Biochimica et Biophysica Acta, 979 (1989) 279–286
Elsevier

BBA 74338

Effects of cholesteryl esters on the accessibility of LH/hCG receptors and membrane lipid fluidity in rat testes

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(Received 14 October 1988)

Key words: Luteinizing hormone/human chorionic gonadotropin receptor; Cholesteryl ester; NMR, ^{1}H-; Membrane fluidity

Incubation of rat testicular membranes with cholesteryl hemisuccinate resulted in an increase in both membrane lipid microviscosity and ^{125}I-labelled hCG specific binding. The purpose of this investigation was to establish which functional groups of cholesteryl hemisuccinate are important for the stimulatory effects. The data obtained showed that only esters of cholesterol with dicarboxylic acids, not those of monocarboxylic acids, increase the accessibility of LH/hCG receptors and membrane rigidity. Experiments with cholesteryl sulfates showed that there are polar groups on C_3 carbon of cholesterol having no stimulatory effect on receptors, although an increase in membrane rigidity occurred. The side-chain of cholesterol is important for the stimulatory action. Androstenolone hemisuccinate was ineffective in this respect. On the other hand, partially modified side-chains (hemisuccinates of β-sitosterol and stigmasterol) did not result in a marked reduction of the stimulatory action. The carboxyl group of cholesteryl hemisuccinate must be 'free': its esterification abolishes the stimulatory effect of cholesteryl hemisuccinate on both the LH/hCG receptor and membrane microviscosity. These results suggest that an intact carboxyl group of ester and the side-chain of cholesterol are indispensable for the stimulatory effect of cholesteryl hemisuccinate on the accessibility of LH/hCG receptors.

Introduction

Cholesterol is one of the main lipid constituents of most mammalian membranes. As a structural membrane component, cholesterol is involved in maintaining the cell integrity and membrane fluidity. Cholesterol has a condensing effect on membrane phospholipids in the liquid-crystalline state, i.e., above the phase transition temperature [1]. The effect of cholesterol on the physical state of plasma membrane has been studied by many investigators and has been shown to influence a variety of membrane functions [2–7]. Depending on the system used, changes in membrane fluidity may have different effects on the accessibility of hormonal receptors. Thus, the exposure of β-adrenergic receptors in rabbit reticulocytes [2] and those of serotonin in mouse brain membranes [3] was enhanced by an increase in membrane rigidity. On the other hand, when lipid rigidity is decreased, it is prolactin receptors of the ventral prostata [4] and β-adrenergic receptors in liver cell membranes [5] that become more exposed. We reported earlier that LH/hCG receptors are increased in a dose-dependent manner after the incorporation of cholesteryl hemisuccinate (CH-S) into rat testicular membranes [6,7]. The reason for the rise of hCG binding sites under the action of CH-S on testicular membranes still remains unclear. The results showed that the rigidifying action of cholesterol on membranes is unlikely to be the sole cause for the increaseed accessibility of LH/hCG [7].

To establish structural features of CH-S important for the stimulatory action on the receptors, a number of cholesterol esters were prepared and their effects on LH/hCG receptors and fluidity of testicular membranes were tested.

Materials and Methods

Materials

Purified hCG (CR 123, 12 780 mg^{-1}) was generously supplied by the Center for Population Research of NICHD, NIH, Bethesda. Na^{125}I was purchased from

Abbreviations: PVP, poly(vinylpyrrolidine); DPH, 1,6-diphenyl-1,3,5-hexatriene, DMSO, dimethyl sulfoxide; CH-S, cholesteryl hemisuccinate; CH-Bu, cholesteryl butyrate; CH-Oc, cholesteryl octanoate; CH-La, cholesteryl laurate; CH-St, cholesteryl stearate; CH-Ma, cholesteryl hemimalonate; CH-Ol, cholesteryl oleate; CH-Ph, cholesteryl hydrogen phthalate; CH-Ad, cholesteryl hemiadipate; STIG-S, stigmasterol hemisuccinate; CMO-Ch, cholest-4-*en*-3-one *O*-(carboxymethyl)oxime.

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Amersham International, U.K. PVP, DPH and CH-S, CH-Bu, CH-Oc, CH-La, CH-St, CH-Ol, CH-Ph were products of Sigma, St. Louis, MO.

