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Oxygenated cholesterols synergistically immobilize acyl chains and enhance protein helical structure in human erythrocyte membranes

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Fourier transform infrared spectroscopy revealed that insertion of 20α-hydroxycholesterol into human erythrocyte membranes (10% of total membrane sterol) immobilized the lipid acyl chains to a degree equivalent to enriching total membrane cholesterol by 50% (Rooney, M.W., Lange, Y. and Kauffman, J.W. (1984) J. Biol. Chem. 259, 8281–8285). Raman spectroscopy showed that the amount of acyl chain rotamers was not significantly altered by the presence of 20α-hydroxycholesterol, indicating that acyl chain immobilization was limited to an inhibition of lateral motion. The presence of 20α-hydroxycholesterol may synergistically enhance the acyl-chain-immobilizing behavior of membrane cholesterol. In addition, protein helical structure was not altered by 20α-hydroxycholesterol. The insertion of 7α-hydroxycholesterol into erythrocyte membranes resulted in an increase in protein helical structure which was comparable to that observed for erythrocyte membranes enriched with pure cholesterol by 50%. However, both acyl chain mobility and conformation were unchanged. These results suggest a synergistic behavior between oxysterols and cholesterol in modifying erythrocyte membrane packing.

Introduction

Oxygenation converts cholesterol into a variety of compounds [1–2]. In the living system, oxygenated sterol compounds are produced as normal components of the metabolic pathway of cholesterol [3–5]. In addition, oxygenated sterol compounds are present in arterial injury which is a precursor to arteriosclerosis [6–15]. Studies on the effects of oxygenated sterol compounds in lipid bilayers [16–19] were preceded several years by experiments investigating their inhibition of microsomal-bound enzymes of cholesterol metabolism [3,20–25] and their effects on peripheral blood cell membrane morphology and function [26–32]. It is postulated that oxygenated sterol compounds participate in the normal endogenous regulation of sterol biosynthesis [33,34] and that in the case of deregulated sterol synthesis, typical of tumor growth, specific oxygenated sterol compounds are involved [35,36]. Substantial evidence indicates that a central property of oxygenated sterol compounds may be their physical insertion into biomembranes [26–30].

Previous studies with model lipid bilayers [16–19] have shown the oxygenated sterol compounds to have physical properties different from those of cholesterol, particularly the miscibility and condensing effectiveness of cholesterol oxygenated at the C7 and C25 positions. A recent

surprising discovery is that the oxygenated sterol compounds have the capacity to modulate synergistically the fluidizing/condensing properties of cholesterol in dipalmitoylphosphatidylcholine bilayers, as indicated by vibrational spectroscopic examination (unpublished data).

Fourier transform infrared and Raman spectroscopies have been used to study molecular conformation and organization of erythrocyte membranes [37–41]. It has been previously shown [37], using these techniques, that cholesterol depletion and enrichment of human erythrocyte membranes alter lipid acyl chain mobility and conformation as well as protein secondary structure. In this study, evidence is presented, using these same techniques, to support further the theory that oxygenated sterol compounds insert into erythrocyte membranes and synergistically modulate the fluidizing/condensing properties of cholesterol. In addition, specific oxygenated sterol compounds are shown to enhance erythrocyte membrane protein helicity to a degree equal to that produced by increasing total membrane cholesterol by 50%.

Experimental procedures

Freshly drawn heparinized blood was washed three times in phosphate-buffered saline (0.15 M NaCl/0.001 M PO_4^- (pH 7.4)) containing 0.25% crystalline bovine serum albumin (Miles Laboratories, Inc., Elkhart, IN) to remove plasma and buffy coat. The cells then were resuspended in lipoprotein-depleted medium at a hematocrit of 5%. The lipoprotein-depleted medium consisted of RPMI 1640 (Gibco), supplemented with an antibiotic-antimycotic mixture (Gibco) and containing 20% (v/v) lipoprotein-depleted serum (final protein concentration was 10 mg/ml, with less than 4 μg/ml cholesterol). Lipoprotein-depleted serum was prepared by ultracentrifugation floatation of lipoproteins of less than 1.25 density. The residual bottom fraction was dialyzed extensively against phosphate-buffered saline, heated to 56°C for 45 min, and sterilized by membrane filtration prior to use. Sterols were dissolved in absolute ethanol at 100-times the desired final concentration. 5 ml of red blood cell suspension were incubated with $2.5 \cdot 10^{-5}$ M sterol (≈ 10 μg/ml) in square centimeter tissue culture flasks (Falcon 3013) at 37°C in a 5% CO_2 water-saturated air environment for 1 h or longer if needed. During the incubation period, the flasks were rocked 40 times per min on a rocker platform (Model 7740, Bellico Glass Co., Vineland, NJ). Ethanol (1% v/v) was added to control cultures.

