Detection of oestrogenic activity of steroids present during mammalian gestation using oestrogen receptor α - and oestrogen receptor β -specific *in vitro* assays

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Abstract

Numerous steroid hormones are present in the foetus but their potential to activate oestrogen receptor (ER) α and/or β is largely unknown. In this study, *in vitro* assays were developed to rapidly and specifically detect ER α or ER β activation by these steroid hormones. Our results showed that several oestrogen precursors and androgens are able to activate both ER α and ER β . Of special interest is that some of these precursors are able to activate ER α and ER β at concentrations that are present during human gestation. Moreover, some precursors (dehydroepiandrosterone (DHEA) and 17-hydroxylated pregnenolone sulphate) and androgens (5-androsten-3 β ,16 α ,17 β -triol and testosterone) showed a more than 100-fold relative preference for ER β transactivation over ER α transactivation

when compared with 17 β -oestradiol. Due to their relatively high levels, the precursor steroids DHEA and pregnenolone may be of particular importance in the regulation of ER β activity *in vivo*. To obtain information about the oestrogenic activity of the total pool of steroid hormones present during mammalian gestation, steroids were extracted from mouse embryos at different prenatal stages and assayed for oestrogenic activity in the established *in vitro* assays. Oestrogenic activity was detected in steroid extracts from all stages tested. This study has demonstrated that oestrogen receptor agonists are present in the murine embryo and that oestrogen precursors may contribute to the total pool of agonists during foetal life.

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Introduction

Oestrogens are steroid hormones with numerous target tissues in both males and females, tissues such as the reproductive tract, gonad, mammary gland, bone tissue, cardiovascular system and the central nervous system. Endogenous oestrogens are synthesised from cholesterol via the conversion of various precursors (Fig. 1A). Cholesterol from the diet is converted by p450-side-chain-cleavage enzyme (CYP11A1) to pregnenolone, which then is converted to either 17-hydroxy (OH)-pregnenolone or progesterone. The conversion to 17OH-pregnenolone is mediated by 17 α -hydroxylase (CYP17), while 3 β -hydroxysteroid dehydrogenase (3 β -HSD) and $\Delta^{5,4}$ -ketosteroid isomerase mediate the formation of progesterone. Progesterone can be further hydrolysed to 17OH-progesterone by 17,20 lyase (CYP17). Subsequently, both 17OH-pregnenolone and 17OH-progesterone can serve as a substrate for CYP17 to form dehydroepiandrosterone (DHEA), which is oxidised to androstenedione by 3 β -HSD. Finally, androstenedione is aromatised to oestrone by aromatase (CYP19) or hydro-

lysed by 17 β -hydroxysteroid dehydrogenase (17 β -HSD) to testosterone. Testosterone can be aromatised to 17 β -oestradiol or converted to dihydrotestosterone by 5 α -reductase. This last conversion occurs mainly in males.

During human gestation, the maternal-placental-foetal unit (MPF unit) is the main source of steroid hormones after 8 weeks of gestation (Solomon 1994). However, the human placenta lacks the CYP17 enzyme (Voutilainen & Miller 1986) and placental steroids downstream of the CYP17 conversion steps are therefore derived from either maternal or foetal precursors (Fig. 1B). The foetal-derived precursors for oestrogen formation in the placenta are DHEA-sulphate (DHEAS) and 16OH-DHEAS formed in the foetal liver and adrenal respectively. In the sulphatase-rich placenta (Pasqualini & Kincl 1985), DHEAS and 16OH-DHEAS are desulphated to DHEA and 16OH-DHEA, which are the substrates for 3 β -HSD to form androstenedione and androstetriol. Androstenedione serves as a precursor for both oestrone and 17 β -oestradiol, while androstetriol is a precursor for oestriol. In addition, 16OH-androstenedione formed from 16OH-DHEAS can be metabolised to oestriol (Numazawa *et al.*

Figure 1 Biosynthesis pathway of steroids in the human and presence of steroids in the human maternal-placental-foetal (MPF) unit. (A) Biosynthesis pathway of steroids. Numbers refer to enzymes: (1) p450-side-chain-cleavage enzyme, (2) 3 β -hydroxysteroid dehydrogenase, (3) aromatase, (4) 17,20 lyase, (5) 17 α -hydroxylase, (6) 17 β -hydroxysteroid dehydrogenase, (7) 5 α -reductase. (Adapted from Gore-Langton & Armstrong 1994 and Ojeda 1996.) (B) Steroids in the human maternal-placental-foetal unit. (Adapted from Solomon 1994 and Carr 1996.)

1985). Most of the steroids present in the foetus are sulphated, which is believed to reduce their biological activity (Pasqualini & Kincl 1985).

Mice differ to some degree from humans with regard to steroid hormone synthesis during gestation. The main oestrogen produced during murine gestation is 17 β -oestradiol versus oestriol in humans (Pasqualini & Kincl 1985). While foetal concentrations of steroid precursors have been well studied in humans (Guez *et al.* 1976, Den *et al.* 1979, Mathur *et al.* 1980, de Peretti & Mappus 1983), little is known of their levels in mice. Differences between mice and humans in the activity of the enzymes involved in steroidogenesis have been found; this could lead to a difference in the relative amounts of precursors present in the two species. Furthermore, binding proteins in serum, which may influence the concentration of

hormone that is biologically available, differ in the two species. In mice, the main oestrogen-binding protein during gestation is α -foetoprotein, whereas in humans α -foetoprotein has been shown not to bind oestrogens. In humans, one of the major oestrogen-binding proteins is sex steroid-binding protein which, like α -foetoprotein, is upregulated by oestrogens (reviewed by Pasqualini & Kincl 1985).