Methods

Crude membrane preparation. Male Wistar rats aged 55–65 days were killed by decapitation. Homogenates of decapsulated testes in ice-cold 50 mM Tris-HCl (pH 7.4) were filtered through six layers of surgical gauze and centrifuged at $1000 \times g$ for 15 min, and the supernatants were further centrifuged at $20000 \times g$ for 30 min [8]. The final pellets were resuspended in the same buffer (200 mg of tissue per ml).

Incubation of tissues with lipids. 3 mg of lipid were disolved in 0.2 ml of DMSO. On stirring the solution, it was diluted with 50 mM Tris-HCl (pH 7.4) + 3.5% PVP to 50 vol. The dispersion was briefly sonicated (15 s, 30 W). The crude testicular membrane fraction in Tris-HCl buffer (1.5 ml) was incubated for 90 min with various concentrations of lipid suspensions at 25°C. Controls were incubated with the same concentration of Tris buffer with PVP and DMSO [3,7]. The testicular membranes were then centrifuged at $20000 \times g$ for 30 min and the pellets were washed twice with Tris-HCl buffer. The final pellets were suspended in Tris-HCl buffer ($200\ mg \cdot ml^{-1}$).

hCG binding assay. Aliquots of the testicular membrane fraction (0.1 ml) were incubated for 16 h at 25°C with 0.1 ml of 50 $mmol \cdot l^{-1}$ Tris-HCl buffer (pH 7.4) + 1 $mg \cdot ml^{-1}$ BSA with or without 100-fold excess of unlabeled hCG and 0.1 ml ^{125}I-hCG (1–1.5 ng, specific activity about 2.3 $T \cdot Bq^{-1}$). After incubation and centrifugation, the pellets were washed twice with 2 ml of cold Tris-HCl buffer [7,9]. The results were expressed in terms of specific binding per mg protein. Protein was determined by the standard procedure [10].

Hormone degradation. At the end of the binding study, the supernatants were collected and the unbound ^{125}I-hCG was assessed for its ability to bind to fresh membrane preparations during 16 h incubation at 25°C. This binding was compared to that in control experiments in which ^{125}I-hCG was preincubated with buffer alone [11]. Aliquots of supernatants were also subjected to precipitation with trichloroacetic acid to monitor the extent of degradation of the radioactive hormone [12]. There were no differences observed in ^{125}I-hCG degradation by testicular membranes incubated without or with esters of cholesterol (Ch-S, CH, CH-Bu, CH-S-M; data not shown).

Cholesterol determination. Lipids from testicular membrane preparations were extracted with chloroform/methanol, 2:1 (v/v) [13]. Cholesterol was estimated with 2,5-dimethylbenzenesulfonic acid in the presence of sulfuric acid (Bio-La-Test, Lachema) [14]. Calibration curves were made for each steroid. The results were expressed as μg of particular steroid per mg protein.

Fluorescence polarization measurements. Fluorescence polarization was measured with a Perkin-Elmer LS-5 luminescence spectrometer, equipped with circulation bath to regulate the sample temperature at 25°C [6,15]. A solution of 2 mmol DPH in tetrahydrofuran was dispersed by 1000-fold agitative dilution in 50 $mmol \cdot l^{-1}$ Tris-HCl buffer. Crude testicular membranes (100 μg protein) were incubated for 1 h at 25°C with 2 ml of DPH in Tris-buffered saline. The fluorescence polarization was computed by the equation:

$$P = \frac{I_{vv} - I_{vh}\,(I_{hv}/I_{hh})}{I_{vv} + I_{vh}\,(I_{hv}/I_{hh})}$$

where I_{vv} and I_{vh} are the fluorescence intensities detected through a polarizer oriented parallel and perpendicular to the direction of vertically polarized light. I_{hv}/I_{hh} represents the ratio when the excitation is polarized horizontally and the emission observed through the analyzer oriented perpendicularly and parallel, respectively. Lipid microviscosity was estimated by the empirical relation, $2P/(0.46 - P)$ [3].

