Human & Experimental Toxicology (1998) 17, 560–563

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Effect of retinoid (vitamin A or retinoic acid) treatment (hormonal imprinting) through breastmilk on the glucocorticoid receptor and estrogen receptor binding capacity of the adult rat offspring

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Hormonal imprinting occurs perinatally when the developing receptor and the appropriate hormone meet each other. The presence of related molecules in this critical period causes misimprinting. Ligands bound to a member of the steroid-thyroid receptor superfamily can disturb the normal maturation of other members of the family, which is manifested in altered binding capacity of the receptor and decreased or increased response of the receptor-bearing cell for life. Excess or absence of the hormone also can cause misimprinting. Treatments once a week for 3 weeks of nursing rat mothers with 6 mg/animal all-trans retinol/dose caused faulty imprinting manifested in significantly reduced density (Bmax) of thymic glucocorticoid receptor in male and female adult progenies alike. 0.03 mg all-trans retinoic acid treatment of nursing mothers was ineffective. Receptor affinity (K_d) was unchanged in both cases as well, as the binding values of uterine estrogen receptors. The results of the experiment call attention to the transmission of imprinter molecules by breastmilk to the progenies, which can cause lifelong alterations at receptorial level and points to the human health aspect. Possible reasons for the differences between retinol and retinoic acid effects and in the sensitivity of receptors are discussed.

Keywords: vitamin A; retinol; retinoic acid; hormonal imprinting; lactation; nursing mother; hormone receptor; glucocorticoids; steroids

Introduction

The perinatal critical period is the time of hormonal imprinting, when the hormone and its receptor are adjusted to each other.[1] As a consequence of this, the receptor maturation is completed and a normal receptor-hormone relation develops for life.[2] In the presence of molecules different from the target hormone, however able to bind to the receptor (members of the same hormone family, synthetic analogues, molecules with related chemical structure), or in the case of excess or lack of the target hormone, faulty imprinting develops, causing a disturbed receptor-hormone relation for life.[3]

Hormones acting on the members of steroid-thyroid receptor superfamily can overlap on each other's receptor perinatally, resulting in misimprinting, which is manifested in morphological, biochemical, receptorial and behavioural alterations alike.[4–15] Retinoid receptors belong to this superfamily. Perinatal vitamin A (ROH) or retinoic acid (RA) treatments can disturb the normal maturation of different steroid receptors, causing changes in the hormone binding, sexual hormone level and sexual behaviour in the adult age.[15–17] In the present experiment the effect of retinoid treatment of lactating rats on the steroid hormone binding capacity of adult offspring, was studied.

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Received 26 January 1998; revised 9 June 1998; accepted 24 June 1998

Methods

Nursing rat mothers of our closed (Charles River originated) breed were treated from the first day after delivery with 6.0 mg/animal sunflower seed oil dissolved vitamin A (all-trans retinol, Sigma, St. Louis, USA) intramuscularly, once a week, for 3 weeks (total dose 18 mg/animal/3 weeks for three dams) or with 0.03 mg/animal sunflower seed oil dissolved retinoic acid (all-trans retinoic acid, Sigma, St. Louis, USA) im. once a week for 3 weeks (total dose 0.09 mg/animal/3 weeks for 3 dams). Controls (three dams) got the vehicle only, in the same quantity.

The animals were fed with granulated (Charles River) chow and water was given *ad libitum*. Weaning was in the fourth week after birth, sexes were separated and the rats were kept in cages containing four rats of the same sex.

Receptor assays were performed when the animals were 3 months old. Thymus (from male and female rats) and uterus were used for receptor kinetic analysis (in case of uterus 9 days after ovariectomy) as models for glucocorticoid receptor and estrogen receptor studies. For each measurement three thymi and five uteri were pooled. Number of measurements are given in the Tables.

Receptor assays

Preparation of cytosol fraction
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 Coomassie Blue method.

