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Polyunsaturated fatty acids alter sterol transbilayer domains in LM fibroblast plasma membrane

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Sterols are asymmetrically distributed between the leaflets of animal cell plasma membranes. Although transbilayer migration of sterols is extremely rapid, s to min, previous experimental manipulations have not altered their transmembrane steady-state distribution. However, the effect of polyunsaturated fatty acids has not been reported. When cultured in a lipid-free, chemically defined culture medium, LM fibroblasts do not synthesize polyunsaturated fatty acids but will incorporate polyunsaturated fatty acids into their plasma membranes if supplied in the medium. Sterol transbilayer distribution in LM plasma membranes was determined from quenching of fluorescence of dehydroergosterol by trinitrophenyl groups selectively attached to the exofacial leaflet. When cells are cultured in lipid-free media, 28.1% of the plasma membrane sterol is located in the exofacial (outside) leaflet. In contrast, when cells are cultured with linoleate- or linolenate-supplemented medium, 71.8% and 75.5% of the plasma membrane sterol is exofacial, respectively.

Sterol; Cell membrane; Membrane lipid; Unsaturated fatty acid; Lipid bilayer asymmetry

1. INTRODUCTION

Cholesterol is a primary determinant of biological membrane structure and function [1-3]. Changes in membrane cholesterol content occurring in certain diseases and in aging, as well as by experimental manipulation, alter a variety of membrane-associated properties [4]. Not only phospholipids and fatty acids, but also sterols are asymmetrically distributed between the leaflets of many biological membranes including plasma membranes from eukaroytic cells such as transformed mouse fibroblasts (LM and derivative cell lines) [4–6], human, rat and mouse erythrocytes [5-8], rat siatic nerve myelin [9], the insect-derived K_c cell line [10], as well as non-nucleate viral [11,12] and mycoplasmal [13] membranes. The mechanism(s) establishing, maintaining and regulating this transmembrane asymmetry of sterol concentration and the significance of this asymmetry for non-receptor mediated sterol transport and for membrane function, especially membrane-bound proteins, remain unknown. Previously, we have shown that the transmembrane sterol concentration gradient in LM cells was remarkably stable to a wide variety of manipulations to the structure and composition of the plasma membrane. Specifically the transmembrane sterol gradient was not affected after (i) clonal selection of LM cells resulting in plasma membrane sterol/phospholipid ratios varying 3-fold [4], (ii) in vivo replacement of choline in plasma membrane phosphatidylcholine by demethylated congeners [4], (iii) membrane leaflet selective fluidization with charged anesthetics [14],

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Abbreviations: 18:2, 9,12-*cis,cis*-octadecadienoic acid (linoleic acid); 18:3, 9,12,15-*cis,cis,cis*-octadecatrienoic acid (linolenic acid); BSA, bovine serum albumin

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and (iv) induction of lateral phase separations by changes in temperature [4].

The current interest in cholesterol transport [15] and in the role of polyunsaturated fatty acids (PUFA) of the ω-6 and, more especially, ω-3 classes in the prevention of atherosclerotic heart disease (reviewed in [16,17]), suggested an examination of the effect of PUFA on membrane structure and transbilayer sterol distribution. The LM plasma membrane is an excellent model system since it contains no PUFA under the usual culture condition but the lipid composition is amenable to manipulation by supplementation of the medium. The fatty acyl composition of LM cells was dramatically altered by supplementing the culture medium with linoleic and linolenic acids. Herein, we report that increased unsaturation of the phospholipid fatty acyl chains reversed the transmembrane sterol gradient of LM fibroblast plasma membranes.

2. MATERIALS AND METHODS

2.1. *Reagents and fluorescence probe molecules*

The fluorescent sterol dehydroergosterol ($\Delta^{5,7,9(11),22}$-ergostatetraen-3β-ol) was synthesized and purified as described [18]. High-performance liquid chromatography of dehydroergosterol and comparison with dehydroergosterol standards purchased from Frann Scientific, Inc. (Columbia, MO) confirmed the purity of dehydroergosterol. Other reagents were purchased from the following sources: Sigma (St. Louis, MO), trinitrobenzenesulfonic acid, diamide (azocarboxylic acid bis [dimethylamide]); Eastman Chemical Co. (Rochester, NY), 1,6-diphenyl-1,3,5-hexatriene; Aldrich (Milwaukee, WI), malonaldehyde bis(dimethyl acetal); Nu Check Prep (Elysian, MN), linoleic (18:2) and linolenic (18:3) acid; Miles Research Laboratories (Elkhart, IN), fatty acid poor BSA, fraction V.

