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DIFFERENTIAL EFFECTS OF ETIOCHOLANOLONE ON PHOSPHOLIPID/CHOLESTEROL STRUCTURES CONTAINING EITHER TESTOSTERONE OR ESTRADIOL

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SUMMARY

Artificial lipid spherules (ovolecithin–cholesterol–dicetylphosphate, 70:10:20, mole %) were prepared to contain varying amounts of either testosterone or 17β-estradiol preincorporated into their structures. Such spherules captured marker ions in the aqueous spaces between lipid layers, and it was found that the presence of from 0.1 to 7.5 % 17β-estradiol retarded the release of CrO_4^{2-} from the structures. Preincorporation of testosterone induced little change in the overall permeability of the spherules to anions. Spherules with 17β-estradiol preincorporated proved to be more resistant to leakage of anions induced by the subsequent addition of etiocholanolone or deoxycorticosterone. No such protective effect could be shown with spherules containing 17β-estradiol when progesterone or diethylstilbestrol were added subsequently. Addition of similar amounts of 17β-estradiol or testosterone to already formed spherules did not retard the release of anions induced by etiocholanolone or deoxycorticosterone. Differences in response to preincorporation of 17β-estradiol or testosterone could be explained by the stabilizing effect of 17β-estradiol in that artificial spherules were also affected by (in order) 17α-estradiol, estriol, and estrone. The data suggest that 17β-estradiol, at a minimum molar preincorporation ratio of 1:1000, reduces the overall permeability of artificial lipid structures, and renders these less permeable to subsequent disruption by etiocholanolone. This property may, at least in part, be related to the pharmacologic effects of estrogens.

INTRODUCTION

Previous studies from this laboratory have shown that artificial lipid spherules responded to the addition of steroid hormones by gross changes in their overall permeability to previously sequestered anions, cations, glucose, or glycine[1–3]. If the structure of these model, liquid crystalline structures be accepted as that of a series of concentric lipid lamellae which are separated by aqueous compartments, then release of previously trapped ions or markers would represent exchange diffusion of the markers across the lipid layers[4]. By such means, it has been shown that etiocholanolone and other 5β-H steroids, but not their 5α-H isomers, increase the overall

permeability of the spherules. Other steroids, such as progesterone, deoxycorticosterone, and diethylstilbestrol also acted in this direction. In contrast, cortisone, cortisol, and their acetates, retarded the diffusion of markers from the spherules. Furthermore, it was shown that preincorporation of cortisone, from 0.5 to 1 % (molar composition), rendered spherules less permeable than controls, either to simple incubation or to addition of lytic steroids[1–3].

These studies supported previous suggestions that steroids might exert their pharmacologic effects by virtue of an action upon the lipid membranes of cells and organelles[5,6]. One of these pharmacologic effects is the induction of fever in man by etiocholanolone and other 5β-H steroids[7]. Fever is not provoked by the 5α-H isomers, and is indeed abolished when cortisol is admixed with etiocholanolone[8]. Antagonism between cortisol and etiocholanolone has thus been demonstrated *in vivo*, and in their effects upon isolated lysosomes[9] and artificial spherules[2]. Were these opposing effects to be more than fortuitous, then hormonally determined differences in response to pharmacologic effects of etiocholanolone should extend to artificial systems as well.

Hormonally determined differences in response to etiocholanolone have, indeed, been recently described. KIMBALL *et al.*[10] have shown that mature women are far more resistant to the pyrogenic effects of injected etiocholanolone than men. Women also manifest milder systemic reactions and respond with less leucocytosis; they require less antipyretic treatment. Since it appeared possible that sex hormones might mediate this difference in response, we studied the effect of preincorporating either estradiol or testosterone into artificial lipid structures. Were the hypothesis correct that the antagonistic effects of etiocholanolone and cortisol might reflect their action upon an as yet unidentified lipid membrane, then the differences in response to etiocholanolone of men and women might also represent differences in the response of such lipid membranes containing different hormones.

