Octylphenol does not mimic diethylstilbestrol-induced oestrogen receptor- α expression in the newborn mouse uterine epithelium after prenatal exposure

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Abstract

This study examined whether the endocrine disruptor octylphenol (OP) mimics the synthetic oestrogen diethylstilbestrol (DES) in ability to induce oestrogen receptor- α (ER- α) expression in the newborn mouse uterine epithelium after prenatal exposure. Pregnant mice were given daily s.c. injections with DES (10 or 100 μ g DES/kg maternal wt) or OP (100 or 250 mg/kg maternal wt) or with vehicle alone from day 11.5 to 16.5 of pregnancy. ER- α expression was evaluated on histological sections by detecting ER- α mRNA with the *in situ* hybridization technique and ER- α protein using immunohistochemistry. The immunostaining was quantitated using a microspectrophotometer. Oestrogen-like activity of the DES and OP batches used for *in vivo* exposure was confirmed in

an *in vitro* assay based on transient gene expression of an oestrogen-dependent reporter plasmid. In mice exposed prenatally to vehicle alone, the uterine epithelium did not express either ER- α mRNA or protein, while both were highly expressed in the stroma. Exposure to either DES dose induced the expression of both ER- α mRNA and protein in the epithelium, whereas it was unchanged in the stroma. In contrast, neither OP dose induced the expression of ER- α mRNA or protein in the epithelium and expression was unchanged in the stroma. Our data stress the importance of *in vivo* studies when investigating endocrine disruptors.

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Introduction

During the last decades, increasing incidents of abnormally developed reproductive organs and reproductive dysfunctions have been reported in human and wildlife populations (Crisp *et al.* 1998). Environmental chemicals are being increasingly suspected for their possible participation in inducing such effects. They are proposed to possess hormone-like properties such as mimicking natural hormones and/or inhibiting the action of hormones. The chemicals constitute a large group of man-made and naturally occurring compounds in the environment and are often referred to as endocrine disruptors. It is well documented that prenatal exposure to the synthetic oestrogen diethylstilbestrol (DES) induces various reproductive alterations in humans (Bibbo *et al.* 1977, Gill *et al.* 1979, Wilcox *et al.* 1995) and mice (McLachlan *et al.* 1975, 1980, Newbold 1995). Numerous reproductive changes in wildlife populations suggested to be caused by

endocrine disruptors resemble those induced by DES (Crisp *et al.* 1998). Therefore, suspected endocrine disruptors are traditionally examined for their ability to mimic oestrogen-like effects.

The mechanisms by which endocrine disruptors produce an effect are uncertain. However, induction of oestrogen-like effects might involve the oestrogen receptors. So far two subtypes of nuclear oestrogen receptors, oestrogen receptor- α (ER- α) and oestrogen receptor- β (ER- β) (Kuiper *et al.* 1996, Mosselman *et al.* 1996, Tremblay *et al.* 1997) have been identified, and the existence of a membrane-associated oestrogen receptor has been reported (Nemere & Farach-Carson 1998). A number of fast and low-cost *in vitro* screening systems based on binding to and induction of effects via nuclear oestrogen receptors have been developed in order to assess the oestrogenicity of endocrine disruptors (Pelissero *et al.* 1993, Makela *et al.* 1994, White *et al.* 1994, Soto *et al.* 1995, Arnold *et al.* 1996, Coldham *et al.* 1997, Kuiper

et al. 1998, Legler *et al.* 1999). Such assays are very useful for preliminary screening but obviously present difficulties in extrapolation to the *in vivo* situation in which uptake, distribution and metabolism is involved in the biological activity of a compound. Another drawback of *in vitro* assays is the limited number of cellular signalling pathways as compared with the *in vivo* situation. Thus, when studying endocrine disruptors and their possible influence on reproductive function, *in vivo* experiments seem to be unavoidable.