2.2. *Cell lines and tissue culture*

LM cells (CC11.2), a tumorigenic mouse fibroblast subline of the L-929 cell, were obtained from the American Type Culture Collection (Rockville, MD). Cells were cultured in suspension and maintained in logarithmic growth at approx. 1×10^6 cells/ml in serum-free, chemically defined medium at 37°C in 500 ml bottles (250 ml/bottle) as described [19].

2.3. *Lipid supplementation*

LM cells were cultured for 24 h as above except that the media contained 0.119 mg delipidized (procedure of Goodman [20] as modified by Wilcox et al. [21]) bovine serum albumin/ml with 5 μg bound fatty acid (18:2; 18:3)/ml as described [22,23]. The free fatty acids were bound to the delipidized bovine serum albumin according to the published procedure in [24]. Fatty acid content of the complex was determined by titration [25]. Dehydroergosterol (5 μg/ml) was dissolved in ethanol and added to cultures 72 h prior to treatment with trinitrobenzenesulfonic acid and isolation of plasma membranes. Ethanol did not exceed 0.5% (v/v) final concentration, and did not adversely affect cell growth.

2.4. *Trinitrobenzenesulfonic acid labeling and plasma membrane isolation*

Cells were cultured without supplement, with dehydroergosterol, or with BSA-fatty acid and split into two aliquots, one which received no treatment and one which was treated with buffer containing trinitrobenzenesulfonic acid under nonpenetrating conditions (4°C for 80 min) to trinitrophenylate outer leaflet amine moieties. Treatment of cells with buffer alone versus no treatment gave identical results. This procedure has been extensively documented [4–6, 14, 26–32]. The cells were homogenized and plasma membranes isolated by differential and sucrose gradient centrifugation as described [19].

2.5. *Determination of sterol/phospholipid ratio and fatty acid composition*

All organic solvents were glass distilled and all glassware was washed with sulfuric acid/dichromate before use. Total membrane lipid from approx. 1.5 mg membrane protein, determined by the Lowry method [33], was extracted by the Bligh and Dyer method [34] as modified in [35]. Sterols were separated from phospholipids by silicic acid chromatography [19]. Phospholipid was quantitated by the method of Ames [36]. Sterol content and composition was determined by high-performance liquid chromatography [18] with the following modifications: 4.8 mm × 30 mm guard column and 4.8 mm × 150 mm column packed with 3 μm octadecyl silica (Alltech Assoc., Applied Science Labs, Deerfield, IL), with 20% methanol in acetonitrile (HPLC grade, Fisher Scientific, Pittsburgh, PA) at 1 ml/min, monitoring eluant absorbance at 215 nm (Series 4 HPLC and LC95 spectrophotometric detector with 18 μl flow cell, Perkin-Elmer, Inc., Norwalk, CT). Retention times were: dehydroergosterol, 11.0 min; desmosterol, 14.1 min; stigmasterol (added as internal standard), 23.5 min. Phospholipid fatty acid composition was determined as described [37]. Fatty acids and sterols were quantitated using a 3390A recording integrator (Hewlett Packard Inc., Palo Alto, CA). Peak areas were converted to mass using the experimentally determined response factor for each sterol.

2.6. *Fluorescence measurements and transbilayer sterol distribution*

The transbilayer distribution of dehydroergosterol was determined by the selective quenching of outer monolayer dehydroergosterol fluorescence by trinitrophenylated amine moieties in the outer monolayer [4–6] resulting from trinitrobenzenesulfonic acid labeling at 4°C.

The absorbance-corrected fluorescence intensity of dehydroergosterol excitation 324 nm, emission 380 nm) and diphenylhexatriene (excitation 362 nm, emission 424 nm) (1:1000 molar ratio to membrane lipid) was determined for unlabeled and trinitrophenylated plasma membranes (50 μg protein/ml phosphate-buffered saline, pH 7.4) using a computer-centered spectrofluorimeter [38] as described [29]. The fluorescence lifetime of dehydroergosterol in the plasma membranes was determined against a reference of 1,4-bis[2-(4-methyl-5-phenyloxazolyl)] benzene (dimethyl POPOP) in absolute ethanol [39] using a T format SLM 4800 spectrofluorimeter (SLM Instru-

ments, Champaign-Urbana, IL) as described [29]. Emission was measured above 380 nm using cutoff filters (Schott Optical Co., Duryea, PA).

