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Fetal Digoxin Treatment Enhances the Binding Capacity of Thymic Glucocorticoid Receptors in Adult Female Rats

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ABSTRACT. 1. Hormonal imprinting is provoked in the perinatal critical period in the presence of the appropriate hormone or molecules similar to it. As a consequence of hormonal imprinting, the developing receptor finishes its maturation normally (in the presence of the adequate hormone) or abnormally (under the effect of foreign molecules that are able to bind to the receptor).

2. Digoxin—which has a steroid character—caused faulty imprinting by treatments at the 15th, 17th and 20th days of pregnancy. In the adult (3-month-old) animals, the density of thymic glucocorticoid receptors was significantly elevated, whereas the density of uterine estrogen receptors was not, without any change in receptor affinity.

3. The experiments call attention to the steroid receptor imprinting effect of fetal digoxin treatment that must be considered in regard to this treatment at this period and later in regard steroid treatments. GEN PHARMAC 30;5:647–649, 1998. **© 1998 Elsevier Science Inc.**

KEY WORDS. Cardioactive glycosides, digoxin, hormonal imprinting, steroid receptors, thymus, uterus

INTRODUCTION

Hormonal imprinting takes place mainly neonatally at the first encounter between the developing receptor and the appropriate hormone (Csaba, 1980). After the first encounter, the receptor accomplishes its maturation and reaches the binding capacity characteristic of the adult. However, in this critical period, the discriminating capacity of the maturing receptor is not perfect; consequently, molecules similar to the "target" hormones can cause misimprinting that permanently affects the binding capacity of the receptor (Csaba, 1986, 1991). These molecules could be members of the same hormone family, synthetic hormone analogues or molecules not related to the hormone, yet able to bind to the receptors (Csaba, 1991, 1994). In regard to steroid receptors, steroid hormones can influence (misimprint) in this critical period of receptor development one another's receptors (Bern *et al.*, 1975, 1992; Csaba *et al.*, 1986; Inczefi-Gonda *et al.*, 1986a); even endogeneous or exogeneous molecules that are not steroid hormones but are bound by a member of the steroid-thyroid receptor superfamily [e.g., vitamins A and D or the peroxysome proliferator clofibrate Csaba *et al.*, 1995; Gaál and Csaba, 1997; Mirzahosseini *et al.*, 1996] can disturb the normal development of the steroid hormone receptors, causing a lifelong change in their binding capacity. However, excess of the appropriate hormone in the perinatal critical period also can cause faulty imprinting (Csaba, 1991).

Some molecules, not having receptors in the steroid receptor superfamily but having a steroidlike structure, also provoke faulty imprinting. Such molecules are, for example, the aromatic hydrocarbons (benzpyrene or dioxin), which can cause persistent disturbances in steroid hormone binding, steroid hormone level and sexual behavior alike and provoke morphological changes after neonatal or prenatal treatment (Birnbaum, 1995; Csaba and Inczefi-Gonda, 1984, 1993; Csaba *et al.*, 1991a, 1991b, 1993; Janz and Bellward, 1996). Because digoxin, which could be present in the maternal or fetal organism endogeneously (digoxin-like molecules) or as a consequence of the treatment of the mother or the fetus (Castaneda-Hernadez, 1989; Chavkin *et al.*, 1996; Di-Grande *et al.*, 1993; Ghione *et al.*, 1993; Kovacs *et al.*, 1989; Trigo *et al.*, 1995) also has a steroid character, it seemed worthwhile to study its effect on the steroid hormone receptor binding capacity of adults, after fetal exposure.

MATERIALS AND METHODS

Females of our closed-breed Charles River–originated Wistar rats were treated intramuscularly with a daily dose of 3 μg digoxin (Richter, Budapest) at the 15th, 17th and 20th days of pregnancy. Start of pregnancy was determined by vaginal smears. After weaning at the 4th week, the offspring received Charles River chow. Thymic glucocorticoid and uterine estrogen receptor binding capacity was studied when the animals were 3 months old.

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) animals were used for one measurement. Three measurements in case of thymus and six measurements in case of uterus were performed in each group.

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.

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TABLE 1. Saturation analysis of the thymic glucocorticoid and uterine estrogen receptors (±SD) of prenatally digoxin treated and nontreated adult female rats

Group	Receptor	K_d	B_{max}
Control	Glucocorticoid	2.54 ± 0.52	2.47 ± 0.03
Treated	Glucocorticoid	2.77 ± 1.42	4.58 ± 0.65*
Control	Estrogen	6.04 ± 4.13	6.43 ± 2.99
Treated	Estrogen	6.63 ± 7.14	8.22 ± 4.73

*$P < 0.03$.

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, specific activity 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 hr for total binding (*T*). Nonspecific binding (*NS*) was measured in the presence of 100-fold molar excess of dexamethasone acetate (Sigma, St. Louis, MO, 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, specific, activity 3.5 TBq/mmol) were incubated with rat uteri cytosol (300 μg protein) in a 0.1 ml total volume, at 0°C for 18hr for total binding (*T*). To estimate nonspecific binding (*NS*), another set of tubes containing 100-fold excess of unlabeled estradiol (Organon, Oss, Holland) for each radioligand concentration was used. All assays were performed in duplicate.