At the end of the incubation period, the erythrocytes were washed three times in phosphate-buffered saline (4°C), and ghosts were prepared as described in Ref. 37. Ghosts were then ultracentrifuged at $350\,000 \times g$ for 1 h at 4°C. The oxygenated sterol compound content of red blood cell ghosts prepared in this manner is approx. 10% of the total membrane sterol [26,27].

Approx. 25 μl of sample were transferred at 4°C to a Harrick cell (Ossining, NY) equipped with either $CaFl_2$ or ZnSe windows. The temperature was controlled with a Haake circulator within 0.2°C and monitored with a Digilab chromel-constantan thermocouple. Sample thickness (path length) was 50 μm. The samples were allowed to stabilize for 15 min prior to data acquisition. Spectral recording was performed with a Nicolet 7199 Fourier transform infrared spectrometer using parameters previously described [37]. A liquid-nitrogen-cooled HgCdTe detector gave the best signal-to-noise but had a spectral cut-off at approx. 700 cm^{-1}. Wave-number stability was accurate to ± 0.01 cm^{-1}. Band positions were determined at peak maxima by a Nicolet computer algortihm using approx. nine transform points per wave number. The temperature dependence of band certainty and techniques for background subtraction were described previously [37].

For Raman spectroscopy, ultracentrifuged samples were aspirated into Kimax capillary tubes of 2 mm diameter. Temperatures were controlled by placing the capillary tube along a copper circulating tube (0.25 inch diameter). Scattering at 90° from the incident laser light to the sample was focussed to a Spex 1401 double monochromator, and RCA C31034 Ga-As photomultiplier, and modified Spex counting electronics. A Nova 2/10 minicomputer controlled the scanning rate (1 s gate, 1 cm^{-1} step) and signal averaging (typically 4–9 spectra per average). The laser (Ar^+, Spectra Physics 164) was excited at 4880 nm and delivered at least 250 mW at the sample. The packed ghosts were essentially transparent, thus preventing any

significant laser heat. Spectral resolution was approx. 3 cm^{-1} for split widths of 150 μm. Intensities were accurate to within 0.15 as determined from peak height intensity ratios.

Results and Discussion

Infrared symmetryc C-H stretch regions of erythrocyte membranes containing 20α-hydroxycholesterol (approx. 10% of total membrane sterol substituted), normal cholesterol and 7α-hydroxycholesterol (10%) are presented in Fig. 1 for temperatures of 10 and 40°C. The most significant observation is the smaller thermotropic shift (0.1 cm^{-1}) of the C-H stretch band of the 20α-hydroxycholesterol preparation compared to shifts of the control and the erythrocyte membranes containing 7α-hydroxycholesterol. This small shift of the 20α-hydroxycholesterol-containing erythrocyte membranes is essentially the same as that seen for erythrocyte membranes enriched with pure cholesterol by 50% of normal cholesterol [37]. The previous study [37] inferred that the effect of increasing the erythrocyte membrane cholesterol content on the C-H stretch band was due to a decreased mobility of the membrane acyl chains rather than an overlapping cholesterol spectra. This was consistent with previous Fourier transform infrared studies of bilayers containing dipalmitoylphosphatidylcholine and cholesterol. It can then be hypothesized that the substitution of only 10% of the total membrane cholesterol by 20α-hydroxycholesterol is sufficient to modify the membrane acyl chain packing in a manner similar to increasing the cholesterol content by 50% above normal. A synergism between cholesterol and 20α-hydroxycholesterol in modifying membrane packing is thus proposed. This result agrees with observations that some oxygenated sterol compounds synergistically enhance the influence of cholesterol on the dipalmitoylphosphatidylcholine bilayer packing (M. Rooney et al., unpublished data).