Glucocorticoid receptor–thymus cytosol
Five hundred μg cytosolic protein was incubated with 10 nM [^{3}H]dexamethasone acetate (Amersham, UK spec. act. 1.8 Tbq/mmol) and increasing concentration of dexamethasone acetate (0, 10, 20, 40, 80, 160 and 1000 nM: Sigma, USA) in duplicates in a total volume of 100 μl at 0°C for 18 h. Bound glucocorticoid was separated by the charcoal method and counted in Turner cocktail (0.5% PPO, 0.05% POPOP, 30% efficiency). Radioactivity was measured by a Beckman scintillation counter. Radioactivity measured in the presence of 1000 nM dexamethasone was regarded as nonspecific binding.

Estrogen receptor–uterus cytosol
Three hundred μg cytosolic protein was incubated with 5 nM 2, 4, 6, 7 [^{3}H]estradiol (IZINTA, Hungary, 3.2 Tbq/mmol spec activity) and increasing concentration of estradiol (0, 2, 5, 10, 20, 50 and 1000 nM, Organon, Oss, Holland). Conditions of incubation, termination of the reaction and counting were identical to those of receptor assay on thymus cytosol.

Analysis of results
Analysis of the results were carried out by the computer program EBDA and LIGAND written by McPherson;[18,19] EBDA was used to process the new raw data. LIGAND (non-linear curve fitting program) was used to obtain final parameter estimates. Anova was performed as statistical analysis of the final parameters considering the replicate measures on each measurement of pooled tissues (Origin computer program).

Results

Thymus
Retinol treatment of nursing mothers significantly decreased the adult female and male offspring glucocorticoid receptor density (maximum binding capacity of receptors=B_{max}) related to the control thymus. Receptor affinity (K_d) increased, however the values were not significant. Retinoic acid did neither change the receptor density nor the affinity, related to the control group. However there was a significant ($P<0.01$) difference between the thymic receptor density of retinol and retinoic acid treated animals' progenies (Tables 1 and 2, Figures 1 and 2).

Uterus
There were no significant differences in the density or affinity of uterine estrogen receptors in the adult progenies of retinol or retinoic acid treated nursing mothers (Table 2).

Table 1

	Thymus, female	
Groups (n)	K_d mean (E-09)±s.e.	B_{max} mean (E-09)±s.e.
Control (5)	5.91±1.180	3.07±0.491
Retinol (4)	3.79±0.121	1.58*±0.076
Retinoic acid (3)	4.98±1.952	3.31†±0.378

*P<0.05 to control. †P<0.01 to retinol

Table 2

	Thymus, male	
Groups (n)	K_d mean (E-09)±s.e.	B_{max} mean (E-09)±s.e.
Control (4)	3.93±0.395	1.59±0.311
Retinol (3)	2.64±0.661	0.33*±0.196
Retinoic acid (3)	2.98±0.420	0.29†±0.232

*P<0.05 to control. †P<0.01 to retinol

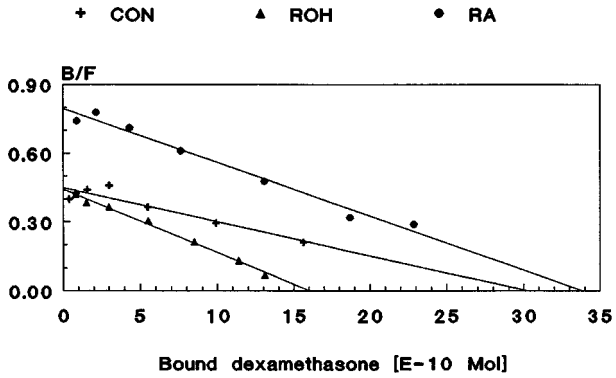

Figure 1 Scatchard plots of dexamethasone binding of female thymus glucocorticoid receptor. Details are given in Table 1

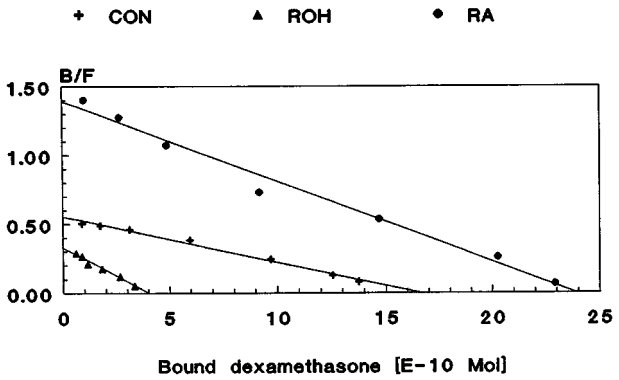

Figure 2 Scatchard plots of dexamethasone binding of male thymus glucocorticoid receptor. Details are given in Table 2