These studies have, in fact, shown that artificial lipid spherules prepared with 17β-estradiol were rendered more resistant to etiocholanolone-induced changes in their overall permeability to sequestered anions than were spherules which contained an equivalent amount of testosterone preincorporated. It was possible to alter the permeability properties of lipid spherules by the addition of as little as 0.1 % estradiol.

MATERIALS AND METHODS

Artificial phospholipid–cholesterol spherules of varying lipid composition were prepared by methods previously described[2,3]. In brief, ovolecithin, cholesterol, and dicetylphosphate (70:10:20, mole %) were dissolved in chloroform, dried under reduced pressure to remove solvent, and permitted to swell in 0.145 M K_2CrO_4 for 6 h at 23°. Anions not trapped within the spherules (16 μmoles of lipid/ml) were removed by dialysis against equimolar NaCl–KCl by methods described before[2,3]. The dialysed spherules, now containing only sequestered ions, were dispensed as 1-ml aliquots into smaller dialysis sacs to which 0.05 ml of various steroids in dimethylsulfoxide was added. 1 sample of each set was incubated with the non-ionic detergent Triton X-100 (see ref. 2), this detergent at 0.2 % (v/v) physically disrupts the spherules, and is used to measure the maximum rate of leakage possible under these conditions. To control samples, 0.05 ml of dimethylsulfoxide was added. Leakage of anions from

the spherules, through the sacs, into smaller test tubes, was measured at 30 and 60 min.

In studies where steroids were to be preincorporated, appropriate amounts of these were dissolved in chloroform and added to the lipids before drying under reduced pressure; they were thus available for incorporation into the spherules during their formation and swelling. Amounts of steroids to be preincorporated were added at the expense of an equivalent reduction in the cholesterol content, as it is entirely possible to form such spherules in the absence of cholesterol[2,3].

For the sake of convenience, trivial names of steroids have been used. These are: etiocholanolone (3α-hydroxy-5β-androstan-17-one), testosterone (17β-hydroxy-4-androsten-3-one), 17β-estradiol (3,17β-dihydroxy-1,3,5,(10)estratriene), 17α-estradiol (3,17α-dihydroxy-1,3,5,(10)estratriene), deoxycorticosterone (21-hydroxy-4-pregnen-3,20-dione), pregnanedione (5β-pregnan-3,20-dione), androsterone (3α-hydroxy-5α-androstan-17-one), estrone (3-hydroxy-1,3,5,(10)estratriene-17-one), and estriol (3, 16α,17β-trihydroxy-1,3,5,(10)estratriene).

Finally, it is to be noted that CrO_4^{2-} was chosen as a marker anion for judging changes in overall permeability of the spherules for the sake of convenience of its measurement. That leakage of this anion readily parallels release of other anions, some cations, glucose, and glycine has been extensively documented in previous studies of the effects of steroids on the spherules[1-4].

RESULTS

Effect of etiocholanolone on spherules prepared with 17β-estradiol or testosterone

Spherules of ovolecithin–cholesterol were prepared with either estradiol or testosterone preincorporated, and subsequently incubated either in the presence of solvent (dimethylsulfoxide) or of etiocholanolone (5 mM). Spherules formed with 1% estradiol (molar composition) released less anions than did those containing testosterone (Fig. 1), an effect observed both in control and in etiocholanolone-treated samples. Not only were the absolute amounts of anion less, but the rates of release (see slopes in Fig. 1) were decreased. Since the total amount of anions trapped in the two preparations did not vary significantly, it is clear that the presence of

TABLE I

RELEASE OF CrO_4^{2-} FROM LIPID SPHERULES (OVOLECITHIN–CHOLESTEROL–DICETYLPHOSPHATE) PREPARED WITH EITHER 1% TESTOSTERONE OR ANDROSTERONE, *vs.* CONTROLS

Formed spherules incubated with	*Steroid preincorporated**			
	1% testosterone	*None*	*1% androsterone*	*None*
Dimethylsulfoxide**	8.3	7.9	9.4	7.4
Etiocholanolone (5 mM)	18.7	17.4	12.2	15.8
Deoxycorticosterone (5 mM)	46.3	44.8	35.7	35.4

* Means of two experiments. Values expressed as % of total CrO_4^{2-} trapped which were released at 30 min.