The effects of oestrogens are mediated through oestrogen receptors, which are ligand-dependent transcription factors. In mammals, two oestrogen receptors are known to exist, oestrogen receptor (ER) α (Green *et al.* 1986) (NR3A1 according to the Nuclear receptors nomenclature committee (1999)) and ER β (Kuiper *et al.* 1996, Mosselman *et al.* 1996) (NR3A2). Both ER α and ER β are expressed during embryogenesis in many species including

humans (Takeyama *et al.* 2001) and mice (Lemmen *et al.* 1999). However, the function of oestrogens and their receptors during normal development of the foetus remains unclear. When either ER α (Lubahn *et al.* 1993) or ER β (Krege *et al.* 1998) or both receptors (Couse *et al.* 1999, Dupont *et al.* 2000) are knocked out, no gross prenatal phenotypes have been observed in mice, suggesting that oestrogen receptors are not essential for prenatal development. On the other hand, prenatal exposure of animals or humans to the synthetic oestrogen diethylstilboestrol (DES) have been reported to induce abnormalities in various organs (Herbst *et al.* 1971, McLachlan *et al.* 1975). This indicates that prenatally expressed oestrogen receptors are functional with respect to transducing the effects of this synthetic oestrogen. In humans, an increased risk for developing breast and prostate cancer as well as cryptorchidism and low sperm counts has been hypothesised to be linked with exposure to exogenous oestrogens during gestation (Sharpe & Skakkebaek 1993, Miller & Sharpe 1998, Toppari & Skakkebaek 1998). The possible contribution of endogenous steroid hormones to the development of such abnormalities remains unclear (Shibata & Minn 2000).

The present study was carried out in order to elucidate the oestrogenic potential of steroid hormones present in the human MPF unit. For this purpose, novel cell lines stably transfected with the human ER α and ER β together with an oestrogen-responsive reporter construct were made. Steroid hormones and their precursors known to be present in the human MPF unit were tested in the developed cell lines in order to separately determine their ability to activate ER α or ER β . In order to assess the combined oestrogenic activity of the total steroid pool, steroids extracted from mouse embryos were tested in the cell lines. Our results show that several steroids, usually not considered to be oestrogenic, were able to activate ERs, some at physiological levels present in the human foetus. In addition, oestrogenic activity could be detected in steroid extracts from embryonic mice, using the same assay system. These findings confirm the presence of biologically active oestrogens in mammalian embryos.

Materials and Methods

Reagents

Androstenedione (4-androstene-3,17-dione, 98%), 16OH-androstenedione (4-androsten-16 α -ol-3,17-dione), 3 β -androstenediol (5 β -androstane-3 β ,17 β diol), cholesterol (5-cholesten-3 β -ol), DHEA-3-sulphate (5-androsten-3 β -ol-17-one sulphate, 98%), dexamethasone (9 α -fluoro-16 α -methyl-11 β ,17 α ,21-trihydroxy-1,4-pregnadiene-3,20-dione), DES (99%), 5 α -dihydrotestosterone (5 α -androstan-17 β -ol-3-one), 17 β -oestradiol (1,3,5[10]-estatriene-3,17 β -diol, 99%), oestrinol (1,3,5[10]-estatriene-3,16 α ,17 β -triol, 99%), oestrone (1,3,5[10]-estatrien-3-ol-17-one, 99%),

pregnenolone (5-pregnen-3 β -ol-20-one, 98%), progesterone (4-pregnene-3,20-dione), testosterone (4-androsten-17 β -ol-3-one), tamoxifen (1-p- β -dimethylaminoethoxy-phenyl-trans-1,2-diphenyl but-1-ene, 99%) and all-trans retinoic acid were obtained from Sigma-Aldrich (Zwijndrecht, The Netherlands). DHEA (5-androsten-3 β -ol-17-one, 99%) was from Fluka Chemie (Sigma-Aldrich). Pregnenolone sulphate (pregnenolone-S; 5-pregnen-3 β -ol-20-one-sulphate), 17OH-pregnenolone (5-pregnen-3 β ,17-diol-20-one), 17OH-pregnenolone-S (5-pregnen-3 β ,17-diol-20-one-sulphate), 5-androsten-3 β ,16 α ,17 β -triol and 16OH-DHEAS (5-androsten-3 β ,16 α -diol-17-one-sulphate) were obtained from Steraloids Inc. (Newport, RI, USA). ICI 182 780 was a gift from Dr A Wakeling, Zeneca Pharmaceuticals (Cheshire, UK). 2,3,7,8-Tetrachlorodibenzo-p-dioxin was kindly provided by Dr M van den Berg (RITOX, Utrecht, The Netherlands). The synthetic androgen receptor agonist R1881 was a gift from Dr A Brinkman (Erasmus University Rotterdam, The Netherlands). The synthetic progestin Org 2058 was a gift from Dr H J Kloosterboer (NV Organon, Oss, The Netherlands). For the exposure of the cells, all chemicals were diluted in either ethanol (100%; Sigma-Aldrich) or dimethylsulphoxide (DMSO; 99.9%; Acros, Geel, Belgium). The antibiotic hygromycin B was from Roche Molecular Biochemicals (Mannheim, Germany) and neomycin (G 418) was obtained from Life Technologies (Breda, The Netherlands). β -Glucuronidase/sulphatase from *Helix pomatia* was obtained from Sigma-Aldrich.

