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

J G Lemmen, C E van den Brink, J Legler, P T van der Saag and B van der Burg

Hubrecht Laboratory, Netherlands Institute for Developmental Biology, Uppsalalaan 8, 3584 CT Utrecht, The Netherlands

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-3β,16α,17β-triol and testosterone) showed a more than 100-fold relative preference for ER β transactivation over ER α transactivation when compared with 17β-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.


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 17α-hydroxylase (CYP17), while 3β-hydroxysteroid dehydrogenase (3β-HSD) and Δ5,4-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 3β-HSD. Finally, androstenedione is aromatised to oestrone by aromatase (CYP19) or hydrolysed by 17β-hydroxysteroid dehydrogenase (17β-HSD) to testosterone. Testosterone can be aromatised to 17β-oestradiol or converted to dihydrotestosterone by 5α-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 3β-HSD to form androstenedione and androstenetriol. Androstenedione serves as a precursor for both oestrone and 17β-oestradiol, while androstenetriol is a precursor for oestradiol. In addition, 16OH-androstenedione formed from 16OH-DHEAS can be metabolised to oestriol (Numazawa et al.
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 17β-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 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 ERβ (Kuiper et al. 1996, Mosselman et al. 1996) (NR3A2). Both ERα and ERβ are expressed during embryogenesis in many species including...

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) 3β-hydroxysteroid dehydrogenase, (3) aromatase, (4) 17,20 lyase, (5) 17α-hydroxylase, (6) 17β-hydroxysteroid dehydrogenase, (7) 5α-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.)
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 ERα and ERβ 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 human MPF unit. For this purpose, novel cell lines (Shibata & Minn 2000). The possible contribution of endogenous steroid hormones to the development of such abnormalities remains unclear (Shibata & Minn 2000).

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Materials and Methods

Reagents

Androstenedione (4-androstene-3,17-dione, 98%), 16OH-androstenedione (4-androstene-16α-ol-3,17-dione), 3β-androstanediol (5β-androstan-3β,17β-diol), cholesterol (5-cholesten-3β-ol), DHEA-3-sulphate (5-androsten-3β-ol-17 one sulphate, 98%), dexamethasone (9α-fluoro-16α-methyl-11β,17α,21-trihydroxy-1,4-pregnadiene-3,20-dione), DES (99%), 5α-dihydrotestosterone (5α-androstane-17β-ol-3-one), 17β-oestradiol (1,3,5[10]-estriene-3,17β-diol, 99%), oestradiol (1,3,5[10]-estriene-3,17α,17β-triol, 99%), oestrone (1,3,5[10]-estriene-3-ol-17-one, 99%), pregnenolone (5-pregnen-3β-ol-20-one, 98%), progesterone (4-pregnen-3,20-dione), testosterone (4-androsten-17β-ol-3-one), tamoxifen (1-p-β-dimethy lamino-ethoxy-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-3β-ol-17-one, 99%) was from Fluka Chemie (Sigma-Aldrich). Pregnenolone sulphate (pregnenolone-S; 5-pregnen-3β-ol-20-one-sulphate), 17OH-pregnenolone (5-pregnen-3β,17-diol-20-one), 17OH-pregnenolone-S (5-pregnen-3β,17-diol-20-one-sulphate), 5-androsten-3β,16α,17β-triol and 16OH-DHEAS (5-androsten-3β,16α-diol-17-one-sulphate) were obtained from Steraloids Inc. (Newport, RI, USA). CI 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 HJ 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 × 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 ERs and reporter transactivation would then be solely via these ERs. If the promoter of a certain oestrogen target gene had been chosen responsive elements other than ERs would have been present and reporter activation could have occurred without oestrogen receptor activation. In stable transfectants, the human ERα expression plasmid pSG5-HEGO (kindly provided by Dr P Chambon, IGBMC, Strasbourg, France) was used. The human ERβ full-length plasmid, pSG5-hERβ (530 amino acids), was provided by Dr J-A Gustafsson (Karolinska Institute, Huddinge, Sweden).