** 0.05 ml.

17β-estradiol at the time of spherule formation retarded the release of marker anions.

Identical amounts of testosterone or estradiol (0.16 μmole/ml) were added to already formed, dialysed spherules before these were exposed to solvent or etiocholanolone. Under such conditions, the addition of estradiol did not appear to retard the release of anions from etiocholanolone-treated spherules. Nevertheless, in control samples, addition of estradiol to the formed spherules resulted in inhibition of anion release from solvent-treated spherules (Fig. 2).

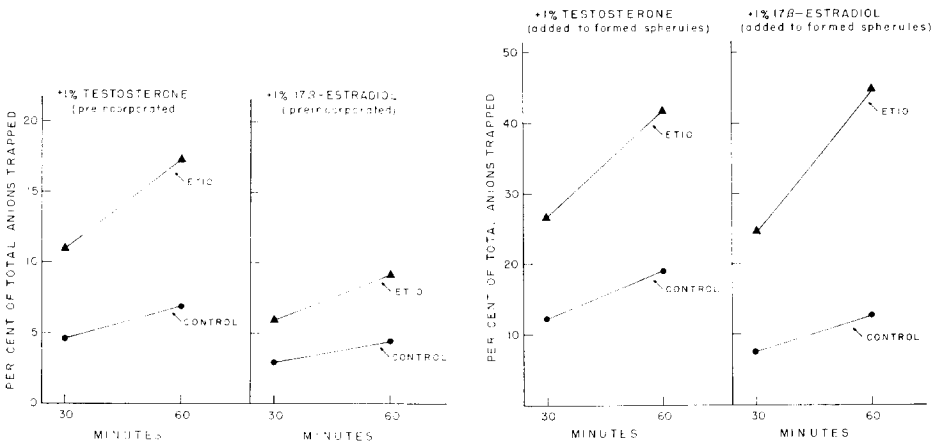

Fig. 1. Release of CrO_4^{2-} from lipid spherules prepared with 1% of either testosterone or 17β-estradiol preincorporated. Spherules prepared with ovolecithin–cholesterol–dicetylphosphate–steroid (70:9:20:1, mole%). Etiocholanolone (ETIO) was added at a final concn. of 5 mM (in 0.05 ml of dimethylsulfoxide/16 μmoles of lipid); an equal volume of dimethylsulfoxide was added to controls. Amounts of anions released (30, 60 min at 37°) are expressed as the total amounts of anions trapped. Total CrO_4^{2-} trapped: spherules + testosterone = 11.4 μmoles/ml; spherules + 17β-estradiol = 8.4 μmoles/ml. Points represent means of 3 experiments.

Fig. 2. Release of CrO_4^{2-} from lipid spherules prepared with ovolecithin–cholesterol–dicetylphosphate (70:10:20, mole%). To the already formed and dialysed spherules, either testosterone (a) or 17-β-estradiol (b) (0.16 μmole) were added, followed immediately by etiocholanolone (ETIO) at a final concn. of 5 mM (in 0.05 ml of dimethylsulfoxide/16 μmoles of lipid); and equal volume of dimethylsulfoxide was added to controls. Amounts of anions released (30, 60 min at 37°) are expressed as the total amounts of anions trapped. Total CrO_4^{2} trapped: spherules in (a) = 7.47 μmoles/ml; spherules in (b) = 9.86 μmoles/ml. Points represent means of 3 experiments.