DNA constructs

The oestrogen-responsive reporter gene construct (3 $\times$ ERE-TATA-Luc) which contains three copies of a consensus oestrogen-response element (ERE) containing oligonucleotide and a TATA box in front of the luciferase cDNA is described in more detail elsewhere (Legler *et al.* 1999). This synthetic construct was chosen since it contained only EREs and reporter transactivation would then be solely via these EREs. If the promoter of a certain oestrogen target gene had been chosen responsive elements other than EREs would have been present and reporter activation could have occurred without oestrogen receptor activation. In stable transfectants, the human ER α expression plasmid pSG5-HEGO (kindly provided by Dr P Chambon, IGBMC, Strasbourg, France) was used. The human ER β full-length plasmid, pSG5-hER β (530 amino acids), was provided by Dr J-Å Gustafsson (Karolinska Institute, Huddinge, Sweden).

Stable cell lines

Human embryonic kidney 293 (293 HEK) cells were obtained from the American Type Culture Collection

(ATCC; Manassas, VA, USA). A stable cell line was made with $3 \times \text{ERE-TATA-Luc}$ cotransfected with a hygromycin B resistance gene (te Riele *et al.* 1990). Subsequently ER α or ER β were transfected in this cell line together with a neomycin resistance gene (Sonneveld *et al.* 1998) using the calcium phosphate precipitation method (Banerji *et al.* 1981). Several clones from ER α and ER β cell lines were tested for their response to 17 β -oestradiol. One clone for 293ER α -ERELuc (clone 35) and one clone for 293ER β -ERELuc (clone 52) were selected for their consequent high response to 17 β -oestradiol and are described further in this paper.

The 293 HEK stable cell lines were cultured in a 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F12 medium (DF; Gibco, Life Technologies, Breda, The Netherlands) supplemented with 7.5% foetal calf serum (Integro, Linz, Austria), 50 $\mu\text{g/ml}$ hygromycin B and 200 $\mu\text{g/ml}$ G418. The cells were rinsed twice with phosphate-buffered saline (PBS) without calcium and magnesium, trypsinised and suspended in phenol red-free DF medium containing 3×10^{-8} M selenite, 10 $\mu\text{g/ml}$ transferrin and 0.2% bovine serum albumin (BSA), supplemented with 5% dextran-coated charcoal-stripped foetal calf serum (DCC-FCS). They were plated in 96-well tissue culture plates (NUNC, Life Technologies, Breda, The Netherlands) (45 000 cells per cm^2) at a volume of 200 μl per well. Forty-eight hours later the medium was refreshed and the compounds to be tested (dissolved in ethanol or DMSO) were added directly to the medium at a 1:1000 dilution. After an additional 24 h, the medium was removed and 50 μl lysis solution was added directly to the wells (1% (v/v) Triton X-100, 2.5×10^{-2} M glycylglycine, 1.5×10^{-2} M MgSO_4 , 4×10^{-3} M EGTA and 1×10^{-3} M dithiothreitol). The luciferase activity of 25 μl of the cell lysates was measured with the lucite luciferase reporter gene assay kit (Recorder no. 6016911; Packard Instruments, Groningen, The Netherlands) according to the manufacturer's instructions using 25 μl lucite solution, using a Topcount liquid scintillation counter (Packard Instruments).

RT-PCR

RT-PCR was carried out as described previously (Quaedackers *et al.* 2001). Briefly, clones of the 293 HEK stable cell lines were cultured in 100 mm dishes and total RNA was isolated using the acid-phenol method (Chomczynski & Sacchi 1987). COS-1 cells (ATCC), transiently transfected with human (h) ER α and hER β , were used as positive controls. cDNA was made with Superscript reverse transcriptase from 1 μg DNase-treated total RNA according to the manufacturer's instructions. Aliquots of 5 μl cDNA and 1 μg DNase-treated total RNA were used in a PCR for hER α , hER β and β -actin. For PCR of hER α , forward primer 5'-GACAAGGGAA

GTATGGCTATGGA-3' and 5'-TTCATCATTTCCC ACTTCGTAGC-3' reverse primer were used. For PCR of hER β , forward primer 5'-TAGTGGTCCATCGCC AGTTAT-3' and reverse primer 5'-GGGAGCCACACT TCACCAT-3' were used. Twenty-five PCR cycles were used (1 min 96 °C, 1 min 55 °C and 1 min 72 °C). All cDNA samples used tested positive in the β -actin PCR (results not shown). The DNase-treated total RNA samples were negative in all three PCR reactions (results not shown).