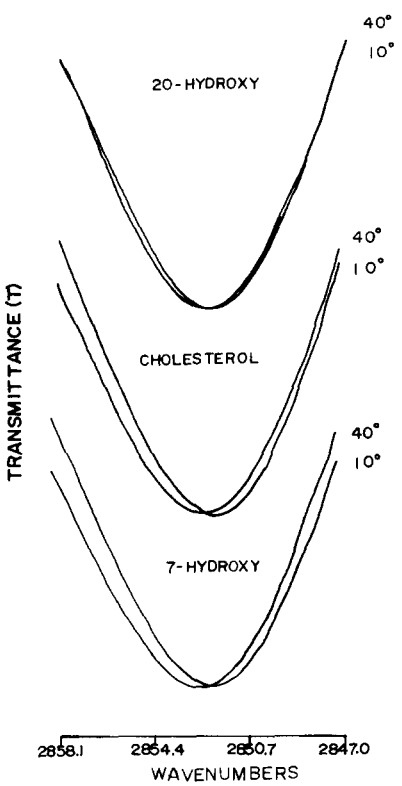

Fig. 1. Infrared symmetric C-H stretch bands of erythrocyte membranes inserted with 7α-hydroxycholesterol (top), cholesterol (middle) and 20α-hydroxycholesterol (bottom) at 10 and 40°C.

Phospholipid headgroup vibrations in the infrared fingerprint region [42,43] are shown for the three preparations in Fig. 2. In contrast to the above C-H stretching regions, the 7α-hydroxycholesterol-containing membranes now indicate an effect of the oxygenated sterol compounds while both the 20α-hydroxycholesterol-containing membranes and the control membranes have spectra that are quite similar. The strong bands (1220–1260 cm^{-1}) in the fingerprint region of the 7α-hydroxycholesterol-containing erythrocyte membranes appear to display more absorption intensity, compared to the 20α-hydroxycholesterol-containing and normal cholesterol-containing erythrocyte membranes. Unemura et al. [42] have shown that the presence of 50 mol% cholesterol shifts the dipalmitoylphosphatidylcholine antisymmetric phosphate stretch band from 1250 cm^{-1} to 1230 cm^{-1}. Normal erythrocyte membrane preparations indicate an absorption at approx. 1260 cm^{-1} while membranes containing 20α-hydroxycholesterol or 7α-hydroxycholesterol have absorptions at about 1230 cm^{-1}. It can be inferred that there are a greater number of sterol lipid contacts in the membrane preparations containing oxygenated sterol

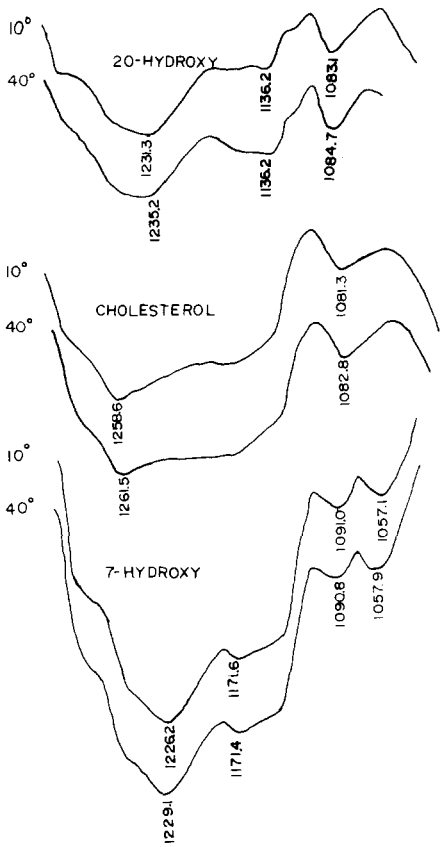

Fig. 2. Infrared phosphate (symmetric, asymmetric) diester stretch bands of erythrocyte membranes inserted with 7α-hydroxycholesterol (top), cholesterol (middle) and 20α-hydroxycholesterol (bottom) at 0 and 40°C.

compounds than in membranes containing only cholesterol.

