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EFFECTS OF ENDOCRINE DISRUPTORS ON THE EXPRESSION OF NUCLEAR RECEPTORS IN MURINE EMBRYOS

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Introduction

The mammalian dam and fetus exchange materials through the placenta during pregnancy. Fetuses show gene expression of a wide range of nuclear receptors during this time in a stage- and region-specific manner, which are suggested to have important roles in fetal development. The nuclear receptor superfamily constitutes a class of ligand-dependent transcription factors that regulate gene expression during many biological processes, including development, cellular proliferation and differentiation [1-4]. This superfamily includes receptors for steroid hormones, retinoids, vitamin D and an increasing number of orphan receptors for which the ligand has not yet been identified [5-8]. In contrast to the ligand-known receptors, the biological functions of the orphan receptors are only beginning to be understood. Several experiments in gene-knockout mice have demonstrated their biological relevance as the orphans chicken ovalbumin upstream promoter-transcription factor 1 (COUP-TF1), chicken ovalbumin upstream promoter-transcription factor 2 (COUP-TF2) and estrogen receptor-related receptor-b (ERRb) are essential genes, and SF-1/Ftz-F1 and DAX1 are required for differentiation of the adrenal glands and gonads [3, 9, 10]. However, the details of their functions and movement of their products within the cell and expression domains are still uncertain.

In the present study, we investigated the sex and region-specific expression of nuclear receptors, including estrogen receptor a (ERa), ERß and several orphan receptors such as SF-1, DAX1, COUP-TF1, at the middle and late stages of pregnancy in ICR mice, and the effects the endocrine disruptor, diethylstilbestrol (DES) on their expression. Using RT-PCR, we attempted to detect the expression of these genes in adult ovaries, oviducts, uterine tissues, placentae and fetuses. Orphan receptors were detected in all tissues examined and DES did not affect their expression, while ERa and ERß showed different patterns between DES-treated and untreated groups.

Materials and methods

Animals

ICR mice were used in this experiment. The day on which a vaginal plug was detected was considered as 0.5 days post coitum (dpc). Several densities of DES solution was prepared by dissolve in corn oil. The pregnant mice were given a daily injection of DES or vehicle alone in the afternoon (12:00 to 14:00) from 8.5 dpc to 15.5 dpc according to their treatment groups; normal (untreated), 0 (vehicle control), 1 and 100 μg/kg of body weight (BW) DES. Pregnant mice at 12.5 and 18.5 dpc were sacrificed under diethyl ether anesthesia in the afternoon (12:00 to 14:00). The adult ovaries, oviducts, uterine tissues, placenta and whole embryos were dissected, trimmed, weighed, frozen in liquid N_2 and stored at -80 °C until RNA extraction. Sections of 12.5 dpc whole embryos were also frozen separately for DNA extraction followed by sex determination.

Treatment of pregnant female mice with diethylstilbestrol (DES)

To determine the appropriate dose for experiments, DES dissolved in corn oil was subcutaneously injected into pregnant female mice at 0 (vehicle control), 1, 10, 25, 50, 100, 200, 400, 800 or 1000 μg/kg BW daily from 8.5 to 15.5 dpc, and the treated mice were sacrificed at 18.5 dpc as described above. The numbers of normal placentae and fetuses were counted.

DNA extraction and sex determination

Genomic DNA of 12.5 dpc fetuses was extracted using extraction buffer containing 200 mM Tris-HCl (pH 7.5), 250 mM NaCl, 25mM EDTA and 0.5% SDS. Extracted genomic DNA was purified by isopropanol and dissolved in TE buffer. Sex determination of 12.5 dpc fetuses was performed by nested PCR using extracted DNA, which was obtained from whole fetal tissues. Primers amplifying the polymorphic X chromosome microsatellite locus (DXNds-3) [12] and Y chromosome Sry gene [13] were used for nested PCR assay. Inner and outer primer pairs were synthesized by Genset (Kyoto, Japan) using DNA sequences identical to those described by Kunieda *et al.* [14]. PCR was performed asdescribed by Greenlee *et al.* [15].

RNA preparations and reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was extracted using a RNeasy Mini Kit (Qiagen Inc., CA, USA), according to the manufacturer's protocol. Extracted total RNA was reverse transcribed with oligo(dT) primer using a T-Primed First-Strand kit (Amersham Pharmacia Biotech, Inc., Piscataway, NJ, USA) to synthesize first strand cDNA. The cDNA was quick-chilled on ice to denature the RNA-cDNA duplex, and stored at -20 °C until subsequent PCR. PCR mixtures were prepared using a Platinum PCR Super Mix kit