Determination of receptor number

The receptor number was determined by Scatchard analysis as described previously (Quaedackers *et al.* 2001). Briefly, cells were plated out in 6-well plates (45 000 cells per cm^2) in DF medium with 5% DCC-FCS. Two days later, at approximately 80% confluency, cells were rinsed with PBS and incubated for 1 h in phenol red-free DF medium containing 3×10^{-8} M selenite, 10 $\mu\text{g/ml}$ transferrin and 0.2% BSA with increasing amounts of tritiated 17 β -oestradiol (Amersham, Roosendaal, The Netherlands) with and without 200-fold excess of unlabelled 17 β -oestradiol. A sample of the medium was counted in a liquid scintillation counter to determine the exact exposure concentration of tritiated 17 β -oestradiol. Medium was discarded and the cells were rinsed with PBS and incubated for 1 h in 0.5 M NaOH to lyse the cells. A sample of the cell lysates was counted in a liquid scintillation counter and another sample of the lysate was used for estimation of the protein content using the BioRad protein assay according to the manufacturer's instructions (BioRad, München, Germany). The dissociation constant (K_d) and the number of receptor sites (B_{max}) were calculated by performing a Scatchard analysis, by plotting bound/free 17 β -oestradiol as a function of bound 17 β -oestradiol. The K_d is the concentration of 17 β -oestradiol where 50% of the receptor sites are occupied and was calculated from the slope of the curve ($K_d = -1/\text{slope}$); the B_{max} is given by the x intercept.

Steroid extractions and desulphation

Steroids were extracted from mouse embryos aged 12–16 days post coitum (dpc). Ovary and liver from adult females, as well as embryos treated *in utero* (24 h) with 1 mg DES per mother, were used as positive controls. The method used for steroid extraction was adapted from Maume *et al.* (2001). Briefly, individual embryos (100–800 mg) were homogenised by sonification in 300–1500 μl methanol and extracted twice with an equal amount of chloroform. The organic chloroform fraction was extracted twice with an equal volume of hexane to remove lipids. Acetate buffer (1 ml) was added and

Table 1 Relative transactivation activity of various hormones towards ER α and ER β in 293 cell lines

Ligand	ER α		ER β		Ratio EC25ER β / EC25ER α ^c	Relative preference for ER β in respect to E2 α ^d
	EC25 ^a	Relative potency to E2 ^b	EC25	Relative potency to E2		
5-Androsten-3 β ,16,17 β -triol	4.2×10^{-9} M	8×10^{-5}	1.6×10^{-9} M	2×10^{-2}	0.4	288
5 β -Androstenediol	5.6×10^{-9} M	6×10^{-5}	2.3×10^{-8} M	2×10^{-3}	4.1	28
Androstenedione*	nr	—	3.3×10^{-6} M	1×10^{-5}	—	—
16OH-androstenedione	nr	—	nr	—	—	—
Cholesterol	nr	—	nr	—	—	—
DHEA	2.6×10^{-8} M	1×10^{-5}	1.3×10^{-8} M	3×10^{-3}	0.5	230
DHEAS	1.5×10^{-6} M	2×10^{-7}	3.9×10^{-6} M	1×10^{-5}	2.6	44
16OH-DHEA	2.0×10^{-8} M	2×10^{-5}	3.9×10^{-8} M	1×10^{-3}	2.0	58
Oestradiol	3.4×10^{-13} M	1	3.9×10^{-11} M	1	115	1
Oestrilol	3.7×10^{-12} M	9×10^{-2}	2.1×10^{-11} M	2	5.7	20
Oestrone	7.1×10^{-12} M	5×10^{-2}	7.6×10^{-11} M	5×10^{-1}	10.7	11
Pregnenolone	2.9×10^{-5} M	1.2×10^{-8}	5.4×10^{-5} M	7×10^{-7}	1.9	61
Pregnenolone-S*	7.8×10^{-5} M	4.4×10^{-9}	8.4×10^{-5} M	4×10^{-7}	1.1	105
17OH-pregnenolone*	1.5×10^{-6} M	2×10^{-7}	3.0×10^{-6} M	1×10^{-5}	2	58
17OH-pregnenolone-S*	2.0×10^{-6} M	1×10^{-7}	1.2×10^{-6} M	3×10^{-5}	0.6	192
Progesterone	nr	—	nr	—	—	—
Testosterone	8.1×10^{-5} M	4×10^{-9}	6.0×10^{-5} M	6×10^{-7}	0.7	164
Dihydrotestosterone	3.6×10^{-8} M	9×10^{-6}	1.0×10^{-7} M	4×10^{-4}	2.8	41

^aThe EC25 values were calculated by determining the concentration by which 25% of maximum activity was reached using the sigmoidal fit equation. For test compounds not reaching plateau levels (indicated with *) necessary to calculate the EC25, and EC25 was calculated using the concentration of the test compound eliciting the same level of luciferase induction as the EC25 for 17 β -oestradiol (E2).

^bThe potency relative to E2 is the EC25 of 17 β -oestradiol divided by the EC25 of the tested hormone.

^cThe ratio of the EC25 of the hormone for ER β divided by the EC25 of the hormone for ER α , a number smaller than 1 represents a relative preference for transactivating ER β .

^dThe relative preference for ER β in comparison with 17 β -oestradiol. The EC25ER β /EC25ER α ratio for 17 β -oestradiol divided by the EC25ER β /EC25ER α ratio of the hormone.

nr, not reached.

extracted twice with an equal volume of ethyl acetate. The ethyl acetate fraction containing free steroids was evaporated under an N₂ gas flow and the extract was then transferred to a conical glass vial. The test tube was rinsed with ethyl acetate, which was transferred to the vial and subsequently evaporated under an N₂ gas flow. The extract was dissolved in 10 μ l DMSO. The water fractions remaining after ethyl acetate extraction were stored at -20°C until further analysis. Steroid conjugates in the remaining water phases were deglucuronidated and desulphated overnight using β -glucuronidase/sulphatase from *Helix pomatia* (400U/ml) at 37°C (Legler *et al.* 2002). 17 β -Oestradiol glucuronide (1×10^{-6} M) served as a positive control. The deconjugated water fractions were extracted three times with ethyl acetate, evaporated as above and dissolved in 10 μ l DMSO. The animals used for the extraction were isolated with the approval of the Royal Netherlands Academy of Arts and Sciences Animal Ethics Committee (protocol HL01.04 and HL01.09).

