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SEX DIFFERENCES IN ADRENOCORTICAL STRUCTURE AND FUNCTION—XX. THE EFFECTS OF GONADECTOMY AND TESTOSTERONE OR ESTRADIOL REPLACEMENT ON CHOLESTEROL CONTENT AND DISTRIBUTION IN THE GLAND[1]

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Summary—Sex differences in the distribution of free and esterified cholesterol in the mitochondria and lipid droplets of the rat adrenal glands, as well as their dependence on gonadal hormones were studied. For these purposes intact, gonadectomized and gonadectomized-testosterone or estradiol replaced rats were employed.

The concentration of free cholesterol [FC] as well as esterified cholesterol [EC] in full homogenates of decapsulated glands was higher in male than in female rats. Neither orchiectomy nor testosterone replacement had an effect on FC and EC concentration. Ovariectomy increased FC and EC concentration in full adrenal homogenates, an effect reversed by estradiol replacement. Similar changes were found in the lipid droplets. The concentration of FC and EC in the adrenal mitochondria was higher in male than in female rats. Orchiectomy increased the concentration of FC and decreased the concentration of EC. Testosterone administration resulted in further increase in FC concentration and restored EC concentration to the level of the control group. On the other hand ovariectomy increased FC and EC concentration, an effect reversed by estradiol replacement.

Results obtained clearly showed that sex differences in FC and EC concentration in rat adrenal gland depend mainly on estradiol which lowers FC and EC concentration in the adrenal lipid droplets and mitochondria.

INTRODUCTION

In the adrenocortical cells free cholesterol (FC), originating from serum lipoproteins or synthesis *in situ*, can either be metabolized to steroid hormones or esterified with fatty acids in the reaction catalysed by acyl–CoA–cholesterol acyl transferase (EC 2.3.1.26) [1–12]. Esterified cholesterol (EC), located mainly in cytosolic lipid droplets, provide a depot from which free cholesterol is liberated by the action of hormone-sensitive cholesterol esterase (EC 3.2.1.13) [3, 4, 10, 11, 12]. The conversion of FC to steroid hormones is preceded by its transport into the mitochondria where the side chain of cholesterol is cleaved in the reaction requiring NADPH + H^+ and molecular oxygen, and catalysed by cholesterol desmolase [2, 6, 7]. Recent reports suggest that the rate limiting step in steroid hormone synthesis is the supply of cholesterol for the mitochondrial side-chain cleavage enzyme rather than the activity of the enzyme itself, and in addition this step is stimulated by ACTH through the mediation of cyclic adenosine-monophosphate (cAMP) [1–4, 6, 7].

Gonadal hormones influence not only the corticotropin releasing hormone (CFR) content in hypothalamus, and corticotropin (ACTH) output from the pituitary gland, but also affect adrenal steroidogenesis and peripheral metabolism of the adrenal steroids [13, 14, 15]. The consequence of this multi-side action of gonadal hormones in rat is higher secretion of the main glucocorticoid—corticosterone—in female than in male [13, 15–18]. However so far the effects of gonadal hormones on the size and distribution of the pools of steroid hormone precursor—cholesterol within the adrenocortical cells were not thoroughly investigated.

The present report was designed to study the possible sex differences in the concentration of cholesterol in the adrenal glands of male and female rats as well as cholesterol distribution between the lipid droplets and mitochondria. An attempt was also made to explain the dependency of these differences upon gonadal hormones.

EXPERIMENTAL

Adult rats of Wistar strain were maintained under standardized conditions of light (14 h on/10 h off) and temperature (22 ± 2°C) with free access to laboratory pellets and tap water. Gonadectomy was per-

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formed on animals weighing 140–150 g. Ten weeks after the surgery some of the orchiectomized rats were injected with a single dose of depo testosterone (Testoviron Depot, Schering A. G., Berlin/Bergkamen) 5 mg per 100 g body weight, and ovariectomized rats with estradiol cypionate [17β-cyclopentylopropionate ester of estradiol, The Upjohn Co., Kalamazoo, MI, U.S.A.) 100 μg per 100 g body weight. The investigations were performed 2 weeks after gonadal hormones replacement.

The animals were sacrificed by decapitation under quiescent conditions and the adrenal glands were quickly removed, decapsulated and placed in ice-cold 0.154 M KCl.