Stable cell lines

Human embryonic kidney 293 (293 HEK) cells were obtained from the American Type Culture Collection
A stable cell line was made with $3 \times 10^8$ ERE-TATA-Luc cotransfected with a hygromycin B resistance gene (te Riele et al. 1990). Subsequently ERα or ERβ 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 ERα and ERβ cell lines were tested for their response to 17β-oestradiol. One clone for 293ERα-ERE-Luc (clone 35) and one clone for 293ERβ-ERE-Luc (clone 52) were selected for their consequent high response to 17β-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), 50µg/ml hygromycin B and 200 µg/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 $3 \times 10^{-8}$ M selenite, 10 µg/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 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) ERα and hERβ, 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 hERα, hERβ and β-actin. For PCR of hERα, forward primer 5’-GACAAGGGAA GTATGGCTATGGA-3’ and 5’-TTCATCATTCGCC ACTTCCGTAGC-3’ reverse primer were used. For PCR of hERβ, forward primer 5’-TAGTGTCATCGCC ACTTAT-3’ and reverse primer 5’-GGAGGCCACACT 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 $3 \times 10^{-8}$ M selenite, 10 µg/ml transferrin and 0·2% BSA with increasing amounts of tritiated 17β-oestradiol (Amersham, Roosendaal, The Netherlands) with and without 200-fold excess of unlabelled 17β-oestradiol. A sample of the medium was counted in a liquid scintillation counter to determine the exact exposure concentration of tritiated 17β-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 17β-oestradiol as a function of bound 17β-oestradiol. The $K_d$ is the concentration of 17β-oestradiol where 50% of the receptor sites are occupied and was calculated from the slope of the curve ($K_d = -1/$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...
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 µl 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). 17β-Oestradiol glucuronide (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 µl 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 17β-oestradiol curve was fitted using the sigmoidal fit (y=a₂+a₁/1+exp(-(x-a₂)/a₃)) in Slidewrite Plus for Windows version 3.0, which determines the fitting coefficients by an iterative process minimizing the c² merit function (least squares criterion). The minimal detection limit was calculated as the concentration of 17β-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 17β-oestradiol (E2).

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

The ratio of the EC25 of the hormone for ERβ divided by the EC25 of the hormone for ERα, a number smaller than 1 represents a relative preference for transactivating ERβ.

The relative preference for ERβ in comparison with 17β-oestradiol. The EC25ERβ/EC25ERα ratio for 17β-oestradiol divided by the EC25ERβ/EC25ERα ratio of the hormone. nr, not reached.

### Table 1 Relative transactivation activity of various hormones towards ERα and ERβ in 293 cell lines

<table>
<thead>
<tr>
<th>Ligand</th>
<th>ERα EC25a</th>
<th>Relative potency to E2b</th>
<th>ERβ EC25</th>
<th>Relative potency to E2</th>
<th>Ratio EC25ERβ/EC25ERαc</th>
<th>Relative preference for ERβ in respect to E2a4</th>
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</thead>
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<tr>
<td>5-Androsten-3β,16,17β-triol</td>
<td>4·2 × 10⁻⁹ M</td>
<td>8 × 10⁻⁵</td>
<td>1·6 × 10⁻⁹ M</td>
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<td>288</td>
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<td>6 × 10⁻⁵</td>
<td>2·3 × 10⁻⁸ M</td>
<td>2 × 10⁻₃</td>
<td>4·1</td>
<td>28</td>
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<td>Androstenedione*</td>
<td>nr</td>
<td>—</td>
<td>3·3 × 10⁻⁶ M</td>
<td>1 × 10⁻⁵</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>16OH-androstenedione*</td>
<td>nr</td>
<td>—</td>
<td>nr</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>nr</td>
<td>—</td>
<td>nr</td>
<td>—</td>
<td>—</td>
<td>—</td>
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<tr>
<td>DHEA</td>
<td>2·6 × 10⁻⁸ M</td>
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<tr>
<td>DHEAS</td>
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<td>1 × 10⁻⁵</td>
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<td>16OH-DHEA</td>
<td>2·0 × 10⁻⁸ M</td>
<td>2 × 10⁻⁷</td>
<td>3·9 × 10⁻⁸ M</td>
<td>1 × 10⁻³</td>
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<td>115</td>
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<td>9 × 10⁻²</td>
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<td>1</td>
<td>5·7</td>
<td>20</td>
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<tr>
<td>Oestrone</td>
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<td>5 × 10⁻²</td>
<td>7·6 × 10⁻¹⁻ M</td>
<td>5 × 10⁻¹</td>
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<td>7 × 10⁻⁷</td>
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<td>1 × 10⁻⁵</td>
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<td>58</td>
</tr>
<tr>
<td>17OH-pregnenolone-S*</td>
<td>2·0 × 10⁻⁶ M</td>
<td>1 × 10⁻⁷</td>
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<td>3 × 10⁻⁵</td>
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<td>192</td>
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<tr>
<td>Testosterone</td>
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<td>4 × 10⁻⁹</td>
<td>6·0 × 10⁻⁵ M</td>
<td>6 × 10⁻⁷</td>
<td>0·7</td>
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<td>1·0 × 10⁻⁷ M</td>
<td>4 × 10⁻⁴</td>
<td>2·8</td>
<td>41</td>
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</table>
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 hERα or hERβ were developed. For this purpose, 293 HEK cells were chosen because they do not express endogenous oestrogen receptors and have been shown to lack 3β-HSD and 17β-HSD activity (Suzuki et al. 2000). Metabolism of the tested hormones by 3β- and 17β-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 ERα and 288 for ERβ. For ERα, 18 clones with luciferase induction exceeding ten times the solvent control following stimulation with 1 × 10⁻⁹ M 17β-oestradiol were selected. For ERβ, 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 ERα and >50-fold for ERβ) were examined in more detail with full 17β-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 ERα (293ERα) and clone 52 for ERβ (293ERβ)).