(Gibco BRL, Rockville, MD, USA) according to the manufacturer's protocol. To examine the nuclear receptor expression during mouse pregnancy, primers to amplify the identical sequences of ERa, ERß, SF-1, DAX1, COUP-TF1 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were used. The primers for ERa and ERß were designed according to the report of Tachibana *et al.* [11]. The other primers were designed according to mouse receptor sequences and and were synthesized by Genset (Kyoto, Japan). PCR was carried out in a Gene Amp 2400 thermal cycler (Perkin Elmer, Norwalk, CT, USA). ERa and ERß cDNAs were denatured at 95 °C for 5 min, than PCR was performed for 45 cycles of 95 °C for 30 sec, 65 °C for 1 min, and 72 °C for 25 sec, with a final extension step at 72 °C for 5 min [11]. SF-1, DAX1, COUP-TF1 and GAPDH cDNAs were denatured at 94 °C for 5 min, than PCR was performed for 35 cycles of 94 °C for 60 sec, 55 °C for 1 min, and 72 °C for 60 sec, with a final extension step at 72 °C for 5 min. The PCR products were electrophoresed in 2% agarose gels and stained with 1 µg/ml ethidium bromide solution. Ready-load 100 bp DNA ladder (Gibco) was used as a molecular weight standard. The gels were visualized and photographed with a digital recorder (FAS III system; Toyobo, Tokyo, Japan).

Results and Discussion

To determine the appropriate dosage for experiments, DES dissolved in corn oil was subcutaneously injected into pregnant female mice at 0, 1, 10, 25, 50, 100, 200, 400, 800 or 1,000 µg/kg BW daily from 8.5 to 15.5 dpc. These mice were sacrificed at 18.5 dpc and the number of normal fetuses was counted (Table 1). All mice in the normal (no administration), vehicle control, 1 and 10 µg/kg BW daily had fetuses. Although the numbers of fetuses decreased dose-dependently in the DES-exposed groups, no obvious differences were observed in the body weight of embryos. In the mice treated at doses of 50, 100, 200 and 400 µg/kg BW daily, embryos were obtained, but both their number (embryos/dam) and body weight were decreased dose-dependently. Pregnant mice with reduced embryo numbers showed many absorbed dots in the uterus, suggesting fetal death during pregnancy caused by DES administration. In the mice treated at doses of 25, 800 and 1,000 µg/kg/day, no fetuses were obtained because of miscarriage or fetal death. Based on these findings, we useda dosage of 100 µg/kg BW daily for the following investigation, as this was considered to allow stable fetal yield, while large effects were expected. In addition to this dosage, 1 µg/kg BW daily was used to examine low dose effects suggested to be the fundamental effects of endocrine disruptors.

The 18.5 dpc pregnant mouse tissues examined showed expression of both ERs mRNAs, whereas fetuses showed diferent expression patterns in an organ- and sex-related manner. ERa

expression was detected in the placenta, eyes, liver, lung, heart, umbilical cord, urogenital system, bone, thymus and gastrointestinal tract, with weak expression in the tissue complex of mesencephalon, cerebellum and spinal cord in 18.5 dpc female fetuses, while no expression was observed in males.

ERb was detected in the thymus and gastrointestinal tractsin both males and females, although heart, skin, the urogenital system, and bone showed ERb expression only in females, while only males showed expression in the brain, eyes, kidney, liver and lung. Treatment with DES at 100 mg/kg BW stimulated ERa expression in female fetal epiphysis and olfactory lobe complex, while that of ERb was suppressed in all organs which it was detected in untreated mice.

The 12.5 dpc pregnant mice showed ERa mRNA expression in all tissues examined, i.e. the ovaries, oviducts and uterus. The 12.5 dpc whole fetuses and placentas of both sexes also showed ERa expression in all treatment groups, but its level of expression was weak in the group treated with 100 mg/kg BW DES. ERb was not detected in any tissues of untreated 12.5 dpc pregnant females or their fetuses, but was detected in the ovaries and whole male fetuses of vehicle control and DES-treated groups. The orphan receptors, SF-1, DAX1 and COUP-TF1, were detected in all tissues examined.

These results indicated that fetal ERa and ERb mRNA expression are controlled in a stage-, tissue- and sex-specific manner, and further that ERb expression increased from the middle to later stages of pregnancy. ERa and ERb, therefore, probably have some important roles during fetal development, especially in later stages, which differ with sex. DES treatment may disturb these functions mainly by increasing ERb expression level in the middle stage of pregnancy but reducing it at in later stages. In contrast, SF-1, DAX1 and COUP-TF1 mRNA showed stable expression suggesting that they are essential not only for fetal development but also for the pregnant dam.

Acknowledgements

This work was supported by a Grant-in-Aid for Creative Scientific Research to N. M. from the Japan Society for the Promotion of Science (13GS0008), by a Grant-in-Aid to N. M. for Scientific Research (13027241) of the Ministry of Education, Sports and Culture in Japan, and by a Grant to N. M. from the Itoh Memorial Foundation.

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