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LYSOSOMAL ENZYME ACTIVATION BY STEROID HORMONES *IN VIVO*

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SUMMARY

A series of 23 hormonal steroids and stilbestrol were examined for effects on β-glucuronidase activity of autolysed lysosome preparations from rat preputial glands. Compounds were administered into the saphenous vein of ovariectomized, or ovariectomized and adrenalectomized, animals 15 min before dissection of preputial glands. Lysosome-rich fractions of homogenates were obtained by differential centrifugation, and β-glucuronidase activity was measured before and after autolysis.

Minimum doses of sex hormones required to induce statistically significant ($P < 0.05$) increases in enzyme activity were (in μg/kg l.b.w.): estradiol-17β 0·05, testosterone 1·0, progesterone 5. Minimum dose of cortisol required for lysosomal stabilization was 100 μg/kg. Simultaneous administration of a sex hormone with a corticosteroid yielded less labilization of lysosomes than the sex hormone alone.

For induction of lysosome labilization in this assay system, steroids required two oxygenic functions per molecule: one at C-3 and a second at either C-17 or C-20. Stabilization required a third group at C-11. Groups in these positions in the α-configuration were generally inactive; only β-substituents were associated with significant changes in β-glucuronidase activity.

INTRODUCTION

NATURAL and synthetic sex hormones labilize lysosome membranes and activate lysosomal enzymes *in vitro* and *in vivo*, while corticosteroids have the reverse effect [1, 2]. While there have been several attempts to establish structure-activity relationships for effects of steroids on lysosomes [1], few satisfactory conclusions have been drawn due to the wide variety of experimental conditions used by different workers.

Szego *et al.* [3] have shown that rat preputial glands are particularly rich in lysosomes and respond dramatically to exogeneous sex hormones within a few minutes of administration. The present work was conducted to compare effects of various steroids on preputial gland lysosomal enzymes *in vivo*.

MATERIALS AND METHODS

Animals

Sprague–Dawley rats were ovariectomized at 6 weeks of age and used in experiments 1–2 weeks later. For some experiments, both adrenal glands were also removed. Each steroid was tested on groups of 10 animals.

Steroids

All compounds were checked for purity before use by thin-layer chromatography. Solutions were prepared in the minimum volume of ethanol, then diluted with normal saline. Ethanol concentration of the final solutions was 1% (v/v). Steroid solution (0·5 ml), or saline containing 1% ethanol, was administered into

the saphenous vein of rats under sodium pentobarbital anaesthesia (50 mg/kg body weight).

The following compounds were tested:

(a) *Estrogens.* Estradiol-17β (1,2,3,5(10)-estratriene-3, 17β-diol), estradiol-17α (1,3,5(10)-estratriene-3,17α-diol), ethinylestradiol (17α-ethinyl-1,3,5(10)-estratriene-3,17β-diol), stilbestrol (3,4-di-p-hydroxyphenylhex-3-ene).

(b) *Androgens.* Testosterone (17β-hydroxy-4-androsten-3-one), dihydrotestosterone (17β-hydroxy-5α-antrostan-3-one) dehydroepiandrosterone (3β-hydroxy-5-androsten-17-one), etiocholanolone (3α-hydroxy-5β-androstan-17-one), epitestosterone (17α-hydroxy-4-androsten-3-one), fluoxymesterone (9α-fluoro-11β,-17β-dihydroxy-17α-methyl-4-androsten-3-one).

(c) *Progestogens.* Progesterone (4-pregnene-3,20-dione), pregnanolone (3β-hydroxy-5β-pregnan-20-one), allopregnanolone (3β-hydroxy-5α-pregnan-20-one), epipregnanolone (3α-hydroxy-5β-pregnan-20-one), medroxyprogesterone acetate (17α-acetoxy-6α-methyl-4-pregnen-3,20-dione), norethisterone (17β-hydroxy-17α-ethinyl-4-estren-3-one), norgestrel (17β-hydroxy-17α-ethinyl-18-methyl-4-estren-3-one), lynestrenol (17α-ethinyl-4-estren-17β-ol).

(d) *Corticosteroids.* Cortisol (11β,17α,21-trihydroxy-4-pregnene-3,20-dione), corticosterone (11β,21-dihydroxy-4-pregnen-3,20-dione), deoxycorticosterone (21-hydroxy-4-pregnen-3,20-dione), dexamethasone (9α-fluoro-11β,17α,21-trihydroxy-16α-methyl-1,4-pregnadiene-3,20-dione), prednisolone (11β,17α,21-trihydroxy-1,4-pregnadiene-3,20-dione).