Preparation of steroid esters. Cholest-5-en-3β-ol hydrogen propanedioate (CH-Ma) was prepared as follows. From a solution of cholesterol (310 mg) and malonic acid (170 mg) in benzene (20 ml), 5 ml of azeotropic mixture was distilled off. A solution of dicyclohexylcarbodiimide (280 mg) and 4-dimethylaminopyridine (20 mg) in tetrahydrofuran (5 ml) was added with stirring at 20°C. After 24 h the mixture was diluted with ethyl acetate (15 ml), washed with diluted hydrochloric acid (5%) and water and dried over sodium sulfate. The product was purified twice on a thin layer of silica gel (10% diethyl ether in benzene and then 1% acetic acid and 10% diethyl ether in benzene). The product was crystallized from acetone and heptane; m.p. 157–163°C (dec.), $[\alpha]_D^{20} - 31°$ (*c* 0.9, chloroform). Mass spectrum: 369 *m/e* (b.p., $M^+ - 103$). For $C_{30}H_{48}O_4$ (472.7) calculated: 76.22% C, 10.24% H; measured: 75.98% C, 10.28% H.

Cholest-5-en-3β-ol hydrogen hexanedioate (CH-Ad) was prepared similarly to CH-Ma; m.p. 131–132°C. For $C_{33}H_{54}O_4 \cdot H_2O$ calculated: 72.73% C, 11.28 H; measured: 72.54% C, 10.86% H.

22*E*-Stigmasta-5,22-dien-3β-ol hydrogen butanedioate (stigmasterol hemisuccinate, STIG-S) was prepared in the same way. M.p. 157–159°C (diethyl ether), $[\alpha]_D^{20} - 33°$ (*c* 0.9, chloroform). For $C_{33}H_{52}O_4$ (512.7) calculated: 77.29% C, 10.22% H; measured: 77.01% C, 10.40% H.

The preparation of cholesteryl hemisuccinate methylester (CH-S-M), cholest-5-en-3β-ol sodium sulfate (CH-Sf-Na), cholest-5-en-3β-ol pyridinium sulfate (CH-Sf-Py), stigmast-5-en-3β-ol hydrogen butanedioate (β-

sitosterol hemisuccinate, β-SIT-S), CMO-Ch, 17-oxo-5-androsten-3β-ol hydrogen butanedioate (androstenolone hemisuccinate, A-S) and 5-oxo-A-nor-3,5-secocholestan-3-oic acid (SEKO) was described elsewhere [16–23]. The structure of the above agents was confirmed by elemental analysis, ^{1}H-NMR and infrared spectra (Tables I and II).

Student's *t*-test was used for statistical evaluation. The results are expressed as means ± S.E.

Results

Various cholesterol esters were synthesized and their effects on specific binding of ^{125}I-hCG and microviscosity of rat testicular membrane fractions were tested to establish functional groups of CH-S important for their action. Steroidal esters were prepared by known methods with their structure always being verified by a combination of analytical methods (elemental analysis, infrared, ^{1}H-NMR spectra). Any new compounds were characterized (melting point mass spectrum, specific rotation). The physicochemical properties of other compounds compared well with those given in the literature (as far as reported at all). While infrared bands (Table II) could be taken as evidence for the presence of particular groups, prominent peaks in the ^{1}H-NMR spectra (Table I) were typical of each of the substrates. Fig. 1 shows that cholesterol esters with dicarboxylic acids have stimulatory effects on specific binding of ^{125}I-hCG to testicular membranes ($P < 0.01$). This effect on the accessibility of LH/hCG receptors is a property not only of esters of aliphatic C_3, C_4 and C_5 carbon acids (CH-Ma, CH-S and CH-Ad) but also of esters of aromatic dicarboxylic acid (CH-Ph) as well as polar groups of chains shorter than hemisuccinate and bound as oxime on C-3 of cholesterol (CMO-Ch) ($P < 0.01$). On the other hand, incubation of testicular membranes with increasing concentrations of cholesterol esters of monocarboxylic C_2, C_4 and C_8 carbon acids had no effect on the accessibility of LH-hCG receptors (Fig. 2). Similarly without any effect on receptors was non-esterified cholesterol (CH) (Fig. 2), as well as cholesterol esters with both saturated (CH-La, CH-St) and unsaturated (CH-Ol) fatty acids (data not shown).