One adrenal gland from each animal was homogenized in 1.5 ml of Krebs–Ringer bicarbonate buffer pH 7.4 in a Potter–Elvehjem homogenizer. Samples were taken for estimation of protein concentration [19], and the lipids were extracted by the method of Folch *et al.*[20]. The aliquots of the extracts were spotted onto chromatoplates covered with silica-gel H (Merck A. G., Darmstadt, F.R.G.) and developed in petroleum ether–diethyl ether–acetic acid (70:30:1). The extraction was repeated 3 times. The bands corresponding to EC were scrapped off, saponified and extracted with 20 ml of petroleum ether. The extraction was repeated 3 times. Under these conditions the recovery of free and esterified cholesterol from the different cellular compartments exceeded 98% as indicated from the recovery of tracer amounts of [^{14}C]cholesterol and [^{3}H]cholesterol oleate added before the extraction. The concentration of cholesterol in fractions was determined by o-phtalaldehyde method [21].

The other adrenal glands from two animals were combined and homogenized in 4 ml of 0.25 M sucrose, pH 7.4, and the mitochondrial fraction and lipid droplets were obtained as previously described [5]. Lipids were extracted from the mitochondrial pellet and from the lipid droplets with 20 vol of chloroform–methanol (2:1) and FC as well as EC were separated and quantitated as above.

The results were evaluated statistically by the method of Duncan and Student's t-test [22].

RESULTS

Body weights and adrenal weights

Orchiectomy resulted in a decrease of the body weight of the rats whereas the adrenal glands were heavier than in the control group. Testosterone replacement increased the body weight of the rats to the level of the control group, whereas the adrenal glands were lighter than in orchiectomized rats and no difference in the weight of the glands was found in comparison with the control group. On the other hand ovariectomy resulted in an increase in the weight of the body and in the decrease in the weight of the adrenal glands of the rats while estradiol replacement decreased the weight of the body and increased the weight of the adrenal glands to the levels of the control group (Table 1).

Concentration of free and esterified cholesterol in the whole adrenal homogenates

The concentration of FC as well as EC in full homogenates was higher in male than in female rats, whereas the ratio of EC/FC was higher in female rats.

Table 1. The effect of gonadectomy and gonadal hormone replacement on body and adrenal weight and on free (FC) and esterified (EC) cholesterol concentration in the adrenal gland of the rat

	Control males	Orchiectomy	Orchiectomy + testosterone	Control females	Ovariectomy	Ovariectomy + estradiol
Number of rats	7	8	8	7	8	7
Body weight [g]	434 ± 9	376 ± 7^{b}	409 ± 14^{c}	260 ± 5^{C}	340 ± 8^{b}	282 ± 10^{d}
Adrenal weight						
[mg]	53.11 ± 1.52	60.15 ± 2.30^{a}	56.97 ± 1.84	69.74 ± 3.75^{C}	58.75 ± 2.56^{a}	69.63 ± 2.29^{c}
[mg/100 g]	12.25 ± 0.27	15.97 ± 0.52^{b}	$14.02 \pm 0.66^{a,c}$	26.73 ± 1.15^{C}	17.34 ± 0.91^{b}	24.80 ± 0.93^{d}
Whole homogenate						
FC	5.7 ± 0.3	6.5 ± 0.3	6.2 ± 0.2	4.3 ± 0.3^{B}	8.3 ± 0.4^{b}	$5.8 \pm 0.2^{b,d}$
EC	83.3 ± 4.9	80.1 ± 3.0	80.7 ± 2.5	66.1 ± 4.9^{B}	82.7 ± 4.3^{b}	60.4 ± 1.8^{d}
EC/FC ratio	14.7 ± 0.2	12.2 ± 0.2^{b}	$12.9 \pm 0.1^{b,c}$	15.5 ± 0.2^{A}	10.0 ± 0.1^{b}	$10.5 \pm 0.1^{b,c}$
*Mitochondria**						
FC	2.35 ± 0.06	4.70 ± 0.07^{b}	$6.15 \pm 0.11^{b,d}$	1.20 ± 0.01^{C}	2.60 ± 0.03^{b}	$1.61 \pm 0.01^{b,d}$
EC	4.23 ± 0.03	3.08 ± 0.04^{b}	4.34 ± 0.03^{d}	3.27 ± 0.02^{C}	5.89 ± 0.06^{b}	$4.34 \pm 0.04^{b,d}$
EC/FC ratio	1.79 ± 0.04	0.65 ± 0.01^{b}	0.71 ± 0.01^{b}	2.70 ± 0.03^{C}	2.27 ± 0.03^{b}	2.71 ± 0.03^{d}
*Lipid droplets**						
FC	9.98 ± 0.22	9.12 ± 0.18	9.22 ± 0.44	6.67 ± 0.05^{C}	7.12 ± 0.01^{b}	$6.94 \pm 0.04^{b,d}$
EC	42.82 ± 0.50	39.25 ± 0.53	41.38 ± 0.53	35.46 ± 0.06^{C}	47.97 ± 0.38^{b}	35.86 ± 0.23^{d}
EC/FC ratio	4.29 ± 0.03	4.31 ± 0.05	4.51 ± 0.11	5.31 ± 0.05^{C}	6.73 ± 0.05^{b}	$5.17 \pm 0.02^{b,d}$