Lysosome preparation

Paired preputial glands were removed from animals 15 min after administration of steroid solution or saline. Tissues were weighed, minced with fine scissors, then ground in 0·25 M sucrose with a Potter–Elvehjem homogenizer to give a 10% (w/v) suspension. A purified lysosome preparation was obtained from this suspension by differential centrifugation using the method of Szego *et al.* [3].

Enzyme assay

Activity of β-glucuronidase (β-D-glucuronide glucuronohydrolase, EC 3.2.1.31) was determined at pH 4·5 by the method of Musa *et al.* [4] using phenolphthalein β-D-monoglucosiduronic acid (Sigma Chemical Co.) as substrate. Enzyme activity was expressed as units per μg protein [4]. Determinations were conducted on aliquots of washed lysosome pellets suspended in 50 mM acetate buffer in 0·25 M sucrose. One aliquot was taken immediately after preparation and a second aliquot after 30 min autolysis at 37°C in a shaking water-bath.

Experiments

Activation of preputial gland β-glucuronidase by steroids was compared in ovariectomized and ovariectomized–adrenal–ectomized animals using a range of doses. In some experiments, two steroids were administered simultaneously. Mean values and standard deviations were calculated for each treatment group and statistical significance of differences determined by a t-test.

RESULTS

Activity of β-glucuronidase in lysosome preparations from preputial gland homogenates of control animals averaged about 0·8 units/μg protein before lysis

and about 1·9 units/μg protein afterwards. Initial activities of preparations from animals treated with steroids was usually within plus or minus 0·2 units/μg protein of controls before lysis, but afterwards sex hormone-treated animals yielded results much higher than control values, while corticosteroid treatment was generally associated with smaller changes. Table 1, summarises mean changes in β-glucuronidase activity before and after lysis for lysosome preparations from groups of ovariectomised animals treated by 23 different compounds. Three steroids also were tested in both ovariectomized and in ovariectomized-adrenalectomized rats.

In this first series of experiments, steroid doses were selected arbitrarily. Minimally effective doses were then derived by gradually increasing the amount of estradiol-17β, testosterone, progesterone, or cortisol administered. Doses

Table 1. Effect of steroids on preputial gland lysosomal β-glucuronidase

Hormone	Dose	Mean change in β-glucuronidase activity.			
		ovariectomized		ovariectomized and adrenalectomized	
	(μg/kg)	(units/μg protein)	(%)	(units/μg protein)	(%)
none (control solution)	—	1·51	100	1·89	100
estrogens:					
estradiol-17β*	1	3·21	212	3·68	195
estradiol-17α	20	1·52	101	—	—
ethinylestradiol*	1	2·25	215	—	—
stilbestrol*	10	2·65	175	—	—
androgens:					
testosterone*	20	2·85	188	3·15	166
dihydrotestosterone*	20	1·60	106	—	—
dehydroepiandrosterone	20	1·57	103	—	—
etiocholanolone	20	1·56	102	—	—
epitestosterone	20	1·50	100	—	—
fluoxymesterone	20	1·40	93	—	—
progestogens:					
progesterone*	50	2·31	153	—	—
pregnanolone*	50	2·28	151	—	—
allopregnanolone	50	1·42	94	—	—
epipregnanolone	50	1·50	100	—	—
medroxyprogesterone acetate*	20	1·81	120	—	—
norethisterone*	20	2·41	160	—	—
norgestrel*	20	2·89	191	—	—
lynestrenol	20	1·50	100	—	—
corticosteroids:					
cortisol†	200	1·30	86	1·25	66
corticosterone†	200	1·38	91	—	—
deoxycorticosterone*	200	1·71	113	—	—
dexamethasone†	100	1·21	80	—	—
prednisolone†	100	1·29	85	—	—

*Significantly greater than controls ($P < 0·05$).
†Significantly less than controls ($P < 0·05$).

(μg/kg) giving statistically significant ($P < 0{\cdot}05$) changes in lysosomal β-glucuronidase were: estradiol 0·05, testosterone 1·0, progesterone 5, cortisol 100. These doses were then examined times two, ten and twenty. Results are shown in Table 2.