3. RESULTS AND DISCUSSION

Fluorescent sterols, such as dehydroergosterol, are excellent nonperturbing probe molecules for sterols in model [40–42] and biological membranes [4–6]. Dehydroergosterol offers significant advantages over nitroxide or deuterated sterols [42,43]. Because the determination of transbilayer distribution of dehydroergosterol depends on trinitrophenylation of the exofacial amino groups, it was prudent to assess the effect of fatty acid supplementation on the plasma membrane purity. As ascertained by $(Na^+ + K^+)$-ATPase specific activity, the extent of plasma membrane purification (6–9-fold over crude homogenate) was unaltered by either fatty acid supplementation or trinitrobenzenesulfonic acid treatment (table 1). However, the specific activity of $(Na^+ + K^+)$-ATPase was significantly increased in the plasma membranes containing polyunsaturated fatty acids (table 1). Furthermore, penetration of the membrane by trinitrobenzenesulfonic acid was examined by 1,6,-diphenyl-1,3,5-hexatriene fluorescence quenching as well as extent of reaction of exofacial amine groups [26–30]. Quenching of 1,6-diphenyl-1,3,5-hexatriene fluorescence in all unsupplemented and polyunsaturated fatty acid-supplemented membranes was near 50%, as expected for extensive exofacial and no cytofacial trinitrophenylation of amine groups. Insufficient reaction and penetration of the reagent into the cell yields quenching of less than 50% and greater than 50% (to greater than 95%), respectively [26–30]. Exofacial trinitrophenylation does not disturb membrane lipid structure [14].

Supplementation of the nutrient medium with fatty acid, non-covalently bound to bovine serum albumin, resulted in the incorporation of supplemented fatty acids into the LM cell plasma membrane phospholipids. The plasma membrane from cells cultured in lipid free medium contained only saturated and monounsaturated fatty acids (table 2). Palmitic (16:0) and oleic (18:1) acids together comprised 76% of the plasma membrane phospholipid acyl groups. Supplementation of the culture medium with linoleic acid (18:2) resulted in the incorporation of linoleyl groups (5.1 ± 0.4%) with concomitant reduction of palmitoyl groups (from 20.9 to 7.9%). Supplementation with linolenic acid (18:3) resulted in its incorporation (7.1 ± 1.4%) with a simultaneous reduction in oleoyl groups (from 55.0 to 45.2%). Conversion between linoleic acid, the parent member of the ω-6 PUFAs, and linolenic acid, the parent member of the ω-3 PUFAs, was not observed, as was expected since metabolic interconversion between classes does not occur [16]. Thus, at the concentration tested, LM cells bioincorporate into their plasma membrane exogenously supplied fatty acids of a type not normally present. Cell viability was unaffected. This confirms previous results [44].

Supplementation of LM cells with polyunsaturated fatty acids did not alter the sterol/phospholipid molar ratio of the plasma membrane. This ratio was 0.74 ± 0.09, 0.60 ± 0.10 and 0.68 ± 0.08 in plasma membranes from control, linoleate- and linolenate-supplemented cells, respectively. These ratios, consistent with previous observations [45], suggest that any alteration in transbilayer asymmetry of sterol distribution is not due to alterations in sterol/phospholipid ratio. Furthermore, in LM cell sublines selected for phenotypic expression of different sterol/phospholipid ratios, no change in transbilayer sterol distribution was observed over a range of plasma membrane sterol/phospholipid ratios [4]. Thus, even had the sterol/phospholipid ratio differed between nutritional regimens, no effect of this ratio on transbilayer distribution would be expected.

In the plasma membrane from LM cells cultured without fatty acids in the medium, 28.1% of the dehydroergosterol fluorescence was quenched, indicating that the cytofacial leaflet was enriched in sterol (table 3). In contrast, in plasma membranes containing polyunsaturated fatty acids, the transbilayer sterol gradient was exactly reversed, with 71.8 and 75.0% of the dehydroergosterol fluorescence being quenched in the linoleate- and linolenate-containing plasma membranes, respectively. This reversal was not due to changes in other fluorescence parameters since neither fluorescence lifetime nor absorbance-corrected fluorescence intensity of dehydroergosterol is altered by the presence of polyunsaturated fatty acids in the membrane (table 3).

Table 1

Effect of polyunsaturated fatty acid supplementation on specific activity of $(Na^+ + K^+)$-ATPase in LM cell plasma membranes

Medium supplement	Untreated		Trinitrobenzenesulfonic acid	
	Crude homogenate	Plasma membrane	Crude homogenate	Plasma membrane
None	14.2 ± 2.6	84.3 ± 9.2	14.4 ± 3.1	85.7 ± 8.9
Linoleic acid	18.1 ± 3.2	125.3 ± 2.3*	19.6 ± 2.8	141.5 ± 5.2*
Linolenic acid	15.9 ± 1.2	131.8 ± 10.4*	13.2 ± 3.1	120.8 ± 7.6*