In the 96-well-based assay the maximum fold induction by 1 × 10⁻⁹ M 17β-oestradiol for the 293ERα cell line was 41 (coefficient of variation (CV)=14%) and the fold induction of the 293ERβ cell line was 342 (CV=11%) in six independent experiments. The eight times higher fold induction in the 293ERβ cell line compared with the 293ERα 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 293ERα cell line, only ERα mRNA and no ERβ mRNA could be detected while, in the 293ERβ cell line, only ERβ mRNA was detected. The amount of receptor per mg protein determined with Scatchard analysis was 206 fm and 161 fm for 293ERα and 293ERβ respectively. The sensitivity of the reporter cell lines to the natural ligand 17β-oestradiol was tested and is shown in Fig. 3. The 293ERα cell line had an EC50 of 1.3 × 10⁻¹² M and a minimal detectable concentration of 1 × 10⁻¹³ M 17β-oestradiol. The 293ERβ cell line expression had an EC50 value of 1.0 × 10⁻¹¹ M and a minimal detectable concentration of 6 × 10⁻¹³ M 17β-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 17β-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 ERα with the highest concentration (1 × 10⁻⁶ M at-RA). No agonistic activity of at-RA on ERβ or
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 ERβ, but did show a slight agonistic activity towards ERα at the highest concentration tested (1 × 10−8 M). TCDD did not act as an antagonist in the 293ERα or 293ERβ cell lines.

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

MPF hormones were tested both alone and in the presence of 17β-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, 3β-androstanediol and 5-androsten-3β,16α,17β-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 × 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 17β-oestradiol (data not shown). The preference of the hormones for activating either ERα or ERβ was evaluated by calculating the ratio of the EC25 of the hormone for ERβ divided by the EC25 of the hormone for ERα. A number higher than 1 indicates a preference for activating ERβ, while a number smaller than 1 indicates a preference for activating ERα. Surprisingly, most precursor hormones showed a similar preference for activating ERβ and ERα. This in contrast to 17β-oestradiol, oestrone and oestriol, which preferentially activated ERα (Table 1). Also the relative preference of the hormones for either ERα or ERβ was compared with the preference of the classic ligand 17β-oestradiol for these receptors (Table 1). 16OH-DHEA, pregnenolone and 17OH-pregnenolone had a more than 50 times higher relative preference for ERβ than 17β-oestradiol. More than 100 times higher relative preference for ERβ was found for 5-androsten-3β,16α,17β-triol, DHEA, pregnenolone-S, 17OH-pregnenolone-S and testosterone when compared with 17β-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...
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 293ERα and 293ERβ 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 293ERβ cell line, oestrogenic activity was too low for calculating EEQ values. However, oestrogenic activity of the same steroid fractions was detected in the 293ERα cell line and EEQ values could be calculated. The steroid fractions of 12.5 dpc males had an EEQ value of 3.9 × 10⁻¹⁰ 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 0.9–1 × 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 17β-oestradiol to the combination of extracts 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, 17β-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 3β-androstenediol, 5-androsten-3β,16α,17β-triol, dihydrotestosterone and testosterone all stimulated transcriptional activity via both ERα and ERβ. Surprisingly, these ligands were more potent ERβ activators when compared with the most prominent oestrogen receptor ligand 17β-oestradiol, which preferentially activates ERα. The activation of ERβ 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 17β-oestradiol, could be natural ERβ ligands rather than 17β-oestradiol. DHEA stimulated both oestrogen receptors at 1 × 10⁻⁹ M. It is unlikely that this is due to conversion of DHEA to 17β-oestradiol via either androstenediol or androstenedione, because 3β-HSD and 17β-HSD are not expressed in the placenta.
absent from the 293 HEK cells (Suzuki et al. 2000). DHEA has been shown to bind both ERα and ERβ, albeit at concentrations more than 1000 times higher than 17β-oestradiol (Kuiper et al. 1997). DHEA also activates ERα-mediated transcription in yeast (Nephew et al. 1998). In the present study, the 16-hydroxylated form of DHEA was able to transactivate both ERα and ERβ, 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 \(1 \times 10^{-6}\) M, which supports the hypothesis that sulphation 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 \(\times\) \(10^{-6}\) M, 3.6 \(\times\) \(10^{-6}\) M and 5 \(\times\) \(10^{-6}\) 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 \(6 \times 10^{-8}\) 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 \(2.5 \times 10^{-5}\) M in human foetal serum at term (de Peretti & Mappus 1983), which in the present study was sufficient to activate both ERα and ERβ. Surprisingly, the sulphated form of 17-hydroxylated pregnenolone was more potent in inducing an oestrogenic response via ERα than the un-sulphated form. We cannot exclude the possibility that pregnenolone and its sulphated