Finally, antagonism between corticosteroids (cortisol, prednisolone, dexamethasone) and sex hormones (estradiol-17β, testosterone, progesterone) was tested by simultaneous intravenous administration of a corticosteroid and a sex hormone. Results are given in Table 3.

Table 2. Dose response of lysosomal β-glucuronidase to steroid hormones

Hormone	MED:*	Mean change in enzyme activity (% of control) ×1	×2	×10	×20
Estradiol-17β		109	120	171	212
Testosterone		105	113	146	188
Progesterone		106	114	153	192
Cortisol		95	86	82	84

*MED = minimum effective dose (i.v.) to produce statistically significant change in enzyme activity (estradiol-17β 0·05 μg/kg, testosterone 1 μg/kg, progesterone 5 μg/kg, cortisol 100 μg/kg).

Table 3. Antagonism between corticosteroids and sex hormones for lysosomal enzyme activation

Corticosteroid	Dose μg/kg	Mean change in lysosomal β-glucuronidase (% control value) estradiol-17β	testosterone	progesterone
	dose:	0·5 μg/kg	10 μg/kg	50 μg/kg
none	—	171	146	153
cortisol	200	136	122	125
prednisolone	100	121	115	118
prednisolone	50	140	125	131
dexamethasone	100	125	112	110

DISCUSSION

It is apparent from the results that many sex hormones produce marked increases in lysosomal β-glucuronidase activity of preputial glands within 15 min of intravenous administration. In contrast, some corticosteroids have the opposite effect. When administered together, the activating effect of a sex hormone is significantly reduced by a corticosteroid.

Estrogens, androgens and progestogens all possess lysosome labilizing activity, but this varies markedly between individual compounds. Estradiol-17β and ethinylestradiol were the two most active compounds tested, with stilbestrol third. Estradiol-17α, which lacks any significant estrogenic activity, was without effect on lysosomal enzymes. Of androgens, testosterone was the most active, with dihydrotestosterone showing only a weak effect. Dehydroepiandrosterone,

etiocholanolone and epitestosterone were inert at the doses tested, while fluoxymesterone, which is a potent androgen, had a weak lysosome *stabilizing* action. The most potent progestogens for lysosome effects were the three synthetic compounds: medroxyprogesterone acetate, norethisterone, norgestrel. Progesterone and pregnanolone had less effect, while allo-pregnanolone was a weak lysosome stabilizer. Lynestrenol and epipregnanolone were inactive.

Cortisol and corticosterone had stabilizing effects on lysosomal β-glucuronidase activity, while synthetic corticosteroids (prednisolone and dexamethasone) had similar, though more potent effects. Deoxycorticosterone was a lysosome labilizer.

A summary of structure activity relationships of steroids for effects on preputial gland lysosomal β-glucuronidase activity is given in Table 4. It is apparent from these results that lysosome labilizers may be estrane, androstane, or pregnane derivatives, while some pregnanes and androstanes are activators. Similarly, some estranes and androstanes are inert. Activity is largely unrelated to either type or degree of hormonal properties.

All labilizing steroids in this series possessed an oxygenic function at C-3, though a wide range of chemically different groupings occur in these compounds. For example, estradiol-17β possesses a phenolic hydroxyl, testosterone and progesterone a conjugated ketone, dihydrotestosterone an unconjugated ketone, while pregnanolone has a secondary alcoholic hydroxyl. All stabilizing steroids also possess an oxygenic function at C-3, though one of the inactive steroids (lynestrenol) does not.

Substitution of ring B does not appear very important, but 5 out of 6 stabilizing steroids have a β-hydroxyl at C-11 in ring C. Allopregnanolone is the exception, and has only weak activity. An α-hydroxyl at C-17 in ring D is present in 3 stabilizers and a 17β-hydroxyl in a fourth (fluoxymesterone). Compounds with a 17α-hydroxyl are much more potent than those stabilizers without this group. There are oxygenic functions at C-17 in 7 of 10 labilizing steroids and in 5 of 6 inactive compounds. However, only one of the latter group (lynestrenol) has a 17β-hydroxyl, while the only labilizing steroid with a 17α-hydroxyl (medroxyprogesterone acetate) has this esterified.

Labilizing compounds lacking oxygen at C-17 all have a ketone at C-20. This group is also present in 5 of the 6 stabilizers, though in none of the inactive steroids. Four stabilizers and one labilizer also have a hydroxyl at C-21.