Release of marker anions from spherules which had been formed with 1% testosterone was closely similar to that induced by the subsequent addition of testosterone to spherules formed in the absence of steroid. Therefore spherules formed with testosterone were compared to control structures. No difference was found (Table I) between these two preparations of spherules, either in the total amount of anion trapping, or in the subsequent overall permeability to anions. Nor did the incorporation of androsterone (hormonally less active than testosterone) significantly modify anion release.

It was therefore clear that differences in overall permeability between spherules with testosterone and with estradiol preincorporated were due to retardation of anion release by estradiol, rather than to acceleration by testosterone. Accordingly, spherules were prepared in the presence of 0.01 to 7.5% (molar composition) estradiol.

Etiocholanolone-induced release of anions from such spherules, and from those formed in the absence of estradiol, were compared (Fig. 3). It was not possible to compare spherules prepared with amounts of estradiol greater than 7.5 %, because the total amounts of anion trapped in such preparations differed considerably from controls. At all levels of preincorporation (above 0.01 %) the presence of estradiol inhibited release of anions from spherules after addition of etiocholanolone.

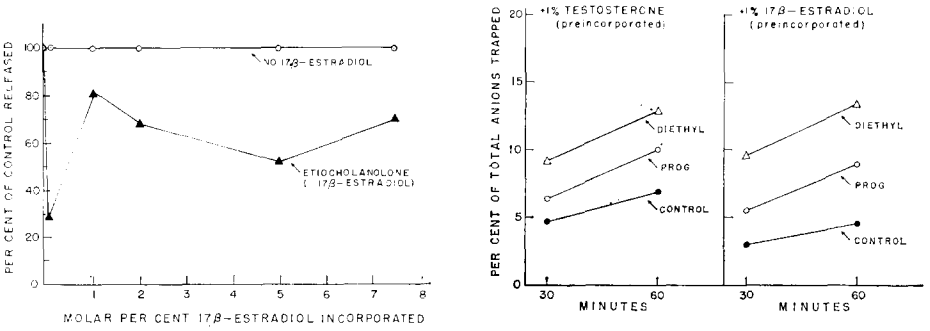

Fig. 3. Effect of etiocholanolone (5 mM) on release of CrO_4^{2-} from spherules prepared with varying amounts of 17β-estradiol. Ovolecithin and dicetylphosphate kept constant as in legend to Fig. 1, cholesterol reduced to accommodate increasing amounts of steroid (total cholesterol + 17β-estradiol = 10 %). Total anions trapped ranged from 7.9 to 14.8 μmoles/ml in controls, and 5.5 to 15.6 μmoles/ml in spherules + estradiol. At 0.01 % preincorporation, the two preparations did not differ at all. Points represent means of 3 experiments.

Fig. 4. Release of CrO_4^{2-} from lipid spherules prepared with 1 % of either testosterone or 17β-estradiol preincorporated. Spherules prepared with ovolecithin–cholesterol–dicetylphosphate–steroid (70:9:20:1, mole %). Diethylstilbestrol (DIETHYL) was added at a final concentration of 2 mM (in 0.05 ml of dimethylsulfoxide per 16 μmoles of lipid), progesterone (PROG) of 5 mM; an equal volume of dimethylsulfoxide was added to controls. Amounts of anions released (30, 60 min at 37°) are expressed as the total amounts of anions trapped. Totals as in Fig. 1; points represent means of 3 experiments.

Effect of several steroids on phospholipid–cholesterol spherules

The effects were also tested of the addition of progesterone and diethylstilbestrol. Whether added to spherules which had been prepared with 17β-estradiol or with testosterone, these hormonally active steroids (diethylstilbestrol is considered as such here by virtue of structure and physiologic functions) had similar actions. Thus their action did not resemble that of etiocholanolone: no differential effects were observed on spherules with steroids preincorporated (Fig. 4). In spherules prepared with 17β-estradiol, solvent alone released less anions than in spherules prepared with testosterone.