Reproductive organs are highly susceptible to hormonal exposure during a critical phase of foetal life, when they form and sex differentiate. It is therefore believed that exposure to DES or endocrine disruptors during foetal life might be detrimental to the development and differentiation of the reproductive organs, although abnormalities may not be expressed until adulthood (Bern 1992). Neonatal exposure of mice to oestradiol or DES, at concentrations which later in life lead to the development of abnormalities in the female genital tract including the uterus, has been shown to induce nuclear oestrogen receptor expression in the uterine epithelium (Andersson & Forsberg 1988, Sato *et al.* 1992, 1996). Prenatal DES exposure and changes in oestrogen receptor expression in mice have only been examined in one study, which showed induction similar to that after neonatal exposure (Newbold 1995). The mechanism behind such DES- and oestradiol-induced changes in oestrogen receptor expression is unknown. However, when studying endocrine disruptors it seems pertinent to examine their capability to mimic various DES-like changes, e.g. induction of oestrogen receptor expression in the uterus.

One of the suspected endocrine disruptors is the alkylphenol 4-*tert*-octylphenol (OP). It is a widely distributed degradation product of octylphenol ethoxylates used in household products such as detergents and emulsifiers, as well as in agricultural and industrial applications. OP is weakly oestrogenic in *in vitro* assays (White *et al.* 1994, Soto *et al.* 1995, Arnold *et al.* 1996) and in the traditional utero-tropic assay it possesses oestrogen-like activity by increasing the uterus weight (Bicknell *et al.* 1995). Based on such assays, OP is regarded as an oestrogenic compound with potential impact on reproductive health. However, detailed examinations of whether OP induces effects resembling oestradiol or DES effects at the cellular level are few. In the present study we tested whether OP mimics DES-induced ER- α expression in the uterine epithelium of the newborn mouse after prenatal exposure. ER- α expression was evaluated by detecting ER- α mRNA by *in situ* hybridization and ER- α protein by immunohistochemistry. Quantitation of immunostaining was evaluated using a microspectrophotometer. The DES and OP batches used for *in vivo* exposure were tested in an *in vitro* assay in order to confirm their oestrogen-like activity.

Materials and Methods

In vivo exposure

Mature mice, strain NMRI (Bomholtgaard, Denmark) were maintained under constant conditions with a photoperiod of 12 h light (beginning at 0800 h) and 12 h darkness. Food and water were supplied and animals were allowed to feed *ad libitum*. The morning after mating, female mice were examined for the presence of a vaginal plug. The day when the plug was present was designated day 0.5 post coitum (p.c.) and the term for delivery was day 19 p.c. The timed pregnant mice were given daily s.c. injections from day 11.5 p.c. to 16.5 p.c. of either DES (Sigma, Copenhagen, Denmark) or OP (Aldrich, Copenhagen, Denmark) dissolved in peanut oil, or of vehicle alone. The daily doses were 10 or 100 μ g DES/kg maternal wt and 100 or 250 mg OP/kg maternal wt. In preliminary experiments an even higher dose of OP (500 mg/kg per day) was tested. This dose turned out to be toxic, as four out of six pregnant mice died. On the day of birth, the female genital system including ovary, oviduct and uterine horns was removed from the newborns and fixed appropriately for either *in situ* hybridization or immunohistochemistry. Those DES-treated mice which did not deliver on the expected day were delivered by caesarean section on day 19 p.c. Tissue from each of the five treated groups of animals will in the following be referred to as DES10, DES100, OP100, OP250 and control respectively.

In situ hybridization

Tissue fixed overnight in 4% w/v paraformaldehyde at 4 °C was embedded in paraffin. Sections (6 μ m thick) were mounted on TESPA (3-aminopropyltriethoxysilane)-coated glass slides and dried overnight at 42 °C. Tissue from three to four animals originating from two to four litters from each group of treated animals was processed in this manner. Uteri from adult mice in which ER- α mRNA expression is high, served as positive control tissue for the *in situ* hybridization procedure.

To detect murine ER- α mRNA, anti-sense and sense ³⁵S-labelled riboprobes for the ligand binding domain were generated as described previously (Lemmen *et al.* 1999). An FspI/XbaI fragment of mouse oestrogen receptor cDNA spanning amino residues 929–1204 was ligated into pBluescript cut with both SmaI and XbaI. To generate the anti-sense riboprobe, the plasmid was linearized with EcoRI and subsequently transcribed with T3 RNA-polymerase in the presence of α -³⁵S-UTP (Amersham, Roosendaal, The Netherlands). The sense probe was transcribed by T7 RNA polymerase using α -³⁵S-UTP after the plasmid was linearized with SacI.