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**Figure 5** Transactivation of ERα and ERβ 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 hERα-containing cell line (293ERα) (●) and transactivation of the reporter construct in the hERβ-containing cell line (293ERβ) (○). Abscissa: log M of hormone. Ordinate: transcriptional activity as percentage of maximal induction by 17β-oestradiol for each oestrogen receptor subtype.
and hydroxylated forms are metabolised to DHEA in 293 HEK cells by CYP17. However, the fact that 17OHPregnenolone, 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, 3β-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

![Figure 6](image6.png)

Figure 6 Oestrogenic activity of embryo extracts; oestradiol equivalents (EEQ) (mol/g) following 24-h incubation in the 293ERα 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.

is known: 1.6 x 10^{-8} M in males and 0.7 x 10^{-8} M in females (vom Saal et al. 1990). The total concentration of testosterone in human cord blood is 1–2 x 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-3β,16α,17β-triol, testosterone and (17OHPregnienolone-S have a similar preference for activating ERβ and ERα. This in contrast to 17β-oestradiol, which preferentially activates ERα. It has been shown that some synthetic oestrogens and naturally existing ligands such as phyto-oestrogens have different relative affinities for ERα versus ERβ when compared with 17β-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 ERβ than to ERα (Kuiper et al. 1997), which partially explains the observed preferential activation of ERβ by these hormones when compared with 17β-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 ERα and ERβ by these hormones could indicate that these processes are mediated by oestrogen receptors. In particular, ERβ, which is prominently expressed in several important brain regions, is a likely candidate. The relatively higher affinity of DHEA for ERβ is also interesting in the light of co-expression of sulphatases (Compagnone et al. 1997) and ERβ at ossification sites in the mouse embryo (Lemmen et al. 1999). Local conversion of DHEAS to DHEA could therefore activate ERβ in these ossification centres. Recently, it was found that ERβ 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 ERβ expression in humans. ERα was barely detectable during this period, suggesting that ERβ 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 17β-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 17β-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 17β-oestradiol production increases during pregnancy, a drop is observed when the 17β-oestradiol

![Figure 7](image7.png)

Figure 7 Luciferase induction of steroid extracts is mediated by ERα. 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 17β-oestradiol (E2; 10^{-7} M) in the 293ERα cell line. m: male; f: female. Ages shown are dpc. Ordinate: transcriptional activity as percentage of maximal induction by 17β-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 293ERα cell line and not the 293ERβ cell line, suggested activation by a hormone that preferentially activates ERα. 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 foetuses 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 ERα 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 ERα and ERβ at physiological concentrations. Interestingly, precursor steroids and androgens showed a relative preference for ERβ transactivation over ERα 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 oestrogenic chemicals and phytoestrogens with estrogen receptor and reproductive phenotypes of mice lacking estrogen receptor beta. PNAS 95 15677–15682.


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