The effects were compared of progesterone and diethylstilbestrol (membrane-active steroids which act *in vivo* on target tissues likely to be exposed to estrogens) with deoxycorticosterone and pregnanedione (membrane-active steroids not sharing these properties) (Table II). These steroids are active on the membranes of erythrocytes, lysosomes, and lipid spherules[2,7,9]; pregnanedione is a potent pyrogen[7]. Release of anions by deoxycorticosterone and pregnanedione was significantly retarded in spherules prepared with 17β-estradiol, in contrast to the effects of progesterone and diethylstilbestrol. Indeed the experiments indicate that spherules containing 17β-estradiol were as resistant to deoxycorticosterone or pregnanedione-mediated changes in anion permeability as they were to the effects of etiocholanolone.

TABLE II

RELEASE OF CrO_4^{2-} FROM LIPID SPHERULES (OVOLECITHIN–CHOLESTEROL–DICETYLPHOSPHATE) PREPARED WITH EITHER 1% TESTOSTERONE OR ESTRADIOL *vs.* CONTROLS

All figures represent means (range) (number of experiments).

Formed spherules incubated with	*Steroid preincorporated*		
	None	*1% 17β-estradiol*	*1% testosterone*
Dimethylsulfoxide*	4.4 (2.5–7.9) (3)	2.1 (1.6–3.0) (6)	5.2 (2.9–8.3) (4)
Etiocholanolone (5 mM)	18.1 (10.6–22.2) (6)	4.1 (2.7–5.9) (4)	13.0 (9.4–18.7) (3)
Deoxycorticosterone (5 mM)	31.3 (14.0–44.8) (5)	6.5 (5.1–8.0) (2)	46.3 (1)
Pregnanedione (5 mM)	5.6 (3.4–8.4) (3)	2.1 (1.9–2.1) (3)	7.3 (4.8–9.7) (2)

* 0.05 ml.

In order to determine which of the estrogens were most effective in retarding release of anions in etiocholanolone-treated spherules, these steroids were preincorporated into the structure at 1:100 and 1:1000 molar lipid ratios (Table III). It is clear that 17β-estradiol was most effective in retarding anion release induced by etiocholanolone, followed by 17α-estradiol, estriol, and estrone.

TABLE III

INHIBITION OF CHROMATE RELEASE FROM LIPID SPHERULES (OVOLECITHIN–CHOLESTEROL–DICETYLPHOSPHATE) PREPARED WITH VARYING AMOUNTS OF ESTROGENS COMPARED TO CONTROLS PREPARED IN ABSENCE OF ADDED STEROID

Steroid preincorporated	*Amount of estrogenic steroid preincorporated*			
	0.1%		*1.0%*	
	Steroid added (3 mM)		*Steroid added (3 mM)*	
	ETIO	*DOC*	*ETIO*	*DOC*
17β-Estradiol*	7.3	13.9	4.1	6.2
None*	18.1	31.1	16.4	24.5
% inhibition**	60	55	75	75
17α-Estradiol*	2.7	7.0	5.1	10.4
None*	6.7	9.1	18.0	36.1
% inhibition**	60	23	65	65
Estriol*	27.9	56.0	—	—
None*	0.5	79.0	—	—
% inhibition**	34	29	—	—
Estrone*	18.1	53.4	—	—
None*	18.1	43.1	—	—
% inhibition**	0	−24	—	—

Abbreviations: DOC, deoxycorticosterone; ETIO, etiocholanolone.
* % total CrO_4^{2-} release at 30 min. All figures represent means of 3 experiments.
** % inhibition *vs.* controls, expressed as 100%.