Data analysis

The 17 β -oestradiol curve was fitted using the sigmoidal fit ($y = a_0 + a_1 / (1 + \exp(-(x - a_2)/a_3))$) in Slidewrite Plus for

Windows version 3.0, which determines the fitting coefficients by an iterative process minimizing the c^2 merit function (least squares criterion). The minimal detection limit was calculated as the concentration of 17 β -oestradiol elicited by the solvent control plus three times the standard deviation. The EC25 and EC50 values were calculated by determining the concentration by which 25 or 50% of maximum activity was reached using the sigmoidal fit equation. For test compounds not reaching plateau levels necessary to calculate the EC25, an EC25 was calculated using the concentration of the test compound eliciting the same level of luciferase induction as the EC25 for 17 β -oestradiol. The compounds concerned are indicated with an asterisk in Table 1. The oestradiol equivalents (EEQ) for the steroid extracts were calculated by interpolating the response of the (diluted) extracts in the fitted curve for 17 β -oestradiol determined in the same experiment. Extracts were diluted so that their response fell within the linear portion of the 17 β -oestradiol curve. Averages of the EEQ values of the steroid extracts from different sexes and ages were compared with the two-paired Student's *t*-test. Data shown are the average of at least three independent experiments with each experimental point performed in triplicate.

Results

Development of stable cell lines

In order to evaluate oestrogenic activity of the hormones from the MPF unit, stable cell lines expressing an oestrogen-responsive reporter construct and either hER α or hER β were developed. For this purpose, 293 HEK cells were chosen because they do not express endogenous oestrogen receptors and have been shown to lack 3 β -HSD and 17 β -HSD activity (Suzuki *et al.* 2000). Metabolism of the tested hormones by 3 β - and 17 β -HSD is therefore negligible. In addition, previous results with transient transfection of these receptor/reporter gene constructs showed high luciferase induction of the reporter construct in these cells (Kuiper *et al.* 1998).

For the development of the stable cell lines, 64 antibiotic-resistant clones were isolated for ER α and 288 for ER β . For ER α , 18 clones with luciferase induction exceeding ten times the solvent control following stimulation with 1×10^{-9} M 17 β -oestradiol were selected. For ER β , 58 clones were selected in the same manner. This procedure was repeated on the selected clones. For each receptor, the three highest responding clones (>100-fold for ER α and >50-fold for ER β) were examined in more detail with full 17 β -oestradiol dose-response curves (data not shown). From these three clones, the cell line with the highest fold induction was chosen for further use in this study (clone 35 for ER α (293ER α) and clone 52 for ER β (293ER β)).

In the 96-well-based assay the maximum fold induction by 1×10^{-8} M 17 β -oestradiol for the 293ER α cell line was 41 (coefficient of variation (CV)=14%) and the fold induction of the 293ER β cell line was 342 (CV=11%) in six independent experiments. The eight times higher fold induction in the 293ER β cell line compared with the 293ER α cell line was due to a lower background level of luciferase activity in that cell line. In both cell lines, the response remained stable for more than 20 passages (data not shown).

Stable cell lines with specific oestrogen receptor expression and high sensitivity and selectivity to oestrogens

Expression of oestrogen receptors in the two cell lines was confirmed with RT-PCR (Fig. 2). In the 293ER α cell line, only ER α mRNA and no ER β mRNA could be detected while, in the 293ER β cell line, only ER β mRNA was detected. The amount of receptor per mg protein determined with Scatchard analysis was 206 fM and 161 fM for 293ER α and 293ER β respectively. The sensitivity of the reporter cell lines to the natural ligand 17 β -oestradiol was tested and is shown in Fig. 3. The 293ER α cell line had an EC₅₀ of 1.3×10^{-12} M and a minimal detectable concentration of 1×10^{-13} M 17 β -oestradiol. The 293ER β cell line expression had an EC₅₀

Figure 2 Expression of oestrogen receptor (ER) α and ER β mRNA in stable transfectants; amplified human (h)ER α and hER β cDNA. (A) hER α is present in the 293ER α cell line and not in the 293ER β cell line. (B) hER β is present in the 293ER β cell line and not in the 293ER α cell line. As controls, COS cells were transfected with hER α or hER β . Water and RNA samples amplified in the same PCR run were negative (data not shown).

value of 1.0×10^{-11} M and a minimal detectable concentration of 6×10^{-13} M 17 β -oestradiol. Next, the response of the stable cell lines to different natural hormones or synthetic ligands for specific hormone receptors was tested with and without 17 β -oestradiol. These compounds were chosen because interference between the receptors they activate and oestrogen signalling has been shown previously (Kalkhoven *et al.* 1994, Safe 1995). R1881 (androgen receptor/progesterone receptor agonist), dexamethasone (glucocorticoid receptor agonist) and Org 2058 (progesterone receptor agonist) did not have either agonistic or antagonistic effects on these cell lines (data not shown). All-trans retinoic acid (at-RA) was used to determine interference of retinoid signalling with the oestrogenic response and showed only a slight agonistic effect on ER α with the highest concentration (1×10^{-6} M at-RA). No agonistic activity of at-RA on ER β or