The concentration of FC and EC is given in μg/mg protein of the cell.
*Four estimations per group.
Statistical evaluation of results:
control females versus control males (Student's t-test): A – $P < 0.02$; B – $P < 0.01$; C – $P < 0.001$.
Multiple range test of Duncan:
a: differ from corresponding control value $P < 0.05$.
b: differ from corresponding control value $P < 0.01$.
c: differ from corresponding value of gonadectomized rats $P < 0.05$.
d: differ from corresponding value of gonadectomized rats $P < 0.01$.

Orchiectomy did not change FC and EC concentration and decreased the EC/FC ratio. Also testosterone replacement had no effect on EC and FC concentration however the ratio EC/FC increased in comparison with the group of orchiectomized animals.

Ovariectomy resulted in an increase in FC and EC concentration in the adrenal gland, and this effect was reversed by estradiol replacement. The ratio EC/FC also decreased after ovariectomy whereas estradiol replacement increased this ratio.

Concentration of free and esterified cholesterol in the mitochondria

The concentration of FC and EC in the adrenal mitochondria was higher in male than in female rats while the reverse was true for the ratio EC/FC.

Orchiectomy resulted in increasing the concentration of FC and decreasing the concentration of EC. Testosterone replacement resulted in further increase in mitochondrial FC concentration, while the concentration of EC was brought to the level of the control group.

The concentration of FC in the mitochondria of ovariectomized rats was higher than in the control group whereas in estradiol injected ovariectomized rats FC concentration was lowered as compared with spayed animals. Ovariectomy resulted also in increasing EC within the mitochondria, and this effect was also reversed by estradiol. The ratio EC/FC was lower in the adrenal mitochondria of ovariectomized rats than in the control group and was brought to the level of the control group by estradiol replacement.

Concentration of free and esterified cholesterol in the lipid droplets

The concentration of EC and FC in the lipid droplets from the adrenal gland was lower in female than male, whereas the ratio EC/FC was higher in female rats. Neither orchiectomy nor testosterone replacement had an effect on FC and EC concentration as well as on the ratio of EC/FC in the lipid droplets. Ovariectomy increases FC and EC in the adrenal gland as well as the ratio EC/FC whereas estradiol replacement lowers the concentration of FC and EC to the level of the control group.

DISCUSSION

Several investigators have reported the figures regarding the concentration of cholesterol in whole adrenal glands of male and female rats, but the results of various reports are very inconsistent. Coleman *et al.*[23], Ostwald *et al.*[24] and Chen[25] have reported higher cholesterol concentration in the adrenal glands of female than male rats, whereas other authors have reported no differences in the adrenal cholesterol concentration in rats of different sex [26]. It has also been reported that orchiectomy or ovariectomy in rats results in an increase of total cholesterol concentration in the adrenal glands [13, 23], although according to some reports [26, 27] there is no change in total cholesterol concentration in the adrenal glands following gonadectomy. There are also some discrepancies between the results of various investigators concerning the effects of testosterone or estradiol injection in intact or gonadectomized rats on cholesterol concentration in the cortex of the adrenal gland. According to some reports [29] testosterone injection lowers total cholesterol concentration in rat adrenals, according to others elevates it [28] or does not have any effect [26]. On the other hand according to various authors estradiol can either lower [26] or elevate [27], and diethylstilboestrol lowers [30] cholesterol concentration in the adrenal gland of the rat.