In situ hybridization was conducted as described by Feijen *et al.* (1994). The sections were subjected to pre-treatment with 20 μ g/ml proteinase K in order to

increase accessibility, and 0.25% v/v acetic anhydride to reduce background. The probe (1×10^5 c.p.m./ μ l) was applied and hybridization was carried out overnight at 55 °C in a moist chamber. The hybridization was terminated by removing the coverslips in 5 $\times$ SSC (SSC: 0.15 M NaCl, 0.015 M Na citrate, pH 7.4), 25 mM DTT at 50 °C. The sections were then washed at high stringency for 30 min at 65 °C in a solution of 50% formamide, 2 $\times$ SSC, 100 mM DTT. Subsequently, non-specifically bound probes were removed by treatment with 20 μ g/ml RNase A at 37 °C for 30 min followed by a wash at high stringency. The sections were washed at room temperature in 2 $\times$ SSC and 0.1 $\times$ SSC each for 15 min and dehydrated in ethanol supplemented with 300 mM ammonium acetate and air-dried. Ilford G5 photo emulsion was used for autoradiography and diluted 1:1 with 2% glycerol-water. The sections were exposed for 2 weeks at 4 °C, developed in Kodak D19, fixed in Kodak UNIFIX, and subsequently counterstained with haematoxylin. The sections were examined using a light microscope.

Immunohistochemistry

The tissue was fixed overnight at 4 °C in 4% w/v formaldehyde, pH 5.0, followed by dehydration and embedding in paraffin. Sections (5 μ m thick) were cut and mounted on SuperFrost-Plus glass slides (Prohosp, Værløse, Denmark) and dried for 1 h at 60 °C. Uteri from 18–19-day-old prepubertal mice were used as positive control tissue for the immunohistochemical procedure, as this tissue is well known for its high expression of oestrogen receptors. For each group of treated animals, tissue from five to eight animals originating from four to five litters was processed for immunohistochemistry. On each day of immunohistochemical staining, sections from at least one animal from the control group were included.

The immunohistochemical procedure for localization of ER- α has been described previously (Nielsen *et al.* 2000). Briefly, the deparaffinated sections were subjected to antigen retrieval in 0.01 M citrate buffer (pH 6.0) by microwaving. The endogenous peroxidase was then blocked by incubating the sections in 0.3% H₂O₂. A monoclonal ER-antibody raised to the N-terminal (A/B) region of the human oestrogen receptor (DAKO A/S, Glostrup, Denmark) diluted 1:100 in 1% w/v BSA was applied to the sections which were left to incubate for 1 h at room temperature. The ER antibody has been shown to recognize ER- α (Al Saati *et al.* 1993) and does not cross-react with ER- β (Pettersson *et al.* 1997). As a control, sections were incubated in parallel with 1% BSA, either without antibody or supplemented with irrelevant mouse IgG at the same concentration as used for the ER antibody. Biotinylated secondary antibodies (LSAB+ Kit) were added for 15 min followed by the application of peroxidase-conjugated streptavidin (LSAB+ Kit) for 15 min. The peroxidase was visualized by applying 3,3'-

diaminobenzidine chromogen solution (DAB+) for 5 min. Reagents used for visualization were obtained from DAKO A/S, Glostrup, Denmark. The reaction was ended by washing the sections in running tap water, whereupon the sections were dehydrated and mounted with Pertex.

