenaline
HH-079	44	Female	Caucasian	Subarachnoidal hemorrhage	Clindamycin, noradrenaline
HH-080	29	Female	Caucasian	Subarachnoidal hemorrhage	Ceftriaxone, vancomycin
HH-082	44	Male	Caucasian	Stroke	Noradrenaline
HH-083	32	Female	Caucasian	Subdural hemorrhage	
HH-086	39	Male	Caucasian	Cerebral contusion	Ceftriaxone, mannitol, noradrenaline

TABLE 2

Sequences of PCR primers and probes

Primer	Sequence	Probe	Sequence
Human genes			
CYP2B6 Forward	5'-AAAGCGGAGTGTGGAGGA-3'	CYP2B6	FAM-5'-AGGAGGAG-3'-BHQ
Reverse	5'-AAGGTGGGTCCATGAGG-3'		
CYP2C9 Forward	5'-GTGCACGAGGTCCAGAGATAC-3'	CYP2C9	FAM-5'-CTTCTCCC-3'-BHQ
Reverse	5'-CAGGGAAATTAATATGGTTGTGC-3'		
CYP2C19 Forward	5'-	CYP2C19	FAM-5'-CAGCAGGA-3'-BHQ
	TGAAGGTGGAAATTTTAAGAAAAGTAA-3'		
Reverse	5'-CCCTCTCCACACAAATCC-3'		
CYP3A4 Forward	5'-CATGGACTTTTAAAGAAGCTTGG-3'	CYP3A4	FAM-5'-CTCTGCCT-3'-BHQ
Reverse	5'-TTCCATGTCAAACATACAAAAGC-3'		
GAPDH Forward	5'-AGCCACATCGCTCAGACA-3'	GAPDH	FAM-5'-TGGGGAAG-3'-BHQ
Reverse	5'-GCCCAATACGACCAATCC-3'		
Mouse genes			
Cyp2b10 Forward	5'-CAATGTTTAGTGGAGGAAGTGGC-3'	Cyp2b10	FAM-5'-CCCAGGGAGCCCCCTGGA-3'-TAMRA
Reverse	5'-CACTGGAAGAGGAACGTGGG-3'		
GAPDH Forward	5'-CCAGAACATCATCCCTGCATC-3'	GAPDH	FAM-5'-CCGCCTGGAGAAACCTGCCAAGTATG-3'-TAMRA
Reverse	5'-GGTCCTCAGTGTAGCCCAAGAT-3'		

FAM, fluorescein labeling; BHQ, black hole quencher; TAMRA, 5-carboxytetramethylrhodamine-labeled probe.