Our experiments have indicated distinct sex differences in the concentration of FC and FC in full homogenates as well as in the mitochondria and lipid droplets of the cortex of the adrenal gland of rats. In the present study decapsulated adrenals have been used, thus our results provide information regarding fasciculata and reticularis zone i.e. the sites where mainly glucocorticoids are synthesized. Under the conditions used FC and EC concentration as well as EC/FC ratio in full homogenates, mitochondria and lipid droplets are lower in female than in male rats. As the experiments with gonadectomy and sex hormone replacement show, these differences can mainly be attributed to the effect of estrogens, which results in lowering adrenal cholesterol concentration, whereas testosterone increased the concentration of FC and EC in the mitochondria, although the effect on the cholesterol concentration in full homogenates and lipid droplets cannot be demonstrated.

Interesting from the point of view of the adrenal steroidogenesis are the measurements of FC concentration in the adrenal mitochondria and EC in the lipid droplets, according to our results lower in female than in male rats. Lower concentration of free cholesterol in the mitochondria of female rats can most probably result from higher conversion of FC into pregnenolone as reported [13, 15, 31]. Also the concentration of cytochrome P-450, which is a terminal electron acceptor in steroid hydroxylation, is higher in female than in male rats [32, 33, 34] and this can also be ascribed to higher concentration of ACTH in blood of female rats [13], since the concentration of cytochrome P-450 in the adrenal mitochondria is controlled by ACTH. However, in the present experiment it has not been possible to correlate precisely the relation of FC concentration in the mitochondria with the adrenal steroidogenesis, as reflected by pregnenolone synthesis (in the mitochondria). It is known that after orchiectomy pregnenolone synthesis increases and this effect is abolished by testosterone replacement [13, 15, 31]. Our results show increased FC concentration in mitochondria of orchiectomized animals. Accordingly neither ovariectomy nor estradiol replacement change the synthesis of pregnenolone in rat adrenal mitochondria [13, 15, 31]. Despite that in present experiments distinct differences in FC concentration in the mitochondria have been found.

Lipid droplets contain mainly cholesterol esters from which free cholesterol is liberated through the action of cholesterol esterase and this cholesterol can be transported into the mitochondria. In this context of interest may also be our earlier results on the activity of cholesterol esterase in the adrenal gland of rats of different sex, as well as the effects of gonadectomy and gonadal hormone replacement on the activity of this enzyme [35]. In male rats the activity of cholesterol esterase is much higher than in female, and this effect is possibly dependent on the suppression of the enzyme activity by estrogens, since the activity increases in ovariectomized rats and decreases upon estradiol replacement, while in male rats neither orchiectomy nor testosterone replacement have an effect on the activity of the enzyme. Our present observations on the concentration of FC and EC in the lipid droplets of male and female rats as well as the effects of gonadectomy and hormone replacement are consistent with these findings since in the adrenal glands of male rats there is no change in EC concentration in the lipid droplets after orchiectomy or hormone replacement, while in female ovariectomy caused an increase in EC concentration in the lipid droplets whereas opposite effect has been observed after estradiol replacement.

The lack of correlation between the concentration of FC in full homogenates, mitochondria and the lipid droplets in control, gonadectomized as well as hormone replaced animals, and pregnenolone synthesis, as discussed earlier, may indicate that the production of pregnenolone as affected by sex hormones, takes place at the stages of steroidogenesis following hydrolysis of cholesterol esters and the transport of free cholesterol into the mitochondria. Some experimental data indicate that the site of action of sex hormones can be the binding of cholesterol to cytochrome P-450 or to the whole enzyme complex of cholesterol desmolase, although other reports indicate that the content of this enzyme can be affected by sex hormones [36, 37]. Testosterone treated rats in which high concentration of cholesterol has been found in the mitochondria can serve as an example to illustrate this viewpoint. It is known that testosterone increases ACTH concentration in the serum [14] and lowers adrenal weight and pregnenolone synthesis in comparison with orchiectomized animals. Thus the effect of testosterone on the adrenocortical cells resembles experiment in which the animals are treated with ACTH and aminoglutethimide, an inhibitor of cholesterol desmolase [1]. In this case one may observe the decrease in EC in the lipid droplets, increased concentration of FC within the mitochondria and despite that lower pregnenolone production.

Thus, our experiments have indicated distinct sex differences in the concentration of FC and EC in full homogenates as well as in the mitochondria and lipid droplets of the zona glomerulosa devoid cortex of the adrenal gland of rat with lower values in females than males. These sex-differences depend mainly upon estradiol which lowers FC and EC concentration in the adrenal lipid droplets and mitochondria while near to physiologic dose of testosterone exerts no marked effect on adrenal FC and EC concentration.

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