LM cells, cultured without lipid supplement or with linoleic or linolenic acid bound to bovine serum albumin, were untreated (one-half) or treated (other half) with trinitrobenzene sulfonic acid under nonpenetrating conditions. After homogenization, the plasma membranes were isolated on discontinuous sucrose gradients and the activity, expressed as nmol phosphate generated/min × mg protein, of $(Na^+ + K^+)$-ATPase was determined. Values represent the mean ± SE ($n = 3$–9). An asterisk indicates a statistically significant ($p < 0.05$) difference from no supplement

The mechanism producing this profound alteration in transmembrane sterol gradient is obscure at present. Previous work utilizing a variety of manipulations has demonstrated the transmembrane steady-state sterol distribution in LM cell plasma membranes to be very stable. These manipulations have included alterations of (i) the phospholipid headgroups, (ii) the sterol/phospholipid molar ratio, (iii) the transmembrane fluidity gradient with leaflet selective charged amphipaths, and (iv) the fluidity and lateral phase separations by changes in temperature [4]. Furthermore, peroxidation of membranes with $H_2O_2/FeCl_2$ and crosslinking of membrane proteins failed to alter sterol asymmetry (Schroeder, F. & Sweet, W.D., unpublished). These data support the notion that transmembrane sterol distribution is a tightly regulated property, the functional consequences of which are unknown. Many membrane functions, such as enzymes, components of receptor-second messenger systems and transporters, may be affected by altered transmembrane sterol distribution. Effects on the flux, and steady-state concentrations, of sterol among the various cellular membranes may be of particular interest. Conditions enhancing an exofacial distribution of

Table 2

Incorporation of exogenous polyunsaturated fatty acids into the plasma membrane of LM cells

Fatty acid	Medium supplement		
	None	18:2	18:3
14:0	0.5 ± 0.3	0.1 ± 0.1	0.3 ± 0.2
15:0	0.5 ± 0.3	0.2 ± 0.1	0.4 ± 0.1
16:0	20.9 ± 2.2	7.9 ± 1.9	20.2 ± 1.7
16:1	3.7 ± 1.6	2.0 ± 0.7	3.9 ± 0.9
18:0	16.2 ± 0.9	20.9 ± 1.1	19.6 ± 1.1
18:1	56.0 ± 4.7	63.2 ± 1.7	45.2 ± 3.2
18:2	ND	5.1 ± 0.4	ND
18:3	ND	ND	7.1 ± 1.4
Other	1.8 ± 0.7	0.8 ± 0.4	3.1 ± 1.3
Double bond index	1.48 ± 0.11	2.57 ± 0.10	1.71 ± 0.12

LM fibroblasts were cultured for 3 days in lipid-free medium or identical medium containing linoleate (18:2) or linolenate (18:3) bound to bovine serum albumin. The plasma membranes were isolated, the lipids extracted and the fatty acids quantitated by gas chromatography as methyl esters. Fatty acid nomenclature states carbon chain length and number of double bonds; other refers to >18 carbon and traces of odd number fatty acid. Double bond index is calculated as Σ (number of double bonds × fractional content of fatty acid)/Σ (fractional content of saturated fatty acid), summed over all fatty acid species. Values are mean ± SE ($n = 6$). ND, not detected

Table 3

Effect of unsaturated fatty acid supplementation on transbilayer distribution and fluorescence parameters of dehydroergosterol in LM cell plasma membrane

Culture medium supplement	% dehydroergosterol fluorescence quenched	Fluorescence lifetime (ns)	Absorbance-corrected fluorescence intensity
None	28.1 ± 5.5	1.5 ± 0.1	60 ± 4
Linoleate	71.8 ± 3.5*	1.5 ± 0.1	54 ± 3
Linolenate	75.5 ± 7.5*	1.5 ± 0.1	ND

Absorbance-corrected fluorescence of dehydroergosterol in isolated LM cell plasma membranes (cells untreated or treated with trinitrobenzenesulfonic acid as described in section 2) was determined at 37°C. Percent quenching (intensity from TNBS treated membranes/intensity from untreated membranes × 100%) equals % dehydroergosterol in the exofacial leaflet. Data represent the mean ± SE ($n = 3$). An asterisk signifies $p < 0.05$ by an unpaired Student's t-test compared to no supplement. ND, not determined

sterol (e.g. polyunsaturated fatty acids) might enhance the removal of cellular cholesterol by extracellular carriers (lipoproteins) for delivery to and disposal by the liver. Dietary polyunsaturated fatty acids lower plasma cholesterol [16]. In contrast, a cytofacial distribution of cholesterol may increase intracellular deposition of cholesterol by enhanced availability of plasma membrane cholesterol to intracellular sterol carrier proteins and transport to intracellular sites thereby. An increased hepatic content of cholestryl esters occurs in rats deficient in essential fatty acids [46,47].

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