Figure 3 Transactivation of ER α and ER β by oestrogens in stable oestrogen reporter cell lines. Data are presented as a percentage of maximal induction by 17 β -oestradiol. Transactivation of the pERE-TATA-Luc reporter construct in the hER α -containing cell line (293ER α) (●) and transactivation of the reporter construct in the hER β -containing cell line (293ER β) (○). Abscissa: log M of hormone. Ordinate: transcriptional activity as percentage of maximal induction by 17 β -oestradiol for each oestrogen receptor subtype. $n=7$ for 17 β -oestradiol and $n=3$ for oestrone and oestriol; mean $\pm$ S.E.M. is shown.

antagonistic effects on either receptor could be detected. 2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD), a ligand for the aryl-hydrocarbon receptor, did not show agonistic activity towards ER β , but did show a slight agonistic activity towards ER α at the highest concentration tested (1×10^{-8} M). TCDD did not act as an antagonist in the 293ER α or 293ER β cell lines.

Several hormones from the MPF unit show oestrogenic activity, with ER β selectivity

MPF hormones were tested both alone and in the presence of 17 β -oestradiol to test for oestrogenic and anti-oestrogenic activity in the developed stable cell lines. Oestrone and oestriol showed strong activation of both ERs at low concentrations (Table 1 and Fig. 3). Strong activation was observed with the androgens dihydrotestosterone, testosterone, 3 β -androstenediol and 5-androsten-3 β ,16 α ,17 β -triol, albeit at concentrations several orders of magnitude higher than the oestrogens (Table 1 and Fig. 4). Cholesterol, 16OH-androstenedione and progesterone showed no oestrogenic activity (Table 1 and Fig. 5). Very weak oestrogenic activity was observed for androstenedione and 17OH-pregnenolone. Strong transactivation of both oestrogen receptors was observed for DHEAS, pregnenolone, pregnenolone-S and 17OH-pregnenolone-S at high ($>1 \times 10^{-6}$ M) concentrations (Table 1 and Fig. 5). DHEA and 16OH-DHEA showed strong activation of both oestrogen receptors at concentrations below 1×10^{-6} M. None of the hormones present in the MPF unit showed any anti-oestrogenic activity when incubated with the EC₅₀ concentration of 17 β -oestradiol (data not shown). The preference of the hormones for activating either ER α or ER β was evaluated by calculating the ratio of the EC₂₅ of the hormone for ER β divided by the EC₂₅ of the hormone for ER α . A number higher than 1 indicates a preference for activating ER α , while a number smaller than 1 indicates a preference for activating ER β . Surprisingly, most precursor hormones showed a similar preference for activating ER β and ER α . This in contrast to 17 β -oestradiol, oestrone and oestriol, which preferentially activated ER α (Table 1). Also the relative preference of the hormones for either ER α or ER β was compared with the preference of the classic ligand 17 β -oestradiol for these receptors (Table 1). 16OH-DHEA, pregnenolone and 17OH-pregnenolone had a more than 50 times higher relative preference for ER β than 17 β -oestradiol. More than 100 times higher relative preference for ER β was found for 5-androsten-3 β ,16 α ,17 β -triol, DHEA, pregnenolone-S, 17OH-pregnenolone-S and testosterone when compared with 17 β -oestradiol.

Steroid extracts from mouse embryos show oestrogenic activity

To determine the oestrogenic activity of the total pool of steroids present during gestation, steroids were extracted

Figure 4 Transactivation of ER α and ER β by androgens. Values shown are means (bars represent S.E.M.) of three independent experiments done in triplicate. Transactivation of the pERE-TATA-Luc reporter construct in the hER α -containing cell line (293ER α) (●) and transactivation of the reporter construct in the hER β -containing cell line (293ER β) (○). Abscissa: log M of hormone. Ordinate: transcriptional activity as percentage of maximal induction by 17 β -oestradiol for each oestrogen receptor subtype.

from embryonic mice. The steroid and water fractions of mouse embryo extracts were tested for oestrogenic activity in the developed stable cell lines. Oestrogenic activity of the steroid fractions from the positive control samples (i.e. ovary and liver from adult female mice and embryos from DES-treated mice) was detected in both the 293ER α and 293ER β cell lines. This indicated that steroids were extracted and the cell lines were responding to them. When testing the steroid fractions extracted from the embryos in the 293ER β cell line, oestrogenic activity was too low for calculating EEQ values. However, oestrogenic activity of the same steroid fractions was detected in the 293ER α cell line and EEQ values could be calculated. The steroid fractions of 12.5 dpc males had an EEQ value of 3.9×10^{-14} M (CV=7%), whereas the steroid fractions of females of the same age had an EEQ of 3.0×10^{-14} M (CV=17%) per g tissue (Fig. 6). Extracts from males and females aged 14.5 and 16.5 dpc had EEQ/g values $0.9\text{--}1 \times 10^{-14}$ M (CV=15–24%). There was no significant difference in the averages of the EEQ values of the steroid extracts between any of the ages or sexes, determined with a two-paired Student's *t*-test. When a subset of fractions (including the positive control samples) was co-incubated with the anti-oestrogen ICI 182 780 (1×10^{-7} M), no oestrogenic activity could be detected. This indicated that the activation of the reporter construct in the stable cell lines was mediated by the oestrogen receptor (Fig. 7). Adding 17 β -oestradiol to the combination of extracts and ICI 182 780 showed activation of the reporter construct, indicating that the combination of

steroid extract and ICI 182 780 was not toxic for the cells. The water fractions of embryo extracts, possibly containing sulphated steroids, were subsequently desulphated, extracted and tested again for oestrogenic activity. None of the desulphated fractions showed any oestrogenic activity in either of the cell lines. The positive control, 17 β -oestradiol glucuronide, was deconjugated during the incubations (data not shown) indicating that the deconjugation reactions were effective.