The following hypotheses of chemical requirements for steroid action on lysosomes can be formulated to account for the observed results: (1) To act on lysosomes a steroid molecule requires a minimum of two oxygenic groups; one of these must be at C-3, though the other may be at C-17 or C-20. Ketones or α-hydroxyls at C-17 are ineffective but enhance the effect of 20-oxo. The group at C-3 may be phenolic, ketonic, or a 3β-hydroxyl (but not 3α-hydroxyl). (2) Compounds with β-hydroxyl at C-11 are lysosome stabilizers. 5α-Pregnanes lacking 11β-hydroxyl are weak stabilizers, though 5β-pregnanes are labilizers. Hydroxyl at C-21 enhances stabilization, but reduces labilization. (3) Conjugation of the oxygenic group at C-3 increases activity; double conjugation (i.e. 1,4-diene) further enhances lysosome stabilization by corticosteroids.

It seems likely that lysosome membranes interact with labilizing steroid molecules by a stereo-specific two-point contact at C-3 and either C-17 or C-20. As 3α- and 17α-hydroxyls yield inactive compounds, it is likely that linkage between membrane and steroid is from the β-side of the molecule. A *cis*-A : B ring junction

Table 4. Structure-activity relations of steroids for lysosome effects

Lysosome action	Compound	Parent steroid	Substituents and groupings				
			Ring A	Ring B	Ring C	Ring D	Other
labilizers	estradiol-17β	estrane	aromatic 3-ol	—	—	17β-ol	—
	ethinylestradiol	estrane	aromatic 3-ol	—	—	17β-ol	17α-ethinyl
	testosterone	androstane	4-ene 3-one	—	—	17β-ol	—
	dihydrotestosterone*	androstane	3-one	—	—	17β-ol	5α-H
	progesterone	pregnane	4-ene 3-one	—	—	—	20-one
	pregnanolone	pregnane	3β-ol	—	—	—	20-one 5β-H
	medroxyprogesterone acetate	pregnane	4-ene 3-one	6α-methyl	—	17α-acetate	20-one
	norethisterone	estrane	4-ene 3-one	—	—	17β-ol	17α-ethinyl
	norgestrel	estrane	4-ene 3-one	—	—	17β-ol	17α-ethinyl 17-methyl
	deoxycorticosterone	pregnane	4-ene 3-one	—	—	—	21-ol 20-one
stabilizers	cortisol	pregnane	4-ene 3-one	—	11β-ol	17α-ol	21-ol 20-one
	corticosterone*	pregnane	4-ene 3-one	—	11β-ol	—	21-ol 20-ol
	dexamethasone	pregnane	1,4-diene 3-one	9α-fluoro	11β-ol	17α-ol 16α-methyl	21-ol 20-one
	prednisolone	pregnane	1,4-diene 3-one	—	11β-ol	17α-ol	21-ol 20-one
	fluoxymesterone*	androstane	4-ene 3-one	9α-fluoro	11β-ol	17β-ol	17α-methyl
	allopregnanolone*	pregnane	3β-ol	—	—	—	20-one 5α-H
inactive	estradiol-17α	estrane	aromatic 3-ol	—	—	17α-ol	—
	dehydroepiandrosterone	androstane	3β-ol	5-ene	—	17-one	—
	etiocholanolone	androstane	3β-ol	—	—	17-one	—
	epitestosterone	androstane	4-ene 3-one	—	—	17α-ol	—
	lynestrenol	estrane	4-ene	—	—	17β-ol	17β-ethinyl
	epipregnanolone	pregnane	3α-ol	—	—	—	20-one 5β-H

*Weak activity.

(5β) enhances binding, but a *trans*-junction (5α) weakens the linkage.

In contrast, stabilization of membranes by steroids appear to require a three point contact at C-3, C-11 and C-20. Weak labilization occurs with contact at C-17 rather than C-20, as with fluoxymesterone, and in the absence of C-11 contact for allopregnanolone. While significant effects occurred in the present experiments within 15 min of administration, it is possible that active metabolites may have been formed from some compounds and explain the few apparent exceptions to the general rules. It must also be remembered that to act on preputial

gland lysosomes, a steroid must first be taken up by the cells, so that compounds active against isolated lysosomes may be inert *in vivo* due to poor cell membrane penetration.

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