Toxicology and Applied Pharmacology 260 (2012) 232–240

Contents lists available at SciVerse ScienceDirect

Toxicology and Applied Pharmacology

journal homepage: www.elsevier.com/locate/ytaap

Potential hazards to embryo implantation: A human endometrial *in vitro* model to identify unwanted antigestagenic actions of chemicals

L. Fischer [a], W.R. Deppert [a], D. Pfeifer [c], S. Stanzel [b], M. Weimer [b], A. Hanjalic-Beck [a], A. Stein [a], M. Straßer [a], H.P. Zahradnik [a], W.R. Schaefer [a,*]

[a] Department of Obstetrics & Gynecology, University Hospital Freiburg, Germany
[b] Department of Biostatistics, German Cancer Research Center, Heidelberg, Germany
[c] Department of Hematology and Oncology, University Hospital Freiburg, Germany

ARTICLE INFO

Article history:
Received 26 January 2012
Revised 22 February 2012
Accepted 26 February 2012
Available online 6 March 2012

Keywords:
Reproduction
Fertility
Endometrium
Progesterone receptor
Endocrine disruptors
Selective progesterone receptor modulators (SPRM)

ABSTRACT

Embryo implantation is a crucial step in human reproduction and depends on the timely development of a receptive endometrium. The human endometrium is unique among adult tissues due to its dynamic alterations during each menstrual cycle. It hosts the implantation process which is governed by progesterone, whereas 17β-estradiol regulates the preceding proliferation of the endometrium. The receptors for both steroids are targets for drugs and endocrine disrupting chemicals. Chemicals with unwanted antigestagenic actions are potentially hazardous to embryo implantation since many pharmaceutical antiprogestins adversely affect endometrial receptivity. This risk can be addressed by human tissue-specific *in vitro* assays. As working basis we compiled data on chemicals interacting with the PR. In our experimental work, we developed a flexible *in vitro* model based on human endometrial Ishikawa cells. Effects of antiprogestin compounds on pre-selected target genes were characterized by sigmoidal concentration–response curves obtained by RT-qPCR. The estrogen sulfotransferase (SULT1E1) was identified as the most responsive target gene by microarray analysis. The agonistic effect of progesterone on SULT1E1 mRNA was concentration-dependently antagonized by RU486 (mifepristone) and ZK137316 and, with lower potency, by 4-nonylphenol, bisphenol A and apigenin. The negative control methyl acetoacetate showed no effect. The effects of progesterone and RU486 were confirmed on the protein level by Western blotting. We demonstrated proof of principle that our Ishikawa model is suitable to study quantitatively effects of antiprogestin-like chemicals on endometrial target genes in comparison to pharmaceutical reference compounds. This test is useful for hazard identification and may contribute to reduce animal studies.

Introduction

Embryo implantation is a crucial step in human reproduction which has not yet been specifically addressed as target of reproductive toxicity. However, it is well known from pharmacology and clinical practice that the implantation process can be interrupted by progesterone receptor antagonists and selective progesterone receptor modulators (SPRMs). Some of these drugs are approved for emergency contraception and abort induction. The mode of action in preventing pregnancy are inhibition or delay of ovulation as well as effects on the endometrium (Baird et al., 2003; Chwalisz et al., 2002; Katkam et al., 1995; Lakha et al., 2007; Spitz, 2009). In this context the endometrium was recognized to be an important fertility-determining factor (Strowitzki et al., 2006) and to be more sensitive to antiprogestins than the hypothalamic–ovarian axis. Based on these findings the concept of endometrial contraception has evolved (Chwalisz et al., 2002; Nayak et al., 2007; Puri et al., 2000).

The human endometrium is unique among adult tissues. It undergoes complex dynamic changes during each menstrual cycle under the control of 17β-estradiol and progesterone. The progesterone receptor plays a crucial role in endometrial function and is an important target for pharmacological agents, particularly in contraception, uterine pathologies, hormone replacement therapy and therapeutic abortions. Remarkably, also non-steroidal drug candidates, environmental chemicals and natural compounds can

Abbreviations: ALPPL2, alkaline phosphatase placental-like 2; BCA, bicinchoninic acid; BPA, bisphenol A; C_q, quantification cycle; DES, diethylstilbestrol; DMSO, dimethylsulfoxide; E2, 17ß-estradiol; EC50, half maximal effective concentration; EDC, endocrine disrupting chemicals; ER, estrogen receptor; EtOH, ethanol; FBS, fetal bovine serum; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; G0S2, G0/G1switch gene; G6PDH, glucose-6-phosphate dehydrogenase; MAA, methyl acetoacetate; MEM, minimum essential medium; 4-NP, 4-nonylphenol; PCR, polymerase chain reaction; P4, progesterone; PR, progesterone receptor; RIN, RNA integrity number; RT-qPCR, reverse transcription quantitative real-time PCR; RU486, mifepristone; SPRM, selective progesterone receptor modulator; SULT1E1, estrogen sulfotransferase; UPL, Universal Probe Library.