Table 3

Groups (n)	*Uterus* K_d mean (E-09) ± s.e.	B_{max} mean (E-09) ± s.e.
Control (3)	5.79 ± 0.032	1.00 ± 0.128
Retinol (4)	5.92 ± 0.622	1.09 ± 0.097
Retinoic acid (3)	6.26 ± 0.103	1.39 ± 0.141

Discussion

Retinol is a nutritional factor (vitamin A), required for many physiological processes. The active metabolites are produced by oxidation of retinol to different retinoic acids which are bound by receptors (RXRs and RARs) belonging to the steroid-thyroid receptor superfamily.[20–21] The members of this superfamily are similar, but not identical. Nevertheless in the perinatal critical period their discriminating capacity is not complete, so their ligands can imprint not only their own, but also other steroid receptors.[1–3]

In our earlier experiments a single neonatal treatment with retinol significantly increased the density of thymic glucocorticoid receptors of both sexes and the affinity of uterine estrogen receptors in adult animals.[16] Neonatal treatments with all-trans retinoic acid also increased uterine estrogen receptor affinity without changing the parameters of thymic glucocorticoid receptors. Similar treatment with retinol caused an enormous reduction of sexual activity in males' and a significant decrease in females' sexual activity.[15] The effect of retinoic acid was also weaker in these cases. Retinoid treatments also influenced the sexual hormone levels for life, causing a reduction of testosterone and progesterone serum levels.[17] Considering these observations it seemed to be reasonable to study the effect of retinoids transmitted by the breastmilk, in the adult progenies. This means that the experimental object was not exposed directly to retinoid effects and it consumed only the quantity which was secreted into the milk of the treated mother.

The results of our experiment demonstrate that the presence of retinol excess in the breastmilk could profoundly and durably influence the binding capacity of the thymic glucocorticoid receptors independently of the sex of the animals

In our earlier experiment[16] retinoic acid had weaker effect, nevertheless it was effective, in the present experiment it seemed to be neutral. This can be explained by the facts, that retinoic acid does not share all of the activities of retinol, though it is more effective in influencing morphogenesis. Moreover it has faster turnover than retinol, not being stored in the liver.[22] This latter fact means that retinoic acid acted in the most critical short period (neonatally), whereas retinol 'bombarded' the receptors continuously. In addition, all-trans retinoic acid specifically activates RAR, while the oxidized metabolites of retinol (retinoic acid isomeres) activate RXR and RAR alike.[20] We have no data considering the secretion ratio of the different metabolites into the breastmilk, which also can influence the strength of imprinting. Compared to our earlier (direct) experiments[16] the effect of retinol was weaker (now it didn't influence K_d) and in this earlier experiment the effect of retinoic acid did not reach that of retinol. It is possible, that the resistance of receptors to retinoic acid at present is only a quantitative problem.

In our earlier experiment[16] thymic B_{max} was increased in the neonatally retinoid treated animals, which is unequivocally decreased now. However this is not unusual. The direction of imprinting depends on the time of administration, the quantity of imprinter, the sex and the receptor bearing organ.[23]

The ineffectivity of lactational treatment in contrast to neonatal–direct–treatment in case of uterine estrogen receptors could be a quantitative problem as well as depend on the different sensitivity of different receptors in different situations. In other experiments[24] the differences between the uterinal and lactational exposure were also demonstrated.

The experiments show that breastmilk can transmit retinol imprinting to the offspring generation, as it was observed earlier in case of an environmental pollutant, benzpyrene.[25] Considering the frequent human medical vitamin A prophylaxis of nursing mothers, the observations highlight its dangerous consequences.

Acknowledgement

This work was supported by the National Research Fund (OTKA) T-017775, Hungary.

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