Androgenic Effects on Adrenocortical Responsiveness in Neonatal Rats

SHINTAROU YOSHIMURA[1], TOSHIRO KUBOTA[2], SATOE SUZUKI[1], SHUJI SASSA[1], HIDEKI KUDO[1] and SHINOBU SAKAMOTO[1]

[1]*Medical Research Institute and* [2]*Comprehensive Reproductive Medicine, Graduate School,*
Tokyo Medical and Dental University, Tokyo 113-8510, Japan

Abstract. *We are increasingly exposed to environmental and occupational hazardous chemicals, which modulate hormonal activity and/or mutagenicity in mammals. In the present study, we investigated the effects of sex-steroid hormones on adrenocortical responsiveness to adrenocorticotropic hormone (ACTH) in neonatal rats. The levels of corticosterone increased with the dose of ACTH in adrenal cells of males and females in vitro. Although castration markedly augmented the responsiveness in male rats, testosterone-replacement in the castrated male rats inhibited the enhancement and, furthermore, the treatment with testosterone suppressed the responsiveness in 14-day-old intact female rats, too. Castration enhanced the level of ACTH receptor mRNA to 3-fold of that in intact male rats at 14 days of age, but replacement treatment with testosterone in castrated male rats lowered the elevated levels. These findings suggest that: 1) the hyporesponsiveness of adrenocorticosteroid in the stress hyporesponsive period of neonates might be dependent on the reduction of ACTH receptor mRNA, and 2) endocrine-disrupting chemicals, with characters of androgens, estrogens or gonadotropin-releasing hormones, might affect the responsiveness to ACTH and the ACTH receptor mRNA expression levels in adrenal cells of neonates.*

We are increasingly exposed to environmental and occupational hazardous chemicals, which modulate hormonal activity and/or mutagenicity in humans and mammals. Worldwide, adverse effects of these chemicals have been reported, *e.g.* "imposex" in snails induced by

butyltin compounds (1) and decreased reproduction in alligators following exposure to "Dicofol" (2). In 1961, Huggins *et al.* reported that a single feeding of polynuclear hydrocarbons induced mammary cancer in rats (3). Herbst *et al.*, in 1971, warned of diethylstilbestrol as a hazardous chemical in pregnant women, *i.e.* maternal therapy using diethylstilbestrol induced adenocarcinomas of the vagina in young women (4), and then Bern *et al.* demonstrated the relationship of the chemical to the disease in neonatal mouse models (5).

Development of the hypothalamo-pituitary-adrenal (HPA) axis is required for adaptation to extra-uterine life in mammals. Adrenocortical hormones in the HPA axis are important for self-preservation. Following a surge in fetal glucocorticoid levels, there is hyporesponsiveness to ACTH in the HPA axis after parturition. This period has been recognized as the stress hyporesponsive period (SHRP) (6, 7). The HPA axis is known to respond moderately to stress at around 1-2 days of age in rats. This responsiveness is reported to decrease and reach a nadir by 4-11 days and then increases to a level similar to that of the adult by 21 days of age (6). Neonatal handling reduced the HPA responsiveness to stress in male, but not in female rats (8, 9). There might be sex differences in stress responsiveness after stimulation in the perinatal environment *in vivo*. The mechanism of this differential gender-specific vulnerability to the same perinatal environment may be related to the perinatal sex steroid milieu.

In the present study, we investigated the effect of endogenous and/or exogenous sex steroid hormones on the adrenocortical response to ACTH in rat neonates of both sexes and the expression levels of ACTH receptor mRNA in rat adrenal glands.

