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Transgenerational effect of a single neonatal benzpyrene treatment on the glucocorticoid receptor of the rat thymus

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Hormonal imprinting is provoked perinatally by the appropriate hormone on its receptor, causing a life-long adjustment of the connection between the two participants. Faulty imprinting is caused by the presence of molecules similar to the hormone in this critical period, which results in a persistent alteration of the receptor. In the present experiment the transgenerational imprinting effect of a steroid-like environmental pollutant, benzpyrene, on the receptor binding capacity of filial thymic dexamethasone and uterine estrogen receptors was studied. The receptor density (B_{max}) of the thymic glucocorticoid receptors of the males was reduced up to the third (F_2) generation. In females this reduction was observed only in the F_1 generation of treated animals. There was no change in receptor affinity (K_d). Uterine estrogen receptors were not subjected to transgenerational imprinting. The experiments demonstrate (1) the possibility of the transgenerational transmission of imprinting effect, (2) the differences of steroid receptors in different organs, and (3) the differences of male's and female's reactions from this aspect. The results call attention to the dangers of perinatal aromatic hydrocarbon exposition to the progeny generations.

Keywords: hormonal imprinting; transgenerational effects; aromatic hydrocarbon; steroid receptors; glucocorticoid receptors; estrogen receptors

Introduction

For the normal development of hormone receptors the perinatal encounter between the appropriate hormone and the developing receptor (hormonal imprinting) is absolutely needed.[1–4] Without this process there is no physiological receptor maturation.[5] The perinatal imprinting determines the binding capacity of hormone receptors for life. However in this critical period the discriminating ability of receptors between the target hormone and related molecules is wrong, which results in false imprinting.[1–4] These related molecules could be synthetic hormone analogues (used in medical therapy), members of the same hormone family, molecules structurally related to the hormones or different from them, however acting also through nuclear receptors (e.g. lipid soluble vitamins[6,7]) or environmental pollutants (contaminants of water, air or food[4,8]). As a consequence of the treatment with related molecules perinatally (faulty imprinting) the binding capacity of receptors is altered for life which is manifested in morphological, biochemical and behavioral abnormalities as well as in malignant transformation in the adult animals.[2–4, 8–18]

Aromatic hydrocarbons are steroid like molecules which can cause faulty imprinting on the receptors of the steroid-thyroid receptor superfamily, administered perinatally. Single benzpyrene treatment at the 15th day of pregnancy or neonatally, caused a significant decrease of dexamethasone binding (Bmax) of adult rat thymic glucocorticoid receptors[19–21] or estradiol binding of uterine estrogen receptors.[22] Single neonatal TCDD (dioxin) administration also decreased steroid hormone binding for life.[23] Sexual behavior and sexual hormone level was also influenced in adult animals perinatally treated with benzpyrene.[13]

The effect of chemical imprinting is profound. It was demonstrated earlier that exposure to diethylstilbestrol (DES) during the critical developmental period led to the persistent induction of two estrogen-regulated genes.[24] This observation made worthy of studying the transgenerational effect of a single benzpyrene treatment, what was the aim of the present experiments.

Methods

Newborn Wistar rats of our closed breed (P generation) were treated with one dose of 20 μg benzpyrene (Sigma, St. Louis, USA) within 24 h

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after birth. Benzpyrene was dissolved in sunflower seed oil, and was given subcutaneously. Controls received the vehicle only. The male and female animals were mated at 3 months of age (treated with treated and control with control, randomly). The offspring of these pairs (F_1 generation) were studied in receptor assay (of thymus and uterus) when males were 6 weeks old and females were 3 months old. Animals not used for receptor study were paired again and progenies (F_2 generation) were studied for receptor assay (of thymus). F_3 generation was not studied, however animals of this generation were paired in adult age for gaining offspring (F_4 generation). These animals were studied again for receptor assay (of the thymus). This means that only one treatment was done neonatally in the P generation and progenies were studied up to the F_4 generation of males and F_2 generation of females, without any further treatments.

Preparation of cytosol fractions
Cytosolic (soluble) fractions were prepared from thymi of males and females and uteri (of females, 8 days after ovariectomy). Organs of four (in the case of thymus) and five (in the case of uterus) were used for one measurement. Five measurements were performed in each group/organ/generation.