Discussion

We found that precursor steroids such as DHEA (–S), 16OH-DHEA, pregnenolone (–S), 17OH-pregnenolone (–S) and androgens like 3 β -androstenediol, 5-androsten-3 β ,16 α ,17 β -triol, dihydrotestosterone and testosterone all stimulated transcriptional activity via both ER α and ER β . Surprisingly, these ligands were more potent ER β activators when compared with the most prominent oestrogen receptor ligand 17 β -oestradiol, which preferentially activates ER α . The activation of ER β by DHEA, pregnenolone and their hydroxylated and sulphated forms is of considerable interest and suggests that these hormones, which circulate at much higher levels than 17 β -oestradiol, could be natural ER β ligands rather than 17 β -oestradiol. DHEA stimulated both oestrogen receptors at 1×10^{-8} M. It is unlikely that this is due to conversion of DHEA to 17 β -oestradiol via either androstenediol or androstenedione, because 3 β -HSD and 17 β -HSD are

Figure 5 Transactivation of ER α and ER β by hormones from the MPF unit. Values shown are means (bars represent S.E.M.) of three independent experiments done in triplicate (for 17OH-pregnenolone, two independent experiments). Transactivation of the pERE-TATA-Luc reporter construct in the hER α -containing cell line (293ER α) (●) and transactivation of the reporter construct in the hER β -containing cell line (293ER β) (○). Abscissa: log M of hormone. Ordinate: transcriptional activity as percentage of maximal induction by 17 β -oestradiol for each oestrogen receptor subtype.

absent from the 293 HEK cells (Suzuki *et al.* 2000). DHEA has been shown to bind both ER α and ER β , albeit at concentrations more than 1000 times higher than 17 β -oestradiol (Kuiper *et al.* 1997). DHEA also activates ER α -mediated transcription in yeast (Nephew *et al.* 1998). In the present study, the 16-hydroxylated form of DHEA was able to transactivate both ER α and ER β , albeit at slightly higher concentrations than DHEA itself. The sulphated form of DHEA was less oestrogenic than DHEA and showed agonistic activity at a concentration of 1×10^{-6} M, which supports the hypothesis that sulphonation reduces the biologic activity of hormones. The data showed that the concentrations at which DHEA, 16OH-DHEA and DHEAS are found in human umbilical cord blood at term (1.3×10^{-6} M, 3.6×10^{-6} M and 5×10^{-6} M respectively; Guez *et al.* 1976, Parker *et al.* 1982, Simmons *et al.* 1994) are sufficient to activate oestrogen receptors. This may suggest that these three

hormones contribute to the total oestrogen exposure of the human foetus.

Pregnenolone and 17OH-pregnenolone and their respective sulphated forms showed oestrogenic activity in both cell lines at micromolar concentrations, with pregnenolone being the most potent ligand. Pregnenolone concentrations, however, only reach about 6×10^{-8} M in cord blood at term (Mathur *et al.* 1980) and it remains to be evaluated whether concentrations in target tissues will reach levels sufficient to activate oestrogen receptors. However, the sulphated form of pregnenolone reaches concentrations of up to 2.5×10^{-5} M in human foetal serum at term (de Peretti & Mappus 1983), which in the present study was sufficient to activate both ER α and ER β . Surprisingly, the sulphated form of 17-hydroxylated pregnenolone was more potent in inducing an oestrogenic response via ER α than the un-sulphated form. We cannot exclude the possibility that pregnenolone and its sulphated

Figure 6 Oestrogenic activity of embryo extracts; oestradiol equivalents (EEQ) (mol/g) following 24-h incubation in the 293ER α cell line of steroid fractions from extracted mouse embryos and positive control samples (ovary and liver from adult female mice; mouse embryos isolated on 12.5 days post coitum (dpc) 24 h after *in utero* DES treatment). m: male; f: female. Ages shown are dpc. Results are average, the CV values are for 12.5 days post coitum (dpc) male 7% ($n=10$) and female 17% ($n=5$), for 14.5 dpc male 15% ($n=9$) and female 24% ($n=7$) and for 16.5 dpc male 17% ($n=6$) and female 21% ($n=8$). For the positive control samples, $n=3$ for adult tissues and $n=2$ for DES-exposed embryos.

and hydroxylated forms are metabolised to DHEA in 293 HEK cells by CYP17. However, the fact that 17OH-pregnenolone, which is only one conversion step from DHEA, is less active than pregnenolone (two steps from DHEA) argues against conversion of these precursors in 293 HEK cells.