Materials and Methods

Chemicals. The following reagents were obtained from commercial sources: bovine serum albumin (fraction V), HEPES, collagenase (type I), deoxyribo-nuclease I, 3-isobutyl-1-methylxanthine

Correspondence to: Shinobu Sakamoto, MD, PhD, Medical Research Institute, Tokyo Medical and Dental University, 1-5-45, Yushima, Bunkyo-ku, Tokyo 113-8510, Japan. Tel: +81-3-5803-5837, Fax: +81-3-5803-5853, e-mail: motoend@tmd.ac.jp

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(IBMX), L-glutamine, testosteronepropionate (T) and 17β-estradiol (E) were from Sigma Chemical Co., St. Louis, MO, USA, Eagle's MEM (Nissui Pharmaceutical Co., Tokyo, Japan), ACTH (Cortrosyn, ACTH1-24, N.V. Organon, The Netherlands) and [³H] corticosterone (80.5Ci/mmol) was obtained from Dupont New England Nuclear Research Products, Boston, MA, USA. Penicillin G potassium and streptomycin sulfate were from Meiji Seika, Tokyo, Japan, and RT kit (Invitrogen Co. CA, USA), PCR Taq (AmpliTaq Gold, Perkin Elmer, CA, USA). Primers for rat MC-2 (ACTH-R), MC-5 and β-actin were from Sawady Technology, Tokyo, Japan.

Animals and procedures. All neonatal male and female Sprague-Dawley rats (SLC, Shizuoka, Japan) were born in the Animal Care Facility of the Tokyo Medical and Dental University (Tokyo, Japan). Pregnant and postpartum rats were kept under standardized conditions with lights on between 06:00 and 20:00 h. Food and water were given *ad libitum*. Male and female neonates from different litters (n=5/group/sex) were sacrificed by decapitation on days 1, 7, 14, 21 and 35 with the date of birth designated as day 1. All procedures were carried out between 09:00-11:00 a.m. to avoid diurnal variations in measured neuroendocrine parameters. To define the effects of androgen on adrenal responsiveness, each animal was given daily subcutaneous injections at 7 μg/g body weight of T or 0.3 mg/g body weight of E for 7 days; *e.g.*, Levine *et al.*, in their experiments, injected 0.5 mg of estradiol benzoate and 1.0 mg of T to rat pups 96 h and 120 h after birth, respectively (10). Control animals were given daily subcutaneous injections of sesame oil as a control vehicle for 7 days according to the same procedure. These animals were sacrificed by decapitation without stress at the age of 14 days. All experimental procedures conformed to the regulations described in the U.S. NIH Guide to the Care and Use of Laboratory Animals.

Adrenal glands were obtained from neonatal (each sex, ages 1,7,14 and 21 days) and pubertal (35 days) rats after decapitation. Day 1 was defined as the period 10-24 h after delivery. Adrenocortical cells were isolated by the method of Kumai *et al.* (11). Briefly, adrenal glands were cut into small pieces and enzymatically dispersed with 1% collagenase and 0.25% deoxyribonuclease in a Ca^{2+}- and Mg^{2+}-free balanced salt solution (CMF) for 40 min at 37°C with shaking. Cells were washed with 0.2% BSA-CMF and filtered. The crude cell suspension was centrifuged for 10 min at 180 x g. The pellet was resuspended with Eagle's MEM supplemented with BSA (0.2%), L-glutamine (0.29 mg/ml), penicillin G potassium (100 U/ml) and streptomycin sulfate (10 mg/ml), which will subsequently be referred to as the culture medium. The number of cells and viability (95%) were determined by the Trypan blue exclusion test.

The adrenal cells ($2x10^5$ cells/ml, $5x10^4$ cells/well) were placed onto 24 multi-well culture plates with culture medium (Costar, Cambridge, MA, USA). The cell cultures were maintained at 37°C in a humidified atmosphere of 95% air and 5% CO_2. After preincubation for 18 h, the cells were cultured for 3 h with or without the indicated concentrations of ACTH in the presence of the phosphodiesterase inhibitor IBMX (0.1 mM). Extracellular accumulation of cAMP and corticosterone in the incubation medium containing adrenal cells, which were obtained and isolated from male and female rats of age 7, 14, 21 and 35 days, at final concentrations of 0, 0.1, 1.0, 10 and 100 ng/ml ACTH, was examined after incubation for 180 min.