All procedures were performed at ice/water temperature. Tissues examined were cut into pieces and homogenized in Tris-HCl containing 1.5 mM EDTA, pH 7.4 (freshly supplemented with 20 mM molybdate and 2 mM dithiotreitol) with a motor driven glass-teflon Potter homogenizer 1.5 ml/1 g wet weight. Homogenates were centrifuged at 100 000 g for 60 min at 4°C and the supernatants were used for receptor assays. Protein content was estimated by the Coomassie-blue method.

Saturation analysis with ^{3}H-dexamethasone
Increasing concentrations (0.625, 1.25, 2.5, 5, 10, 20, 40 nM) of ^{3}H-dexamethasone (Amersham, Buckinghamshire, England, spec. act. 1.5 TBq/mmol) were incubated with thymic cytosol (500 μg protein) in duplicates in a total volume of 100 μl at 0°C for 18 h for total binding (T). Non-specific binding (NS) was measured in the presence of 100-fold molar excess of dexamethasone acetate (Sigma, St. Louis, USA) at each radioactive concentration.

Saturation analysis with ^{3}H-estradiol
Increasing concentrations (0.312, 0.625, 1.25, 2.5, 5, 10, 20 nM) of ^{3}H-estradiol (Izinta, Budapest, Hungary spec. act. 3.5 TBq/mmol) were incubated with rat uteri cytosol (300 μg protein) in a 0.1 ml total volume, at °C for 18 h for total binding (T).

To estimate nonspecific binding (NS) there were another set of tubes containing 100-fold excess of unlabelled estradiol (Organon, Oss, Holland) for each radioligand concentration. All assays were performed in duplicates.

The reaction was terminated both in ^{3}H-dexamethasone and ^{3}H-estradiol saturation analysis by adding 200 μl 0.5% dextran coated charcoal suspended in assay buffer into each tube then pelleting the unbound steroid by centrifugation at 1500 g for 15 min. 200 μl aliquots of the supernatants were transferred for scintillation counting into 4 ml Optiphase 'HiSafe' (Pharmacia, Lund, Sweden) and counted in a Beckman apparatus (38% efficiency).

Tubes in duplicate containing radioactivity in each concentration plus buffer alone were used to generate both total counts and blanks. The difference between T and NS was regarded as specific binding (S) at each concentration of the labelled ligand: S=T-NS.

Analysis of results
Analysis of results was carried out by a computer program written by McPherson[25] named EBDA and by a nonlinear curve fitting program modified by McPherson[26] named LIGAND. The relationship between EBDA and LIGAND: EBDA is used to process the raw data which is then expressed on the appropriate plot. The graphical representation allows initial parameter estimates to be calculated for use by LIGAND. LIGAND is used to obtain final parameter estimates. For statistical analysis of results DATA ANALYSIS V.1.0. (Statistical and Design Services 1985) computer program was used.

Results

Males
In the thymus of the F_1 generation there was a significant ($P<0.05$) decrease of glucocorticoid receptor density (Bmax). Similar reduction was present in the F_2 generation (Figure 1). There was no difference in the receptor concentration of controls and treated in the F_4 generation. The affinity (K_d) was unchanged in each generation studied.

Females
In the thymus of the F_1 generation there was a significant ($P<0.05$) decrease of glucocorticoid receptor concentration (Bmax) (Figure 2). There was no difference between the control and treated groups in the F_2 generation. The affinity was unchanged both in the F_1 and F_2 generations.

In the uterus of the F_1 generation there was no difference in the affinity or density of estrogen receptors at all.