To determine whether the cholesterol side-chain is necessary for the increase of LH/hCG receptors, hemisuccinates of cholesterol derivatives were prepared with partially modified side-chains. Hemisuccinates of β-

TABLE I

Characteristic parameters of ^{1}H-NMR spectra

The spectra were recorded on a Tesla 60 instrument in deuteriochloroform with tetramethylsilane as internal standard. Chemical shifts are given in the δ-scale (p.p.m.), interaction constants (J) and half-height width (W) in Hz.

Compound	3-H [a]	6-H [b]	18-H [c]	19-H [c]	Other signals [d]
CH-Ma	4.65	5.39	0.67	1.01	3.38 [e]
CH-S	4.60	5.35	0.67	1.01	2.63 [f]
CH-S-M	4.60	5.36	0.67	1.00	2.66 [f], 3.67 [g]
Ch-Ad	4.63	5.38	0.67	1.02	2.37 [h]
CH-Sf-Py	4.32	5.37	0.67	1.00	8.13 [i]
β-SIT-S [j]	4.58	5.37	0.68	1.02	2.59 [f]
STIG-S [j]	4.60	5.38	0.70	1.02	2.61 [f], 5.13 [k]
CMO-Ch	–	–	0.68	1.05	1.08 [l], 4.59 [m], 5.75 [n], 6.41 [n]
A-S	4.65	5.42	0.88	1.04	2.59 [f]
SEKO	–	–	0.72	1.10	

[a] *m*, $W = 20$; [b] *m*, $W = 9$; [c] *s*, 3 H; [d] with the exception of the compounds all spectra contain doublets at $\delta = 0.85$ ($J = 6$), δ 0.90 ($J = 6$) of protons at C-26, C-27 and C-21, respectively; [e] *s*, 2 H ($OCOCH_2COOH$); [f] *s*, 4 H ($OCO(CH_2)_2COOH$); [g] *s*, 3 H (OCH_3); [h] *m*, 4 H ($OCO(CH_2)_2COOH$); [i] *t*, $J = 6.5$ (part of a multiplet of pyridinium protons); [j] the doublet of C-26 and C-27 protons is shifted to δ 0.83; [k] *m*, $W = 11.22$ H (C = C – H); [l] *s*, C-19 protons of the other isomer; [m] *s*, 2 H (OCH_2COOH); [n] *m*, 0.5 H, $W = 5$, C-4 proton signal of a *syn*- or *anti*-isomer, respectively).

TABLE II

Characteristic infrared bands of compounds

The spectra were measured on a Zeiss UR20 spectrometer in 5% solution (in chloroform of carbon tetrachloride) or in potassium bromide discs. Wavenumbers in cm^{-1}.

Compound	Method	COOH	COOR	Other function groups
CH-Ma	$CHCl_3$	1724	1736, 1166	
CH-S	$CHCl_3$	1712	1730, 1178	
CH-S-M	CCl_4	–	1743, 1169	1440 (OCH_3), 3030, 1671 (C = C)
CH-Ad	$CHCl_3$	1712	1730, 1189	
CH-Sf-Na	KBr	–	–	1422, 983, 644 (SO_2)
CH-Sf-Py	KBr	–	–	1251, 1235 (SO_2), 1632, 1609, 1539 (C_5H_5N)
β-SIT-S	$CHCl_3$	1712	1721, 1179	
STIG-S	KBr	1712	1729, 1180	973 (*trans*-C = C)
CMO-Ch	$CHCl_3$	1733	–	1766 (COOH), 1619, 1651 (C = C = N), 1108 (-N-O-)
A-S	$CHCl_3$	1711	1731, 1178 1166	1744 (C = O), 1668 (C = C)
SEKO	$CHCl_3$	1712	–	