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 to each tube and then pelleting the unbound steroid by centrifugation at 1,500*g* for 15 min. Aliquots (200 μl) of the supernatants were transferred into 4 ml Optiphase "HiSafe" (Pharmacia, Lund, Sweden) for scintillation counting 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 labeled ligand: $S=T-NS$.

Analysis of results

Analysis of results was carried out by a computer program written by McPherson (1983) named EBDA and by a nonlinear curve-fitting program modified by McPherson (1985) named LIGAND. The relation between EBDA and LIGAND is as follows: EBDA is used to process the raw data, which is then expressed on the appropriate plot. The graphic representation allows initial parameter estimates to be calculated for use by LIGAND. LIGAND is used to obtain final parameter estimates. For statistical anlysis of results, the DATA-ANALYSIS V.1.0. (Statistical and Design Services 1985) computer program was used.

RESULTS AND DISCUSSION

The mothers were treated with digoxin three times in late pregnancy, the last treatment being one day before birth. Digoxin can pass across the placenta (Schmolling *et al.*, 1996), so we can speak

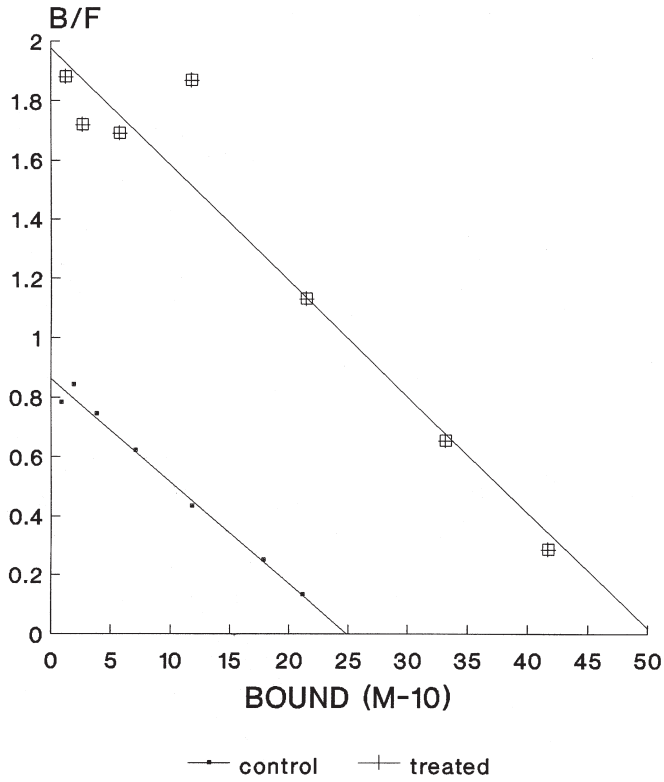

FIGURE 1. Measurement of the dexamethasone binding capacity of thymic glucocorticoid receptor. Scatchard plots of the mean values of saturation analysis data generated by EBDA. Three-month-old female rats neonatally treated or not treated (control) with digoxin. Detailed results are given in Table 1.

of a late fetal (prenatal) treatment. This treatment (imprinting) caused a significant increase (almost a duplication) of thymic glucocorticoid receptor density (B_{max}) without any change in receptor affinity (Table 1; Fig. 1). The density of uterine estrogen receptors also was elevated; however—because of the high standard deviation—it was not significant and the affinity (K_d) also did not change. Considering that only a small quantity of digoxin was given to the mothers and even less could reach the fetus, this means that (1) long-lasting (persistent) faulty steroid receptor imprinting by digoxin treatment is possible and (2) glucocorticoid receptors are more sensitive to it than estrogen receptors.

In earlier experiments, single neonatal ouabain treatment enormously increased the activity of the ouabain ATPase enzyme in adults, also causing a decrease in ouabain binding (Csaba *et al.*, 1983). At the same time, the exogeneously administered digoxin level was higher and the response of the heart to oubain was more intense in the neonatally ouabain treated adults than in the untreated controls. This means that the early treatment with cardioactive glycosides persistently influences other physiological processes, too. However, neonatal treatment with ouabain did not provoke changes in thymic glucocorticoid binding (Inzefi-Gonda *et al.*, 1986b). This shows that the fetal treatment caused a greater effect on receptor formation than did the neonatal one. Considering that digoxin is used for treating fetal arrhythmia (Trigo *et al.*, 1995) or hydrops fetalis (Chavkin *et al.*, 1996) as well as maternal heart problem during pregnancy, the results call attention to the possible side effects of these treatments in human cases, heretofore not known. These side effects could provoke an altered reaction in regard to steroid treatments.

In previous experiments, neonatal triamcinolone or diethylstilbestrol (DES) treatments significantly decreased the activity of ouabain ATPase or ouabain binding (respectively) of the adult animals's heart (Inczefi-Gonda *et al*., 1986b; 1987). This means that mutual neonatal influences can be provoked on each other's receptors by the cardioactive glycosides and steroid hormones.

Digoxin-like endogeneous materials are present in the blood and different organs of fetuses and neonates (Castaneda-Hernadez, 1989; Di-Grande *et al*., 1993; Ghione *et al*., 1993; Kovacs *et al*, 1989). The perinatal adjustment of steroid receptors happens in their presence. However, in our case, digoxin given exogenously provoked imprinting on steroid hormone receptors, imprinting that could be caused by an excess of the material (similar to the endogeneous one) or by a special quality of it (which is different from the endogeneous one). The experiments cannot settle this problem.

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