Assay for corticosterone. Concentrations of the extracellular corticosterone were determined in aliquots taken from the culture medium immediately after incubation. Each sample was stored at -80°C until analysis. The corticosterone concentration was determined in nonextracted medium, as previously described (11). The coefficients of the intra- and inter-assay variations in each radioimmunoassay (RIA) were smaller than 10%.

Expression levels of ACTH receptor mRNA. Neonatal rats (10-40) on days 7, 14, 21, 28 and 35 after birth were decapitated and adrenal glands were removed as quickly as possible and snap-frozen in liquid nitrogen. Total RNA was prepared according to the acidic phenol/chloroform method of Chomczynski and Sacchi (12). The concentration of total RNA was estimated by the absorbance at 260 nm (1 OD unit was estimated at 40 μg/ml) using a spectrophotometer (U2000A Hitachi, Tokyo, Japan). Each sample of total RNA had an optical density ratio of 260: 280 between 1.80 and 2.50. Aliquots were randomly loaded on a 1.5% agarose gel to compare the relative intensity of bands of 28 S and 18 S ribosomal RNAs. Similar intensities were observed in all samples (data not shown). Total RNA (0.1 μg) was converted to cDNA using oligo (dT) according to the manufacturer's recommendations (SUPER SCRIPT Preamplification System, GIBCO BRL). The primers specified for rat ACTH-R and β-actin were, ACTH-R forward 5'-GGGCAGTCTGTATAAGAT-CTTGG-3', reverse 5'-TCA-AAGGGAACAGCGATGTGAAG-3' (product size 338 bp); β-actin forward 5'-AGGCCCAGAGCAAGA GAGGCAT-3', reverse 5'-CATGGCTGGGGTGTTGAAGGTC-3' (product size 227 bp). After an initial denaturation for 7 min at 94°C, PCR was performed at 94°C for 40 sec, 55°C for 40 sec and 72°C for 40 sec for 32 cycles. After the end of the final cycle, the reactions were extended for an additional 7 min at 72°C, then cooled to 4°C. Relative gene expressions were determined by densitometry using an image analyzer (AE-6920-MF Densitograph, ATTO, Tokyo, Japan).

Statistical analysis. All values were expressed as the mean±S.E.M. Differences in these values were analyzed using one-way analysis of variance (ANOVA). Tukey's *post hoc* test was used for multiple comparisons of means and the establishment of statistical significance, which was accepted at a value of $p<0.05$.

Results

Extracellular levels of corticosterone. Extracellular levels of corticosterone in the medium incubated with adrenal cells were evaluated after incubation for 180 min. The levels of corticosterone increased linearly with the dose of ACTH in 14-day-old males and females (Table I). The responsiveness to ACTH in the adrenal cells obtained from 14-day-old female rats was markedly enhanced compared with that in the male ($p<0.01$ and 0.05) (Table I).

Effects of sex-steroids on extracellular corticosterone levels. The *in vitro* responsiveness to ACTH in adrenal cells obtained from 14-day-old neonates following 7-day treatment with E or T was examined in each counter sex (Figure 1). The 7-day treatment with E in male neonates did not affect the response to ACTH in adrenal cells, but the *in vitro* responsiveness to ACTH in adrenal cells of female

Table I. *Effects of various concentrations of adrenocorticotropic hormone (ACTH: 0, 0.1, 1.0, 10.0 and 100 ng/ml of medium) on the levels of corticosterone in medium following 3-h incubation of isolated adrenal cells removed from male and female rats at the age of 14 days.*

ACTH (ng/ml of medium)	Corticosterone ($mg/2x10^5$ cells/3 h)	
	Female	Male
0	4.8±0.4	3.76±0.63
0.1	11.0±0.6	4.03±0.54*
1.0	16.1±1.0	4.78±0.56*
10.0	23.3±0.9	7.56±0.54**
100	30.6±1.3	9.60±0.90**

Data are the mean±S.E.M.
** and *significantly different from that of female; $p < 0.01$ and 0.05, respectively.

neonates was markedly reduced by additional treatments with T ($p < 0.01$) compared with that in the untreated control females (Figure 1).