Three androgens, 3 β -androstenediol, androstenediol and dihydrotestosterone, showed transactivation of both oestrogen receptors at concentrations around 10^{-8} M. As the only androgen, testosterone showed transactivation at concentrations exceeding 10^{-6} M. Of these androgens, only the concentration of testosterone in the mouse foetus

is known: 1.6×10^{-8} M in males and 0.7×10^{-8} M in females (vom Saal *et al.* 1990). The total concentration of testosterone in human cord blood is $1-2 \times 10^{-8}$ M (Mathur *et al.* 1980, Simmons *et al.* 1994) in both males and females. From our results, these levels seem insufficient to induce oestrogen receptor transactivation.

Interestingly, in this study, the hormones DHEA, 5-androsten-3 β ,16 α ,17 β -triol, testosterone and (17OH)-pregnenolone-S have a similar preference for activating ER β and ER α . This in contrast to 17 β -oestradiol, which preferentially activates ER α . It has been shown that some synthetic oestrogens and naturally existing ligands such as phyto-oestrogens have different relative affinities for ER α versus ER β when compared with 17 β -oestradiol, although most ligands bind both oestrogen receptor subtypes with comparable affinity (Kuiper *et al.* 1998). DHEA and dihydrotestosterone have previously been shown to bind two to three times stronger to ER β than to ER α (Kuiper *et al.* 1997), which partially explains the observed preferential activation of ER β by these hormones when compared with 17 β -oestradiol. DHEA, pregnenolone and their sulphates have been indicated to be involved in neural processes such as memory in the adult (Baulieu 1997). The receptors involved in the direct action of DHEA (-S) and pregnenolone (-S) in the brain are, however, unknown (Baulieu & Robel 1998). The activation of ER α and ER β by these hormones could indicate that these processes are mediated by oestrogen receptors. In particular, ER β , which is prominently expressed in several important brain regions, is a likely candidate. The relatively higher affinity of DHEA for ER β is also interesting in the light of co-expression of sulphatases (Compagnone *et al.* 1997) and ER β at ossification sites in the mouse embryo (Lemmen *et al.* 1999). Local conversion of DHEAS to DHEA could therefore activate ER β in these ossification centres. Recently, it was found that ER β is highly expressed in human foetal tissues at 13 and 20 weeks of gestation (Takeyama *et al.* 2001). In particular, the adrenal gland, where DHEAS is produced, was found to be a major site of ER β expression in humans. ER α was barely detectable during this period, suggesting that ER β would be the main effector of oestrogen signalling in humans at this period of gestation.

Given the ability of MPF steroids to activate oestrogen receptors, we were interested in the oestrogenic activity of hormones present in the foetus. In steroid extracts from mouse embryos of different ages, the highest activity, although not significant, was found at 12.5 dpc. If the measured oestrogenic activity derived from 17 β -oestradiol or testosterone and their precursors, the peak of oestrogenic activity at 12.5 dpc seems to contradict the peak at 17.5 dpc of either 17 β -oestradiol in the maternal serum (Barkley *et al.* 1977) or testosterone in foetuses (Weisz & Ward 1980). However, data from the foetal rabbit showed that, although 17 β -oestradiol production increases during pregnancy, a drop is observed when the 17 β -oestradiol

Figure 7 Luciferase induction of steroid extracts is mediated by ER α . Luciferase induction after 24-h incubation of the steroid fractions (pooled for each age and sex) alone and in combination with the anti-oestrogen ICI 182 780 (ICI; 10^{-8} M) or in combination with ICI and 17 β -oestradiol (E2; 10^{-9} M) in the 293ER α cell line. m: male; f: female. Ages shown are dpc. Ordinate: transcriptional activity as percentage of maximal induction by 17 β -oestradiol for each oestrogen receptor subtype.

concentrations are plotted per mg gonadal protein (George & Wilson 1978). This suggests that the increase in gonadal growth counteracts the effect of a rise in 17β -oestradiol production and consequently the net amount of 17β -oestradiol per mg protein drops. A similar effect could occur when the EEQs are corrected for total body weight, as was done in the present experiments. The activation profile of the extracts, capable only of activating the 293ER α cell line and not the 293ER β cell line, suggested activation by a hormone that preferentially activates ER α . Based on the activation profiles identified, this would suggest that the main contribution to the total pool of oestrogenic activity is not by precursors like DHEA but rather by classical oestrogens. Since aromatase activity is present in mammalian foetal gonads of both sexes (Weniger 1990) as well as in the human foetal liver (Schindler 1975), both male and female fetuses are capable of producing oestrogens from precursors. It should be noted that the cell lines used in the present study contained human oestrogen receptors and a slight difference of the extracts to activate murine oestrogen receptors cannot be ruled out. However, studies by Matthews *et al.* (2000) showed that human and mouse ER α react in a very similar manner to steroidal and anti-oestrogenic compounds. We therefore expect that differences between activation of human versus murine oestrogen receptors by the steroid hormones tested will be quantitative rather than qualitative.

In conclusion, DHEA, DHEAS and pregnenolone-S were able to activate ER α and ER β at physiological concentrations. Interestingly, precursor steroids and androgens showed a relative preference for ER β transactivation over ER α transactivation when compared with transactivation by 17β -oestradiol and other oestrogens. Extracts from mouse embryos contained detectable oestrogenic activity, suggesting that endogenous steroids present in the murine embryo are capable of activating oestrogen receptors. The oestrogen precursors examined could contribute to the total pool of endogenous oestrogens during foetal life. These precursors could also be involved in the hypothesised link between exposure to high endogenous oestrogens and breast and prostate cancer or cryptorchidism.

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