Quantitation of immunostained sections and statistical evaluation

The intensity of immunostaining was quantitated with a Vickers M85A scanning and integrating microspectrophotometer (Vickers, York, UK) employing the following procedure. The distal part of the longitudinally cut uteri was sought out by using the built-in differential interference contrast system of the instrument. In this way, feebly stained or even unstained nuclei could easily be identified. In the direction of where the uterine horns originally were connected to the corpus, the nuclei of the first 10–15 epithelial cells and the first 15–20 stroma cells were selected in each of at least two consecutive sections. In this way at least 20 epithelial cell nuclei and 30 stroma cell nuclei were selected for measurement in each specimen. Overlapping nuclei were omitted. Subsequently, the nuclei were scanned with the following machine settings. $\times 40$ objective (scanning area 28.3 μ m²), light at the absorption maximum (460 nm) of the polymerized DAB reaction product and a scanning spot diameter of 0.5 μ m². Within each area, the instrument automatically integrated the individual spot readings. The mean integrated absorbance over all areas was then calculated by reference to a standard calibration graph, constructed by measuring a series of neutral density filters with known absorbance values. The microspectrophotometer was calibrated on each experimental day whereby any day-to-day variation in the sensitivity of the instrument was eliminated. Moreover, measurements of the neutral density filters showed that the instrument was linear within the range of the absorbance values recorded in the nuclei. Finally, the mean polymerized DAB reaction product measured in the absence of the primary antibody (i.e. the control section incubated with 1% BSA without ER antibody) was subtracted from that measured in the presence of antibody to give a measure of the specific antibody binding alone. The slides were decoded at the end of the experiment. The data were processed using version four of the StatGraphics Plus statistical software package (STSC, Rockville, MD, USA). The results for each experimental group are expressed as medians and ranges. The differences between multiple samples were assessed by the Kruskal–Wallis analysis of variance and were identified by a *posteriori* evaluation (Conover 1980) using a 95% confidence limit.

Transient gene expression assay

In order to evaluate whether the DES and OP batches used in this study were biologically active, an *in vitro*

transient gene expression assay was performed. The induction of gene activity was tested in a cell-based transcription assay system, using a reporter gene under transcriptional control of ER- α . 17 β -oestradiol (E₂) (Sigma, Zwijndrecht, The Netherlands) was included as a positive control compound. The oestrogen-responsive reporter gene construct (3 xERE-TATA-LUC) with three copies of a consensus oestrogen response element (ERE) containing oligonucleotide and a TATA box in front of the luciferase cDNA was used (Kuiper *et al.* 1998). The human ER- α expression plasmid pSG5-HEGO (kindly provided by Dr P Chambon, IGBMC, Strasbourg, France) was used. Human 293 embryonal kidney cells obtained from the ATCC (American Type Culture Collection, Rockville, MD, USA) were cultured in a 1:1 mixture of DMEM and Ham's F12 medium (DF) supplemented with 7.5% v/v FCS. Subsequent to trypsinization, the cells were suspended in phenol-free DF medium containing 30 nM selenite, 10 μ g/ml transferrin and 0.2% v/v BSA, supplemented with 5% v/v charcoal-stripped FCS and plated out in 24-well tissue culture plates. After 24 h the cells were transfected by calcium phosphate precipitation (Chu & Sharp 1981) with 1 μ g 3 xERE-TATA-LUC, 0.4 μ g SV2-LacZ (Pfahl *et al.* 1990) internal control plasmid and 0.2 μ g pSG5-HEGO per well. The medium was changed after 16 h and 0.8 μ l of a range of concentrations of E₂, DES and OP (dissolved in ethanol) was added directly to 0.8 ml of medium. The resulting concentrations for E₂ and DES were 10⁻¹³–10⁻⁸ M, and 10⁻⁹–10⁻⁵ M for OP. After 24 h exposure, the cells were lysed in lysis solution (1% v/v Triton X-100, 25 mM glycylglycine, 15 mM MgSO₄, 4 mM EGTA and 1 mM dithiothreitol (DTT)). The luciferase activity of the cell lysates was measured with the Lucite luciferase reporter gene assay system (Packard Instruments, Meriden, CT, USA), according to the manufacturer's instructions. The β -galactosidase activity was measured in order to correct for variations in transfection efficiencies (Pfahl *et al.* 1990).

Results

Influence of DES and OP on ER- α mRNA expression in vivo

In situ hybridization and hence ER- α mRNA expression was demonstrated in all positive control sections of the adult uteri. Sections hybridized with sense probe did not exhibit hybridization (Fig. 1k).

The uterine epithelium of the control mice showed no sign of *in situ* hybridization and thus no presence of ER- α mRNA. Conversely, ER- α mRNA was highly expressed in the stroma (Fig. 1a). In both DES10 (Fig. 1c) and DES100 (Fig. 1e), the epithelium exhibited ER- α mRNA expression, whereas in OP100 (Fig. 1g) and OP250 (Fig. 1i) no expression was observed in this compartment. The ER- α mRNA expression in the stroma of both of the DES- and OP-treated animals (Fig. 1c, e, g, i) was high and did not appear to differ from the expression in control mice (Fig. 1a).