* Corresponding author at: Department of Obstetrics and Gynecology (Endocrinology Laboratory), University Hospital Freiburg, Hugstetter Str. 55, D-79106 Freiburg, Germany. Fax: +49 761 270 29800.
E-mail address: wolfgang.schaefer@uniklinik-freiburg.de (W.R. Schaefer).

0041-008X/$ – see front matter © 2012 Elsevier Inc. All rights reserved.
doi:10.1016/j.taap.2012.02.016

act as PR ligands (Klotz et al., 1997a, 1997b; Madauss et al., 2007; Scippo et al., 2004; Tran et al., 1996; Willemsen et al., 2004). Many plant preparations have been traditionally used for fertility regulation and abort induction. For some of them an anti-implantation activity has been demonstrated (Edwin et al., 2009; Pakrashi and Shaha, 1977; Vasudeva and Sharma, 2008). Therefore the PR must be addressed as target for endocrine disrupting chemicals besides the estrogen receptors and thus should be included in basic research strategies identifying pathways for EDC impact on endocrine tissues (Diamanti-Kandarakis et al., 2009).

However, since embryo implantation in humans is different to animal species chemical risk assessment in this field cannot be based solely on animal studies (Bremer et al., 2007). New human *in vitro* models are therefore needed to assess endometrial effects for chemical-safety testing and drug development.

In our study the human endometrial Ishikawa cell line, widely accepted for analysis of endocrine effects (Boehme et al., 2009; Hannan et al., 2010; Lessey et al., 1996; Naciff et al., 2009, 2010), was used as model system. The described test for evaluation of the effects of antiprogestin compounds on the endometrium is based on a recently developed *in vitro* assay for detection of estrogenic effects by RT-qPCR (Schaefer et al., 2010).

Methods

Ishikawa cell culture. Human endometrial epithelial adenocarcinoma Ishikawa cells (ECACC, Salisbury, United Kingdom) were cultured in minimum essential medium with Earle's salts and without phenol red (Invitrogen, Karlsruhe, Germany), supplemented with 2 mM L-glutamine, 1% non-essential amino acids and 5% charcoal-stripped FBS (all from Invitrogen) in 6 well tissue culture test plates (TPP, Trasadingen, Switzerland) at 37 °C under 5% CO_2. For standard tests of chemicals cells were seeded at a density of 50,000 cells/ml and grown for 3 days to subconfluency prior to addition of test substances. The medium of this priming phase was supplemented with 17β-estradiol (10^{-7} M). For antiprogestin testing, cells were subsequently coincubated in duplicates for 48 h. The test compounds diethylstilbestrol (CAS No. 56-53-1), RU486 (mifepristone, CAS No. 84371-65-3), 4-nonylphenol (technical grade, CAS No. 84852-15-3), bisphenol A (CAS No. 80-05-7), methyl acetoacetate (CAS No. 105-45-3), apigenin (CAS No. 520-36-5) as well as 17β-estradiol (CAS No. 50-28-2) and progesterone (CAS No. 57-83-0) were obtained from Sigma-Aldrich (Taufkirchen, Germany) and applied with 0.1% ethanol or 0.1% DMSO as vehicle. ZK137316 was kindly provided by Bayer-Schering (Berlin, Germany). For this study, 4-NP, BPA and apigenin were selected as exemplary test compounds for environmental chemicals with putative antigestagenic action from the compounds listed in Table 3. DES, RU486, 4-NP, BPA and MAA were also used as appropriate reference compounds for *in vitro* test development in the *ReProTect* project (Pazos et al., 2010). Apigenin is a plant compound whose interaction with the PR was demonstrated previously (Scippo et al., 2004; Willemsen et al., 2004). The tested concentrations were adjusted as appropriate to characterize the concentration–response curves (Schenk et al., 2010). None of the tested compounds was cytotoxic in the AlamarBlue assay at concentrations up to 10^{-5} M (Schaefer et al., 2010).