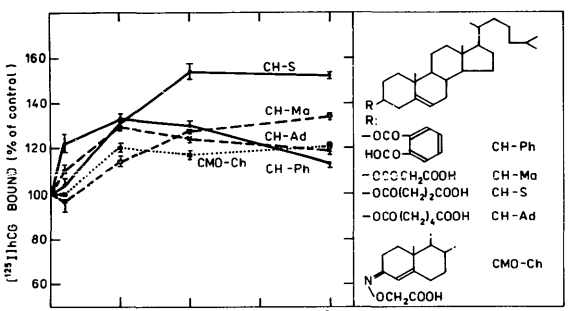

Fig. 1. Stimulatory effect of esters of dicarboxylic acids with cholesterol on the specific binding of ^{125}I-hCG to membrane preparations. Aliquots of rat testicular fraction were incubated for 90 min at 25 °C with various amounts of cholesterol esters in Tris-HCl buffer (pH 7.4) containing 3.5% PVP. Control values were about 25 fmol bound hCG per mg protein. Each point is the mean ± S.E. of five estimations. The experiment was repeated three times with similar results.

sitosterol and stigmasterol possess, in addition to cholesterol, an ethyl moiety at C-24 and a double bond at C-22 in the side-chain. Both sterol had significant stimulatory effects ($P < 0.01$) on the specific binding of ^{125}I-hCG to testicular membrane (Fig. 3). However, for increased accessibility of LH/hCG receptors, the side-chain of cholesterol was indispensable. Membrane exposure to androstenolone hemisuccinate (A-S) did not increase ^{125}I-hCG binding.

The carboxyl group of CH-S has to be intact. Its esterification (CH-S-M) abolished the stimulatory effect of CH-S on the accessibility of LH/hCG receptors (Fig. 4). However, not all polar groups on the C-3 carbon of cholesterol are able to stimulate receptors. Cholesterol

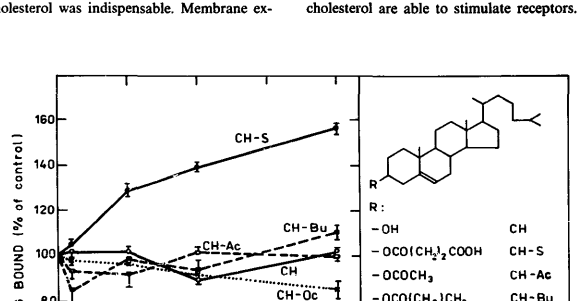

Fig. 2. Effects of esters of monocarboxylic acids with cholesterol on the specific binding of ^{125}I-hCG to rat testicular membranes. Experiments were run in duplicate as described in the legend to Fig. 1. Each value is the mean ± S.E. of five estimations.

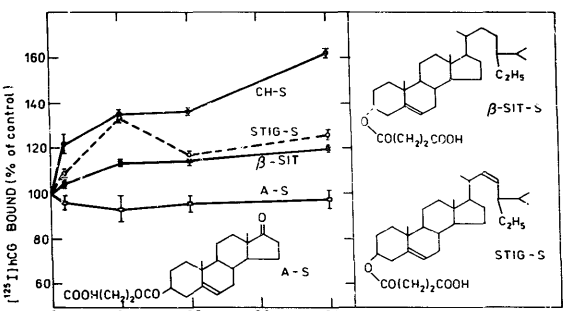

Fig. 3. Variation of the specific binding of ^{125}I-hCG to rat testicular membranes incubated with esters of dicarboxylic acids with steroids. Results are representative of three independent experiments. For details, see legend to Fig. 1.

sulfates (sodium or pyridinium salts) in lower concentrations had no effect, while higher levels had obvious inhibitory effects on ^{125}I-hCG binding. Further experiments showed that the intact steroid skeleton is not indispensable for the stimulatory effect. The split ring A of cholesterol that has a polar group and a side-chain (SEKO), in lower concentrations, had stimulatory effects on LH/hCG receptors ($P < 0.01$). Incubation of testicular membranes with 0.02, 0.1, 0.2 and 0.4 mg · ml^{-1} CH-S increased ^{125}I-hCG binding from 21.5 ± 0.96 fmol bound hCG per mg protein in control membranes to 23.6 ± 0.52, 26.2 ± 1.24, 28.1 ± 1.04 and 33.9 ± 1.44 fmol · mg^{-1}, respectively ($P < 0.01$).