Further, the shift in the symmetric phosphate stretch bond [42,43] from approx. 1080 cm^{-1} to approx. 1090 cm^{-1} when 7α-hydroxycholesterol is added to the membrane, and the appearance of the asymmetric phosphate stretch [42,43] at approx. 1060 cm^{-1} in the same preparation indicates that the presence of 7α-hydroxycholesterol causes an ordering of the phospholipid headgroups in the membrane. The increase in sterol/phospholipid contacts and a unique interaction between 7α-hydroxycholesterol and the membrane phospholipid can be hypothesized to cause the ordering necessary for an increase in the phosphate vibrational frequency. It is known that the insertion of 7α-hydroxycholesterol into erythrocyte membranes causes the formation of echinocytes or 'spur' cells [26,27]. These local spiny projections are probably accompanied by some change in molecular topology such as the formation of short-range molecular domains or clusters. If molecular domains or clusters are formed at the spiny areas, then a specific packing lattice may also be involved. Insertion of 20α-hydroxycholesterol into erythrocyte membranes is known to have no effect on erythrocyte surface morphology [26]. The above evidence supports the claim by Yachnin et al. [29] that sterols oxidized at the 7-position reside at the aqueous membrane interface. The effects of 7α-hydroxycholesterol on these hydrophilic regions of the erythrocyte membrane may be associated with the spiculed surface morphology observed for intact red cells containing 7α-hydroxycholesterol, because a change in surface architecture induced by the oxygenated sterol compounds would also be expected to effect molecular vibrations of the phospholipid headgroup as well.

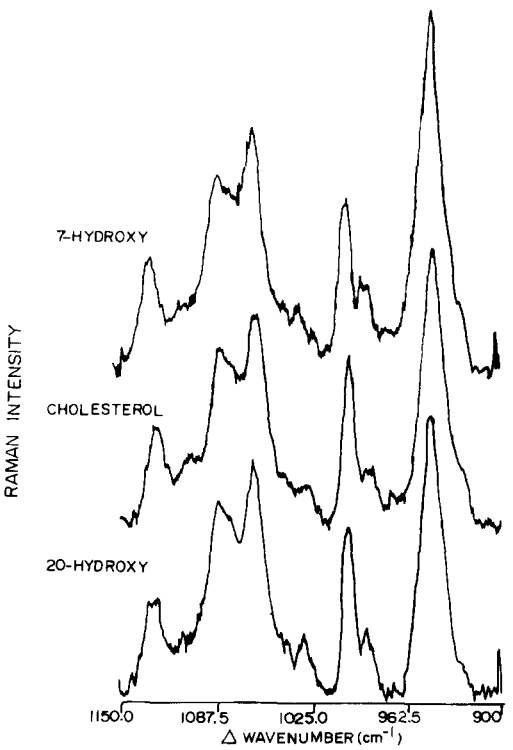

Fig. 3. Raman C-C stretch regions of erythrocyte membrane acyl chains (1025–1150 cm^{-1}) and protein backbone (920–970 cm^{-1}): 7α-hydroxycholesterol (top), cholesterol (middle) and 20α-hydroxycholesterol (bottom) at room temperature.

In Fig. 3, Raman spectra in the acyl chain and protein C-C stretch region are shown for the 20α-hydroxycholesterol-containing, control and 7α-hydroxycholesterol-containing erythrocyte membranes. Proceeding from left to right, the band at about 1128 cm^{-1} represents acyl chains in the extended or '*trans*' rotational conformation, the 1080 cm^{-1} band represents acyl chains in the kinked or 'gauche' conformation, the 1065 cm^{-1} band also represents the *trans* chain, the 1004 cm^{-1} peak represents a resonance Raman enhanced mode and stronger phenylalanine ring vibrations [37–41]. The 940 cm^{-1} band is due to peptide backbone carbon-carbon stretching of helical proteins [44,45]. Protein helical content for protein solutions has been correlated to the relative intensity of the 940 cm^{-1} band to the 1004 cm^{-1} band [46,47]. It can be assumed that the 1004 cm^{-1} band is unchanged in membrane preparation from the same donor (Hammer, B. and Kauffman, J.W., unpublished data) and can be used as an internal reference for membrane spectra. Table I lists the relative intensities between the five bands in each spectrum. The ratios are significantly different from each other in the fourth column (I_{940}/I_{1128}). The data in Table I thus demonstrate that the relative intensity of the 1128 cm^{-1} band of the 20α-hydroxycholesterol-containing erythrocyte membranes is slightly less than the relative intensities of the 1128 cm^{-1} band in the control of 7α-hydroxycholesterol-containing red cell membranes. Snyder et al. [48] have shown, however, that even large decreases in the intensity of the 1128 cm^{-1} band represent only small decreases in *trans* conformation. It can be suggested that although the oxygen at the C20 position may disrupt some *trans* acyl chains, the majority of acyl chain rotamers are unaffected by the presence of 20α-hydroxycholesterol. The effect of 20α-hydroxycholesterol, therefore, is mainly on the lateral mobility of the acyl chains.