Influence of DES and OP on ER- α protein expression in vivo

In uteri from 18–19-day-old prepubertal mice all nuclei, but not the cytoplasm, exhibited stained reaction product. Nuclear staining was absent in control sections incubated without antibody (Fig. 1l) or supplemented with irrelevant mouse-IgG. No significant differences between median absorbance values could be identified when sections from all control mice were run in one sitting and compared with sections from control mice stained on different days. This applied to both epithelial and stromal cell nuclei.

There was no apparent stain in epithelial cell nuclei from control, OP100 or OP250 mice (Fig. 1b, h, j). However, in both DES10 and DES100 mice, several epithelial cell nuclei revealed staining (Fig. 1d, f). In agreement with these observations made by inspecting the sections in the light microscope, only a very low median absorbance was recorded in epithelial cell nuclei from the control group, OP100 and OP250 mice (Fig. 2). Moreover, there was no significant difference between the median absorbance values of these groups. In contrast, the median absorbance of both DES10 and DES 100 epithelial cell nuclei increased significantly (Fig. 2).

In both exposed and control mice, a pronounced staining was seen in virtually all stroma cell nuclei (Fig. 1b, d, f, h, j). No significant differences could be identified when the median absorbance values of stroma cell nuclei in the different experimental groups were compared (Fig. 2).

The DES and OP batches possess oestrogen-like activity in vitro

The tested compounds stimulated transcriptional activity *in vitro* (Fig. 3). For the various concentrations tested, the

Figure 1 ER- α expression in uteri of newborn mice exposed prenatally to vehicle alone (a, b), 10 μ g DES/kg (c, d), 100 μ g DES/kg (e, f), 100 mg OP/kg (g, h) or 250 mg OP/kg (i, j). ER- α mRNA expression is detected by *in situ* hybridization (a, c, e, g, i, k) and ER- α protein by immunohistochemistry (b, d, f, h, j, l). In control mice treated with vehicle alone (a, b) and in OP-treated mice (g–j), neither ER- α mRNA or protein is expressed in the epithelium, whereas a high expression of both is present in the stroma. In DES-treated mice (c–f) both ER- α mRNA and protein is detected in the epithelium, and the stroma exhibits a high expression. Control sections either hybridized with sense probe (k) or incubated in 1% BSA without ER antibody (l) show no expression of ER- α mRNA or protein respectively. The scale bar represents 30 μ m.

Figure 2 Quantitation of ER- α expression in immunostained sections from newborn mouse uteri after prenatal exposure to 10 μ g DES/kg (DES10), 100 μ g DES/kg (DES100), 100 mg OP/kg (OP100), 250 mg OP/kg (OP250) or vehicle alone (Control). Open bars show ER- α expression in epithelial cell nuclei. Hatched bars show ER- α expression in stroma cell nuclei. Results are indicated as medians of specific absorbances (total absorbances minus background absorbances) and ranges. Vertical arrows indicate medians that are significantly different from those calculated for the control animals, as evaluated *a posteriori* using a 95% confidence limit level.

activity is shown as percentage of the maximal induction by E_2 . The results are from two separate experiments in which each concentration was employed in triplicate. The half maximal activity for DES was reached at an approximately 1000 times lower concentration compared with

Figure 3 Transcriptional activity of DES and OP in 293 human embryonic kidney cells. Cells were transfected with ERE-TATA-luciferase reporter plasmid and human ER- α . The transcriptional activity is given as a percentage $\pm$ s.d. of the maximal induction by E_2 . The data are from two separate experiments in which each concentration was employed in triplicate. DES (●) and OP (○).

OP. Nevertheless, both compounds induced transcription and thus demonstrated oestrogen-like activity *in vitro*.