A further experiment showed that the lack of stimulatory effect of several compounds on the accessibility of LH/hCG receptors was due to the lack of incorpora-

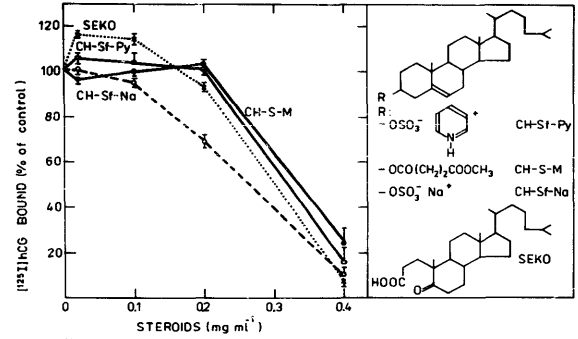

Fig. 4. Specific binding of ^{125}I-hCG in the presence of various concentrations of steroids in rat testicular membrane fraction. Mean values of five estimations (experiments repeated three times) are shown.

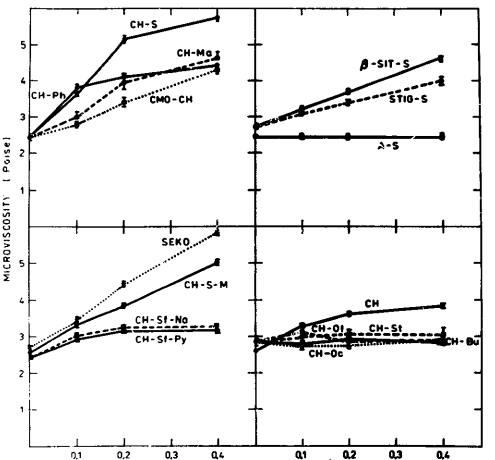

Fig. 5. Dose-dependent changes in membrane lipid microviscosity following incorporation of various steroids. Rat testicular membranes were treated as described in the legend to Fig. 1. The microviscosity was assayed using DPH as a probe. The data represent the mean ± S.E. of four estimations (repeated two or three times).

tion of the steroids into testicular membranes. Table III shows that even steroids which did not change receptors were actively incorporated into membranes.

A series of studies was performed to determine whether the changes in the accessiblity of receptors are linked with alterations in membrane lipid fluidity. As can be seen from Fig. 5, cholesterol esters with dicarboxylic acids (CH-S, CH-Ma, CH-Ph; CMO-Ch), hemisuccinates of β-sitosterol and stigmasterol increased testicular membrane microviscosity ($P < 0.01$), whereas esters of monocarboxylic acids (CH-Bu, CH-Oc, CH-St, CH-Ol) or steroid without side-chains (A-S) were ineffective in this respect. These results are related to the accessibility of LH/hCG receptors. In spite of the increased microviscosity of membrane treated with cholesterol, CH-S-M, cholesteryl sulfates and SEKO ($P < 0.01$), ^{125}I-hCG binding to membranes was not changed or was somewhat inhibited.

TABLE III

Incorporation of esters of steroids into testicular membrane fraction

Aliquots of membranes were incubated 90 min at 25 °C with 0.2 or 0.4 mg·ml^{-1} esters. Each value is the mean ± S.E. of three estimations.

Treatment	Steroid (μg·mg^{-1})	
	0.2 mg·ml^{-1}	0.4 mg·ml^{-1}
Control	91.1 ± 2.9	
CH-S	195.2 ± 4.6	274.5 ± 7.6
CH	154.8 ± 11.0	269.3 ± 7.0
CH-Bu	150.6 ± 9.2	188.4 ± 5.2
CH-S-M	180.8 ± 0.2	362.1 ± 5.9
β-SIT-S	159.2 ± 2.6	258.9 ± 7.9

Discussion

In the present study, modulation of LH/hCG receptors and fluidity of rat testicular membranes by cholesterol esters were studied. Our previous report suggested that in parallel with increasing rigidity of membrane lipid by treatment with CH-S, specific binding of ^{125}I-hCG was also increased. Scatchard analysis of the results showed that changes in hormone binding to membranes are associated with alterations in receptor numbers, rather than with changes in apparent receptor affinity [7]. This is consistent with the concept that the accessibility of cryptic receptors in membranes