RNA isolation and cDNA preparation. Cell lysis of Ishikawa cells was directly performed with TRK lysis buffer from E.Z.N.A.® Total RNA Kit (VWR International, Darmstadt, Germany). Duplicates were combined and total RNA was extracted by using HiBind RNA mini columns (VWR International) with on-column DNase digestion (Qiagen, Hilden, Germany). Total RNA was quantified by measurement of absorbance at 260 (A_{260}) and 280 nm (A_{280}) in a NanoDrop spectrophotometer (PeqLab, Erlangen, Germany). RNA concentrations were > 500 ng/μl. The A_{260}/A_{280} absorbance ratio was between 1.9 and 2.1. The quality of total RNA was further controlled by microcapillary electrophoresis on RNA 6000 Nanochips using a Bioanalyzer 2100 (Agilent Technologies, Böblingen, Germany) and assessed as acceptable when RIN was >8.

cDNA synthesis from total RNA (1 μg) was carried out in a reaction volume of 20 μl containing 50 mM Tris–HCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$, 5 mM dithiothreitol, 0.5 mM deoxynucleoside triphosphate mix, 200 units SuperScript™ III reverse transcriptase (all from Invitrogen) and 1 μl random primers (hexanucleotide mix, 10x; Roche Applied Science, Mannheim, Germany). Initially, RNA was denatured at 65 °C for 5 min, then the reaction mixture was added and reverse transcription was performed at 50 °C for 50 min. The reaction was stopped by denaturing the enzyme at 70 °C for 15 min. The cDNA was stored at −20 °C.

Affymetrix GeneChip microarray. For microarray analysis, carried out to identify the most regulated genes under antiprogestin treatment, three independent Ishikawa experiments were performed. In each experiment one duplicate of the cells was treated with P4 alone (10^{-8} M) and another duplicate of the same cells with P4/RU486 (10^{-8} M, each; cells seeded at a density of 15,000 cells/ml). Total RNA was isolated as described above and characterized by capillary electrophoresis using an Agilent 2100 Bioanalyzer (Agilent Technologies). Synthesis of sense cDNA and labeling were performed with 500 ng of total RNA by using Affymetrix (High Wycombe, Great Britain) and Ambion (Kassel, Germany) kits and protocols. Labeled and fragmented cDNA was hybridized to the Affymetrix Human Gene ST Array 1.0. Data analysis was performed using *Genedata Expressionist* Refiner and Analyst Software (Genedata, München, Germany). Genes that are differentially regulated between the two groups P4/RU486 and P4 alone were identified by applying paired Bayes-t-test (Baldi and Long, 2001). False-discovery rate was estimated by the Benjamini–Hochberg q-value (Benjamini and Hochberg, 1995), and the median fold-change between P4/RU486 and P4 was calculated using the N-fold-change activity in Analyst.

RT-qPCR analysis. RT-qPCR analysis was performed in 384 well plates on a LightCycler® 480 instrument with LightCycler® 480 Probes Master Mix (Roche Applied Science). In this study, we used assays from the Universal Probe Library (Roche) for four target and one reference genes (Table 1). UPL assays are based on small probes and allow the detection of approximately 99% of the human transcripts with a set of 90 probes. The other target genes besides PR were selected from a microarray study on estrogen effects in Ishikawa cells (Boehme et al., 2009) and own microarray experiments (see Section 2.3). G6PDH was identified among eight pre-selected reference genes as appropriate reference gene for the described experimental setting by using a systematic, software-based approach (NormFinder, geNORM). Primers (200 nM, TIB MOLBIOL, Berlin, Germany) and FAM-labeled probes (100 nM) (Table 2) were designed by using the web-based UPL design center (Roche; www.universalprobelibrary.

Table 1
Genes under study in our human endometrial Ishikawa cell model.

	Gene symbol	Biological significance
Target genes		
Estrogen sulfotransferase	SULT1E1	Inactivation of estrogens
Progesterone receptor	PGR	Reproductive function and regulation of endometrium function
Alkaline phosphatase placental-like 2	ALPPL2	Dephosphorylation of molecules
G0/G1switch gene 2	G0S2	Cell cycle regulation
Reference gene		
Glucose-6-phosphate dehydrogenase	G6PDH	Production of NADPH