Effects of castration and androgenic replacement on extracellular corticosterone levels in male rats. Castration markedly augmented the responsiveness to ACTH in terms of corticosterone levels in male rats ($p < 0.05$) (Figure 2), *i.e.* the level of adrenal responsiveness in castrated male rats approached that in the intact female rats. However, the replacement treatment with T in castrated male rats led to a reduction of corticosterone levels ($p < 0.05$), *i.e.* the level of adrenal responsiveness in castrated-androgenized rats approached that of the intact male rats.

Expression levels of adrenal ACTH receptor mRNA in 14-day-old male and female rats, and effects of castration and androgenic replacement in male rats. Relative expression levels of ACTH receptor mRNA (ACTH receptor mRNA/β-actin mRNA) in adrenal glands of the 14-day-old male, female and castrated male rats with or without androgenic replacement treatment are indicated in Table II. The expression level of ACTH receptor mRNA at 14 days in the adrenal cells in male rats markedly differed from that in the female rats ($p < 0.01$). Castration in male rats at the age of 7 days enhanced the ACTH receptor mRNA levels to approximately 3-fold that of the intact 14-day-old male rats ($p < 0.01$). However, T replacement for a subsequent 7 days in castrated 7-day male rats led to a reduction of the levels ($p < 0.01$), *i.e.* similar to that in the intact male rats (Table II).
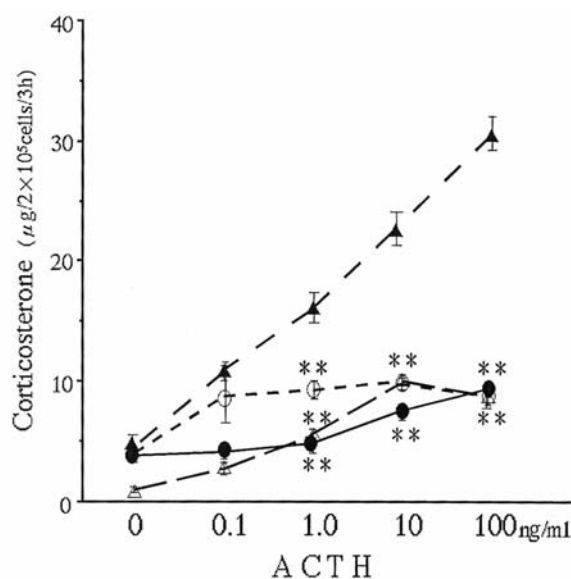

Figure 1. *Effects of 7-day treatment with testosterone (T) or estradiol (E) in vivo on extracellular corticosterone levels of isolated adrenal cells obtained from 14-day female and male rats. Male and female control rats were given daily subcutaneous injections of sesame oil as a control vehicle for 7 days. Intact control male rats (a closed circle and a solid line), intact male rats with 7-day treatment with E (an open circle and a dotted line), intact control female rats (a closed triangle and a dotted line) and intact female rats with 7-day treatment with T (an open triangle and a dotted line). Data are the mean ±S.E.M. **Significantly different from the corresponding value; $p < 0.01$.*
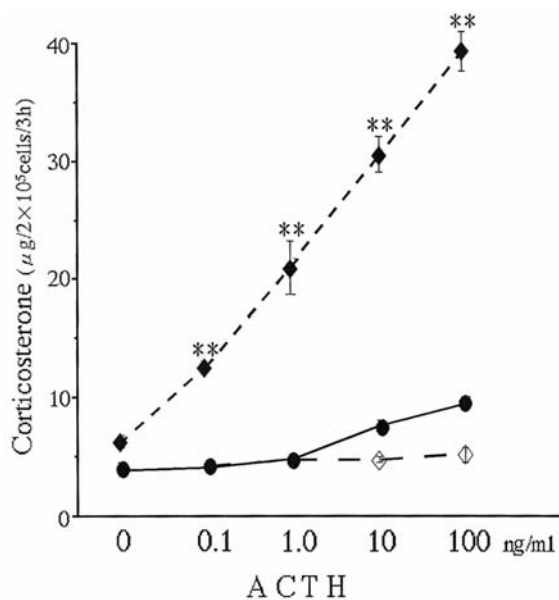