DISCUSSION

These studies indicate that artificial lipid spherules prepared with 17β-estradiol are rendered less permeable to anions following the addition of etiocholanolone than

are spherules prepared with testosterone. At present it is not possible to relate these findings to the observation that women are more refractory to the fever-inducing effects of etiocholanolone than are men[10]. Yet it is becoming appreciated that hormones and their metabolites have wide-ranging pharmacologic effects in addition to their specific physiologic actions on target tissues. Thus estrogens alter hepatic excretory functions[11], diminish responses to immune injury[12], and modify connective tissue reactions both in humans and experimental animals[13]. These actions seem clearly unrelated to the primary effects of estrogens upon their target organs: uterus, vagina, *etc.* Since it has been possible to relate, at least in part, the pharmacologic actions of cortisone and its analogues to the effect of corticosteroids upon lysosomes, cellular, and artificial membranes[1–3, 6, 9], it is indeed possible that the pharmacologic effects of estrogens may also reflect their capacity to modify the properties of lipid structures. Since the order of activity of the various estrogens in pharmacologic effects has not been clearly established[11–13], there is no *a priori* reason to expect this order exactly to mirror their activity on target tissues, such as vagina or uterus.

Whether or not these *in vitro* effects on artificial lipid structures bear more than a fortuitous relationship to *in vivo* actions, they are probably relevant to theories of steroid hormone action. It has previously been shown that several steroid hormones affect the overall permeability of artificial phospholipid–cholesterol structures to anions, cations, glucose, and glycine. Furthermore, they interact with the phospholipids of the structures[1–3], and indeed the changes induced in the artificial spherules bear a close relationship to changes induced by steroids in the permeability of erythrocytes and lysosomes. Yet the concentrations of steroids necessary for these effects were far in excess of the physiologic, or pharmacologic, *i.e.* 10 or 100 μM. These concentrations represent, however, the maximum possible concentration of steroids added to lipid spherules suspended in aqueous media. The actual concentration of steroids present at any given area of the lipid structures remains unknown. Therefore it was of considerable help to have found that the preincorporation of as little as 1% of cortisone into phospholipid–cholesterol structures at the time of their formation could render these structures less permeable to anions after addition of solvent or lytic steroids[1,2]. This finding suggested that a minimum quantity of appropriately structured 'rogue' molecules could greatly modify the overall permeability of lipid structures. Estrogens modified the properties of the spherules when they constituted as little as 0.1% of the structures. The present studies thus reinforce those previously reported in that they indicate a possible lower limit for the amount of extraneous steroid necessary to modify the gross permeability of lipid membranes. Extrapolation of these studies to biological membranes would suggest that estrogens or their metabolites might modify the responses of lipid structures to pyrogenic steroids.

The advantages and disadvantages of considering such phospholipid–cholesterol spherules as appropriate models of the lipid boundaries of cells and organelles have been discussed elsewhere in detail[1–4,15]. Several problems remain unsettled, however, by the studies reported above. Although considerable evidence has been presented that steroids and lipids which are present at the time of spherule formation are actually incorporated into the structures, this evidence is indirect[2,3,15]. Thus changes in the overall permeability of the spherules induced by estradiol might be due to the presence in the suspending media of unincorporated steroid which would be free to interact with added etiocholanolone, thereby reducing its effect. Since, however,

addition of equivalent amounts of estradiol to the already formed, dialysed spherules did not retard etiocholanolone action, this possibility seems remote. Indeed, evidence for the preincorporation of added estrogens is available from the experiments themselves. Thus, although minimum amounts of estrogens did not alter the total quantity of anions trapped by the spherules, as little as 1 μmole estradiol per 1000 of spherule lipid was sufficient to confer overall reduction in anion release. The same argument is not strong in the case of testosterone, since experiments with preincorporation of this steroid did not indicate its capacity to induce functional changes. As has been discussed before[15], negative experiments in the spherule system cannot be used to infer incorporation of added molecules. Despite this reservation, the data clearly suggest that the presence of modest amounts of steroid hormones or metabolites may alter the permeability of artificial lipid structures in directions which can be predicted from studies of steroid pharmacology.

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