The relative increase in the intensity of the Raman band at 940 cm^{-1} (referenced to the 1004 cm^{-1} line) in the 7α-hydroxycholesterol containing erythrocyte membranes (Table I) indicates that protein helical structure in these membranes has increased compared to the control and 20α-hydroxycholesterol-containing red cell membranes. This increase in Raman intensity of the 940 cm^{-1} band of the 7α-hydroxycholesterol-containing red cell membranes is approximately equal to that observed for erythrocyte membranes enriched with pure cholesterol by 50% of normal cholesterol content [37]. This evidence indicates a synergistic influence of oxygenated cholesterols on the total membrane molecular organization, both lipid and protein, since the 7-position sterols are moderately echinocytogenic [26], and a change in membrane surface architecture may be associated with alterations in the spectrin-actin membrane structural proteins [49]. The observed spectral changes in the amount of helical conformation may be assigned to changes in the spectrin-actin proteins leading to echinocytogenicity.

Conclusions

The vibrational spectra indicate that increases in red cell membrane protein helical structure and phosphate headgroup ordering are the results of the insertion of the sterols oxidized at the 7-position into red cell membranes. Insertion of 7α-hydroxycholesterol into the outer cell membrane lamella could expand the local surface area, which changes the surface tension relative to the inner lamella so that local curvature increases to a more favorable conformation and echinocytogenicity occurs. Indirect effects on the conformation of the

TABLE I

Sterol/RBC	Relative Raman intensities (I_{band}/I_{band})									
	940/1004	940/1065	940/1080	940/1128	1065/1004	1080/1004	1128/1004	1065/1128	1065/1080	1080/1128
20-Hydroxycholesterol	1.60	1.22	1.43	2.72	1.32	1.13	0.59	2.23	1.17	1.91
Cholesterol	1.62	1.26	1.50	2.50	1.29	1.08	0.65	1.98	1.19	1.67
7-Hydroxycholesterol	1.99	1.44	1.75	2.97	1.37	1.13	0.67	2.05	1.21	1.69

spectrin-actin complex in the regions of high membrane curvature may be responsible for the observed increase in helical structure, although direct effects of 7α-hydroxycholesterol on glycophorin or other outer lamella proteins are possible.

These results indicate also that insertion of 20α-hydrocholesterol results in an immobilization of the red cell membrane acyl chains. The reason that an increase in protein helical structure was not evident in the 20α-hydroxycholesterol-containing membranes may be due to a lack of change in red cell surface structure as is observed for spiculed membranes containg 7α-hydroxycholesterol. The nature of the changes in protein secondary structure of the 7α-hydroxycholesterol-containing and cholesterol-enriched erythrocyte membranes may be directly related to lipid-protein interactions such as through changes in membrane fluidity; however, they may also be caused by changes in cell membrane topology, as seen with the echinocyte and acanthocyte morphologies of the 7α-hydroxycholesterol-containing and cholesterol-enriched erythrocyte membranes, respectively.

The above results are additionally important, since the effects of inserting 10 mol% oxygenated sterol compounds into the red cell membrane is equivalent to an increase of 50 mol% in the cholesterol content of the red cell membrane [40]. During membrane insertion of the oxygenated sterol compounds (7α-hydroxy or 20α-hydroxycholesterol) an almost equivalent amount of cholesterol is removed [29]. Since the physical dimensions of the oxygenated sterol compound molecules differ only slightly from those of cholesterol, the effect of the oxygenated sterol compounds on red cell membranes must be explained by mechanisms other than the simple bulk volume effect of oxygenated sterol compound molecules inserted in the membranes [29]. We can infer a possible role for oxygenated sterol compounds in cooperative systems in which various oxygenated cholesterols synergistically (or antagonistically, depending on the feature being measured) interact with a larger population of cholesterol molecules to regulate membrane lipid acyl chain and protein stereochemistry.

Acknowledgements

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