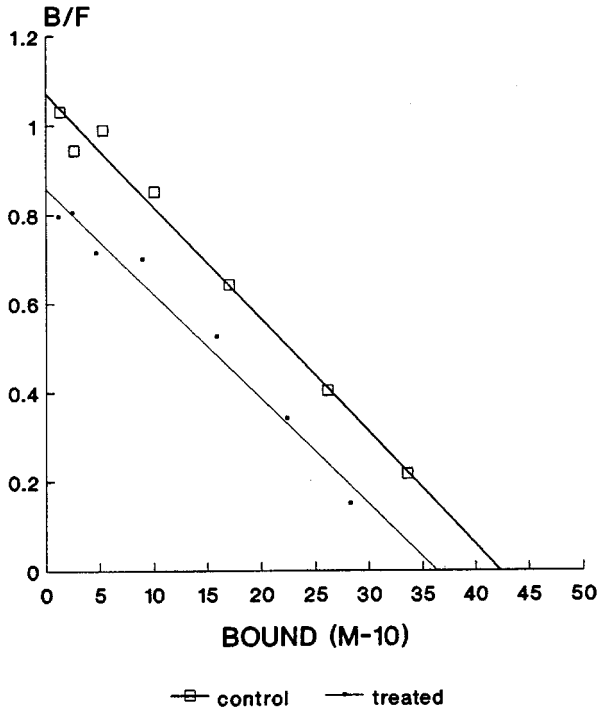

Figure 1 Graphical representation (Scatchard plots) of the mean values of data of male F_2 generation thymus cytosol receptor saturation analysis processed by the program LIGAND. All the K_d and B_{max} values are given in Table 1

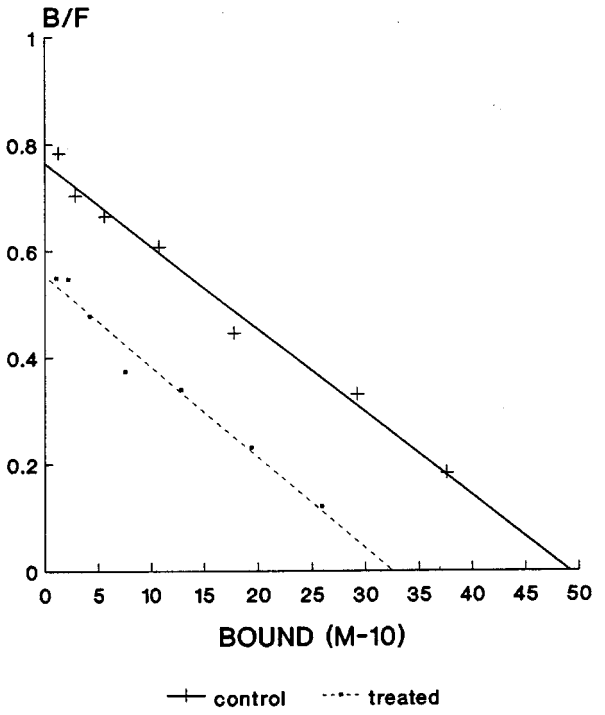

Figure 2 Graphical representation (Scatchard plots) of the mean values of data of female F_1 generation thymus cytosol receptor saturation analysis processed by the program LIGAND. All the K_d and B_{max} values are given in Table 1

The study of the receptors was stopped after the generation where differences were not found between the control and treated animals.

Details of the results are shown in Table 1.

Discussion

The receptor of the aromatic hydrocarbons (e.g. TCDD, benzpyrene) does not belong to the steroid-thyroid receptor superfamily.[27] However, the structure of these aromatic hydrocarbons is similar to the steroid hormones and behaves as steroid hormones[28] and – possibly by this – these molecules can disturb the normal development and function of steroid receptors. Provoking false imprinting, the binding capacity of these receptors is different from the normal one in adult age, after treatment with aromatic hydrocarbons neonatally, and morphological, biochemical and physiological parameters are also altered.[12,19–23,28] In the present experiment the transgenerational receptorial effect of a single neonatal benzpyrene treatment was studied. The results demonstrated that the binding capacity (B_{max}) of thymus glucocorticoid receptors was significantly reduced up to the third generation (F_2) as an effect of the benzpyrene imprinting. This effect disappeared between the third (F_2) and fifth (F_4) generations.