Discussion

Application of ER- α specific antibodies and quantitation based on absorbance measurements has made it possible to distinguish objectively differences in immunostaining of this nuclear oestrogen receptor. Previous comparable studies have not discriminated between the subtypes of the nuclear oestrogen receptors, and evaluations of immunostaining have been based on subjective visual inspection of the sections. Mouse strain differences in neonatal ontogeny of oestrogen receptors in the uterine epithelium have been reported (Bigby *et al.* 1990). Nevertheless, the lack of epithelial oestrogen receptors and relatively high expression in the stroma until around day 5 after birth is common (Cunha *et al.* 1982, Bigby & Cunha 1986, Andersson & Forsberg 1988, Yamashita *et al.* 1989, Greco *et al.* 1991, Sato *et al.* 1992, 1996, Newbold 1995, Eroschenko *et al.* 1996, Nielsen *et al.* 2000). In the present study, a very low absorbance was measured in the uterine epithelium of the control animals. However, upon visual examination the epithelium appeared immunonegative, which is consistent with earlier observations. Our finding that expression of ER- α mRNA and protein is induced in the uterine epithelium after prenatal DES exposure is in accordance with previous reports employing prenatal (Newbold 1995) and neonatal DES exposure (Andersson & Forsberg 1988, Sato *et al.* 1992, 1996). However, the unchanged ER- α immunostaining in the stroma after DES treatment contradicts previous data which by visual inspection describe an apparent decreased immunostaining in this compartment (Sato *et al.* 1992, 1996, Newbold 1995). It is interesting that the stroma and epithelial cells respond differently on DES exposure. Although ER- α is highly expressed in the stroma cells, it does not seem to participate in the regulation of its own expression in these cells. On the other hand, it is suggested that ER- α in the stroma cells could be involved in a paracrine regulation of ER- α in the epithelial cells. In fact, it was recently demonstrated that uterine epithelial progesterone receptors are regulated in a paracrine manner via ER- α in the stroma (Kurita *et al.* 2000).

In contrast to DES, OP does not induce the expression of either ER- α mRNA or protein in the uterine epithelium, although the *in vitro* data confirmed oestrogen-like activity of the OP batch. In the stroma, the immunostaining decreases slightly in a dose-dependent manner but the difference is not statistically significant. Neonatal exposure to the oestrogenic endocrine disruptor methoxychlor has been demonstrated to induce oestrogen receptor protein and mRNA expression in the murine uterine epithelium (Eroschenko *et al.* 1996). It is interesting that two different reported oestrogenic compounds do

not induce the same change in oestrogen receptor expression. However, it is difficult to compare the methoxychlor data and data from our OP-exposed animals due to differences in exposure periods.

Although our *in vivo* data do not demonstrate a DES-like action of OP, we cannot rule out the possibility that it may have oestrogen-like effects in mice. In addition to the numerous *in vitro* studies identifying OP as weakly oestrogenic, other rodent studies have demonstrated its oestrogen-like effects *in vivo*. These studies have primarily focused on effects in males after exposure during either foetal life (Sharpe *et al.* 1995, Majdic *et al.* 1996, 1997, vom Saal *et al.* 1998) or adulthood (Blake & Boockfor 1997, Boockfor & Blake 1997). Foetal exposure was carried out by exposing the mothers orally by adding OP to the drinking water (100–1000 μg OP/l water) (Sharpe *et al.* 1995) or by delivering OP dissolved in oil (2 $\mu\text{g}/\text{kg}$ maternal wt) directly into the mouth daily from 11 to 17 p.c. (vom Saal *et al.* 1998). Others subcutaneously injected the pregnant mothers day 11.5 and 15.5 p.c. with 100 or 600 mg OP/kg maternal wt (Majdic *et al.* 1996, 1997). Adult males were subcutaneously injected with 20 or 80 mg OP three times weekly for 1–2 months (Blake & Boockfor 1997, Boockfor & Blake 1997). The observed effects include changes in the morphology as well as reduced weight of testes and accessory sex organs, decreased sperm production and altered secretion of reproductive hormones (Sharpe *et al.* 1995, Blake & Boockfor 1997, Boockfor & Blake 1997, vom Saal *et al.* 1998). In addition, the expression of P450 17 α -hydroxylase/C17–20-lyase and steroidogenic factor 1 in the testis appears to be affected (Majdic *et al.* 1996, 1997). In neonatal and prepubertal female rats subcutaneously injected with 1–30 mg OP, the uterine weight increases (Bicknell *et al.* 1995) and the oestrous cyclicity is disrupted, identified by the persistent presence of cornified cells (Blake & Ashiru 1997). One explanation for why OP did not exhibit an *in vivo* oestrogen-like effect in the present study could be that the mechanisms behind the induction of ER- α expression differs from those involved in the regulation of uterus weight and oestrous cyclicity. Furthermore, compared with the previous studies in neonatal and prepubertal female rats, the present study employed prenatal exposure, which could result in reduced OP exposure levels due to OP metabolism in the mother or insufficient placental transfer of OP. However, as mentioned, prenatally OP exposed male rodents have been shown to develop abnormalities. When comparing studies of OP-induced effects in prenatally exposed females and males it is important to bear in mind that under normal circumstances the *in utero* level of oestrogens is lower in males compared with females (vom Saal *et al.* 1983). Therefore, male foetuses may be relatively sensitive to changes in levels of oestrogens and/or oestrogen-like compounds. Furthermore, comparison between data obtained in rats and mice and even in different mouse