Figure 2. *Effects of castration and 7-day replacement treatment with testosterone (T) in vivo on extracellular corticosterone levels of isolated adrenal cells obtained from 14-day male rats. Intact control male rats (a closed circle and a solid line), castrated control male rats (a closed diamond and a dotted line) and castrated male rats with 7-day treatment with T (an open diamond and a dotted line). Data are the mean±S.E.M. **Significantly different from the corresponding value; $p < 0.01$.*

Table II. *Effects of castration and the 7-day compensatory treatment with testosterone (T) on the expression of ACTH receptor mRNA (ACTH receptor mRNA/β-actin mRNA) in adrenal cells of 14-day-old male rats. Intact and castrated control male and intact control female rats were given daily subcutaneous injections of sesame oil as a vehicle for 7 days.*

Groups	ACTH receptor mRNA (ACTH receptor mRNA/β-actin mRNA)
Castrated control male rats	0.558±0.014
Testosterone-replaced castrated male rats	0.258±0.020*
Intact control male rats	0.201±0.010*
Intact control female rats	0.356±0.025*,a

Data are the mean±S.E.M.
* and a significantly different from those of the castrated control male rats and the intact control male rats, respectively; $p<0.05$

Discussion

In northern Thailand, alcohol containing seven kinds of plants has traditionally been used by natives to improve libido. We demonstrated that the extracts from the seven plants decreased the number of sperm with reduction of serum testosterone levels in male rats (13). In female rats, a gonadotropin-releasing hormone agonist induced bone loss (14), which was prevented by treatment using a herbal medicine with a slight increase of circulating estrogen levels (15). In virgin mice of the SHN strain, with a high potential for the development of mammary cancer and uterine adenomyosis, the combined administration of conjugated estrogens and medroxyprogesterone acetate completely suppressed the development of uterine adenomyosis, enhanced the bone mineral density in the femur and slightly shortened the latent period of mammary carcinogenesis (16). The components extracted from plants or herbal medicines may contain a biologically active substance such as a phytoestrogen, which is known to be one of the environmental hormones or endocrine-disrupting chemicals. On the other hand, many synthetic steroids are not resolved into harmless products and remain on the earth as endocrine-disrupting chemicals. Such substances are known to induce hormonal disorders, *e.g.* amenorrhea, hypospermatogenesis, infertility and carcinogenesis in hormone-dependent organs (17-20).

In the present study, the adrenocortical responsiveness to ACTH in female neonates seemed to be higher than that in males with testicular testosterone. Prepubertal castration of male rats and hamsters was reported to increase the adrenal

hormone response to physical stress (21). A single injection of testosterone was effective in restoring corticosterone secretion to the level of intact male rats. Castration in adult male rats increased the secretion of ACTH as well as corticosterone in response to both physical and psychological stress (22). It was observed that castration led to an increase in hypothalamic corticotropin-releasing hormone (CRH) levels (23) and the number of immunocytochemically identified CRH neurons (24). These findings suggest that the androgenic effects on the HPA axis are mediated *via* alterations in paraventricular CRH levels. In our study, sex differences were observed in adrenal ACTH receptor (ACTH-R) mRNA expression levels in immature rats. The responsiveness to ACTH and the ACTH-R mRNA expression levels in castrated male neonates were recovered by re-adding androgens. Thus, testicular androgens might have a suppressive effect on the ACTH and/or ACTH-R mRNA of adrenal cells in neonatal rats.

In summary, these findings suggest that: 1) the hyporesponsiveness of adrenocorticosteroid in SHRP might be dependent on the reduction of ACTH receptor mRNA, and 2) endocrine-disrupting chemicals with characters of androgens, estrogens or gonadotropin-releasing hormones might affect the responsiveness to ACTH and the ACTH receptor mRNA expression levels in adrenal cells of neonates.

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