is regulated by membrane fluidity. Heron et al. [3] explained the increased binding of serotonin to brain membranes incubated with cholesterol by vertical displacement of membrane proteins. According to this concept, the bulk of membrane proteins becomes more exposed to the aqueous medium by increasing membranes rigidity [24]. However, this explanation of the increase in LH/hCG receptors is unlikely to be the sole cause of the action of cholesteryl hemisuccinate, since a comparable rigidifying effect of saturated fatty acids on membranes did not result in any increase in ^{125}I-hCG binding [7]. Therefore, it is likely that the effect of cholesterol on the accesibility of LH/hCG receptors may also be related to specific chemical and structural characteristics of cholesteryl hemisuccinate. The present results showed that only cholesterol esters with dicarboxylic acids increased the accessibility of LH/hCG receptors. In addition, the carboxyl group of esters must be 'free': its esterification abolishes the stimulatory effect of cholesteryl hemisuccinate on receptors. The reason for the enhanced hCG binding under the action of CH-S remains unclear. Structural features of CH-S that can be considered important for the interaction of the hormone with the receptor include: a polar hydrophilic chain covalently attached to the C-3 carbon of cholesterol; a steroid nucleus that is hydrophobic and steriochemicaly rigid; and a hydrocarbon side-chain with a high degree of mobility in the phospholipid bilayer [25]. It can be assumed that whatever the role of the carboxyl group as a cholesterol component is, only noncovalent interactions are involved. These experiments showing that hydrophilic sterol groups are required support the view that hydrogen interactions have to be taken into account. The carboxylic group may interact, for example, with amino-acid groups of the LH/hCG receptor at a locality distal to hCG-specific binding sites and thus change the structural integrity of the receptor and expose the closest cryptic binding sites.

Futhermore, since the chain at C-3 of cholesterol is of crucial importance, the polar moiety may be involved in the interaction. ESR studies with cholesterol esters in phospholipid vesicles showed that the spin-labeled ester adopted a 'horseshoe' conformation in the bilayer [26]. Finally, ^{13}C-NMR data on the interaction of spin-labeled cholesteryl palmitate with egg phosphatidylcholine bilayer indicated that the C-5 segment of the ester acyl chain is located near the C-1 and C-2 segments of the phospholipid acyl chain; this confirms the assumption that ester linkage is located near the aqueous interface [27]. The cell LH/hCG receptor, similarly to other membrane receptors, is thought to be an integral membrane protein associated with the membrane by strong hydrophobic bonds [28]. These bonds may play an important role in the interaction of gonadrotropin with the receptor that may be influenced by cholesterol esters. The possibility of a direct interaction between the polar moiety of the carboxyl group and the receptor was indicated by the treatment of membranes with CH-S methyl ester which was without any stimulatory effect on the accessibility of LH/hCG receptors. An important point is that almost all cholesterol esters which increase the accessibility of receptors also increase the rigidity of membrane lipids. These results suggested that changed membrane fluidity plays some role in the stimulatory action of CH-S.

The importance of the carboxyl group of cholesterol esters on the accessibility of LH/hCG receptors was suggested by further experiments with esters of monocarboxylic acids. The attachment of monocarboxylic acids to the 3β-OH group does not affect either the gonadotropin–receptor interaction of membrane fluidity. Cholesterol and cholesterol monocarboxylic ester have been reported to be easily incorporated into membranes [6,29]. Our data demonstrated that in spite of the low relative solubilities of steroid esters in aqueous media the lack of effect on receptor accessibility is not associated with the lack of incorporation of these compounds into the membrane. In addition, most of the esters tested change membrane lipids fluidity, and this may be also taken for evidence of their being incorporated into the membrane bilayer.

The side-chain of cholesteryl hemisuccinate plays an important role in the interaction of the carboxyl group of the ester with the receptor molecule. Androstenolone hemisuccinate (without a side-chain) does not affect membranes. However, moderately changed side-chains (hemisuccinates of β-sitosterol and stigmasterol) still maintained stimulatory activity on the accessibility of LH/hCG receptors. It appears certain from X-ray and neutron diffraction studies that the side-chain extends deeply into the bilayer interior [30], and a fluidity gradient in the sterol phospholipid contact region is therefore to be expected. Our previous studies with different spin-probes, which indicated the highest ordering effect of cholesteryl hemisuccinate at the C-16 carbon depth [7], are consistent with this arrangement.

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