strains should be done with care. Recently, it was shown that among different mouse strains oestrogen responsiveness could differ due to different genetic regulation (Roper *et al.* 1999, Spearow *et al.* 1999).

When studying endocrine disruptors and their possible contribution to the development of structural and functional reproductive abnormalities, it is important to investigate them in the context of relevant parameters. The incidence of uterine structural abnormalities is elevated among mice and humans exposed prenatally to DES (Kaufman *et al.* 1977, Haney *et al.* 1979, McLachlan *et al.* 1980, Newbold 1995). Moreover, in such mice, long-term effects include uterine tumours (McLachlan *et al.* 1980, Newbold 1995) and altered uterine protein secretion (Maier *et al.* 1985). It is therefore relevant to ask whether changes in uterine ER- α expression may be involved in such abnormalities.

It has been shown that an increased level of oestrogen receptors is involved in the development of uterine tumours (Couse *et al.* 1997). In transgenic mice with an aberrant expression of oestrogen receptors, neonatal DES treatment leads to accelerated tumour development in adulthood. The level of oestrogen receptors was hence suggested to be involved in the proliferation of tumours induced by early DES exposure. However, the uterine epithelial oestrogen receptors may not be significant for epithelial cell proliferation. In the uterus of normal adult mice, oestrogen has been shown to stimulate epithelial cell proliferation in a paracrine manner via the stroma cells, in a process in which neither epithelial ER- α nor ER- β is necessary or sufficient (Cooke *et al.* 1997). Consequently, the content of oestrogen receptors in an epithelial cell might not, at least in adults, determine its oestrogen-induced proliferative activity.

DES treatment of neonatal mice induces production of the secretory protein lactoferrin in the uterine epithelium (Yamashita *et al.* 1990, Nelson *et al.* 1994). Cells which express lactoferrin always express oestrogen receptors that are induced before lactoferrin, and epithelial oestrogen receptors are therefore suggested to be a prerequisite for expression of lactoferrin (Yamashita *et al.* 1990). Recently, it has been shown that both stromal and epithelial ER- α are involved in the production of oestrogen-dependent uterine secretory proteins (Buchanan *et al.* 1999). Altered uterine protein secretion throughout adulthood after DES treatment prenatally (Maier *et al.* 1985) or neonatally (Yamashita *et al.* 1990, Nelson *et al.* 1994) may thus involve a perinatal induction of ER- α expression in the otherwise ER- α devoid uterine epithelium. However, recent data suggest that endocrine disruptors and catechol oestrogens are able to induce oestrogen-responsive uterine genes, including lactoferrin via an alternative, unknown pathway which does not require ER- α or ER- β (Das *et al.* 1998, Ghosh *et al.* 1999).

Although OP does not induce ER- α expression, it cannot be eliminated as an endocrine disruptor with

potential oestrogen-like activity affecting the female genital tract. Alterations may be induced via other mechanisms and pathways which do not involve epithelial ER- α and its induction. Moreover, effects induced by exposure in foetal life might not become evident until later in life. It is therefore crucial to extend studies of the prenatally exposed animals to include older animals, as well as widening the range of effect parameters.

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