Transgenerational effects of biologically active materials in the endocrine sphere are well known.[29] Animals treated with diabetogenic drugs (e.g. alloxan) have descendants with signs of diabetes.[30] F_1 generation of neonatally insulin imprinted rats have a changed insulin receptor density, similar to the treated parents.[31] F_1 offspring of neonatally morphine treated rats show permanent defects in the nervous system causing altered behavior and responses to hormonal stresses.[32] Removal of the parathyroid glands from pregnant rats caused a significant depression of blood calcium levels for at

Table 1 Saturation analysis of receptors in the progeny generations of rats (F_1) neonatally treated with a single dose of benzpyrene (dexamethasone binding M^{-9} in the thymus, estradiol binding M^{-10} in the uterus, ± s.d.)

Generation	*Organ*	*Group*	K_d	B_{max}
F_1 male	thymus	control	3.90±1.7	4.56±0.5
F_1 male	thymus	treated	3.85±1.4	3.04±0.3[a]
F_2 male	thymus	control	4.44±1.2	4.41±0.1
F_2 male	thymus	treated	3.46±1.2	3.57±0.4[a]
F_4 male	thymus	control	2.29±0.5	2.58±0.2
F_4 male	thymus	treated	3.20±1.9	2.44±0.6
F_1 female	thymus	control	6.16±0.4	4.56±0.5
F_1 female	thymus	treated	6.69±1.1	3.04±0.3[a]
F_2 female	thymus	control	4.44±0.9	3.15±0.7
F_2 female	thymus	treated	3.83±0.2	3.48±0.5
F_1 female	uterus	control	1.44±0.2	1.01±0.1
F_1 female	uterus	treated	1.77±0.6	0.97±0.5

[a]=$P<0.05$ to the appropriate control

least four generations.[33] Prenatal diethylstibestrol treatment of mice provoked an increased incidence of uterine and ovarian carcinomas in second generation descendants[34] etc (see[29]). This means that there is a possibility of the transmission to the progeny generations of perinatally provoked artificial effects. The steroid receptor level transgenerational effect of an aromatic hydrocarbon (benzpyrene) could also be manifested in alterations of sexual behavior as it was demonstrated by us previously[35] or in other disturbances present in the parental generation,[13,17,23,28] not studied up to now in the progenies.

The effect of the single benzpyrene treatment disappeared after the third (F_2) generation in males and after the F_1 generation in females. This means, that mutation was not caused. Nevertheless the effect was profound. Gray Nelson *et al*.[24] demonstrated the permanent induction of estrogen-regulated genes after perinatal DES treatment. Though this does not explain the transgenerational effect of imprinting by steroid-like materials, it calls attention to the possibility of a switch-over at a gene level. The mechanism of this is not cleared at all, however the change in the methylation pattern of the genes may give some explanation. This methylation pattern could be maintained in a few generation. However, for the exact understanding of the phenomenon, molecular genetical analysis is needed.

It was surprising that in females the transgenerational effect was shorter than in males and, in contrast to the thymic glucocorticoid receptor, uterine estrogen receptors did not show any transgenerational alteration. Since there was no difference in the B_{max} reduction of male and female glucocorticoid receptors, and uterine estrogen receptors are influenced similar to thymic glucocorticoid receptors in the parental generation in earlier experiments,[19–22] it would be very difficult to explain the reasons of this difference. However the tendency is clear and support each other: in females the transgenerational effect on the glucocorticoid receptor is manifested only in the F_1 generation and on the estrogen receptor it is restricted to the P generation. In addition, estrogen receptors of the brain reacted transgenerationally, as it was demonstrated by the above mentioned experiments on sexual behavior, after neonatal benzpyrene treatment.[35] This means that not only a sex-dependence was observed, but the organ representants of the same receptor behaved differently.

It is noteworthy that only the receptor density was changed in the thymus and the affinity (K_d) was stable. This means – and this corresponds with earlier experiments[2–4] that receptor affinity is genetically fixed and the imprinting could influence – with minimal exceptions – solely the evocable number of receptors. However, considering that this effect can last up to the third generation (F_2 in males), and knowing that many molecules similar to benzpyrene are around us as environmental pollutants, this is a warning sign, which must not be disregarded. It is also worthy to note that glucocorticoid and estrogen receptors are members of the steroid receptor superfamily (used for testing of transgenerational imprinting effect) and other members could also be influenced by benzpyrene imprinting of the parental generations.

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