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Lipid Fluidity and Composition of the Erythrocyte Membrane from Healthy Dogs and Labrador Retrievers with Hereditary Muscular Dystrophy

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Erythrocyte membranes and their liposomes were prepared from clinically normal dogs and Labrador retrievers with hereditary muscular dystrophy. The "static" and "dynamic" components of fluidity of each membrane were then assessed by steady-state fluorescence polarization techniques using limiting hindered fluorescence anisotropy and order parameter values of 1,6-diphenyl-1,3,5-hexatriene (DPH) and fluorescence anisotropy values of DL-2-(9-anthroyl)-stearic acid and DL-12-(9-anthroyl)-stearic acid, respectively. Membrane lipids were extracted and analyzed by thin-layer chromatography and gas chromatography. The results of these studies demonstrated that the lipid fluidity of erythrocyte membranes, and their liposomes, prepared from dystrophic dogs were found to possess significantly lower "static and dynamic components of fluidity" than control counterparts. Analysis of the composition of membranes from dystrophic dogs revealed a higher ratio of saturated fatty acyl chain/unsaturated chains (w/w) and lower double-bond index. Alterations in the fatty acid composition such as decrease in levels of linoleic (18:2) and arachidonic (20:4) acids and increase in palmitic (16:0) and stearic (18:0) acids were also observed in the membranes of dystrophic animals. These associated fatty acyl alterations could explain, at least in part, the differences in membrane fluidity between dystrophic and control dogs.

KEY WORDS: Erythrocytes; membrane fluidity; muscular dystrophy; lipid composition; fluorescence polarization.

INTRODUCTION

Hereditary muscular dystrophy of Labrador Retrievers (HMDLR) is a genetic (autosomal recessive) disorder which has clinical and pathological similarities to certain forms of muscular dystrophy in people (1,2). The clinical manifestations of this disease include exercise intolerance, muscle wasting, atrophy and loss of type II skeletal muscle fibers, and hyporeflexia (3,4). The muscle pathology of HMDLR includes features of both neurogenic and myogenic diseases (2,4); however, underlying pathophysiology remains elusive. Currently, the most commonly accepted theory for the basis of muscular dystrophy is that a primary sarcolemmal membrane defect is the underlying factor involved among many pleotrophic effects of this genetic lesion (5). We have recently reported substantial derangements of intracellular electrolyte equilibria, lipid content, as well as water compartment distribution from skeletal muscles of HMDLR (6). Among several other biochemical anomalies associated with this disease (7,8), the calcium content in most muscles from dystrophic dogs was

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significantly increased. Calcium ions play a significant role in the regulation of a variety of functions associated with cell membranes, including the transduction of hormonal signals, neurotransmitter release and action, muscle contraction and relaxation, release of exocrine and endocrine secretory products, transmembrane transport and permeability, and maintenance of membrane viscoelastic properties and cell shape (9). This divalent cation has been shown to be an important effector of membrane phospholipid organization (10) and can interact directly with phospholipid monolayers and bilayers and thereby, decrease the motional freedom of their lipid molecules (11). Although clinical manifestations are most apparent in skeletal muscle, considerable evidence exists that a number of structural and functional alterations are present in erythrocyte membranes of human patients with muscular dystrophy (12, 13), murine dystrophy (14), and HMDLR (15). A functionally significant alteration in the organization or structure of a membrane may perturb its physicochemical properties. The indirect information about the organization of and interactions between lipids, proteins, and glycoconjugates can be obtained by measuring membrane lipid fluidity* by probing different membrane levels with fluorescent labels (16). The purpose of the present study is to explore the changes in lipid dynamics in erythrocyte membranes of HMDLR.

EXPERIMENTAL PROCEDURE

Materials. 1,6-Diphenyl-1,3,5-hexatriene, DL-2-(9-anthroyl)-stearic acid and DL-12-(9-anthroyl)-stearic acid were obtained from Molecular Probes Inc. (Eugene, OR). Lipid standard mixtures for thin layer chromatography were purchased from Matreya Inc. (Pleasant Gap, PA). Fatty acid, fatty acid methyl esters and gas chromatography columns were purchased from Supelco (Bellefonte, PA), and/or Nu Chek Prep Inc. (Elysian, MN). All other materials were obtained from either Sigma Chemical Co. (St. Louis, MO) or Fisher Chemical Co. (Fairlawn, NJ).

* The term "lipid fluidity" as applied to anisotropic bilayer membranes is used throughout this report to express the relative motional freedom of the lipid molecules or substituents thereof. A more detailed description of the sense in which the term is used has previously been published (31). Briefly, as evaluated by steady-state fluorescence polarization of lipid fluorophores, "fluidity" is assessed by the parameters of the modified Perrin equation described under "Experimental Procedure." An increase in fluidity corresponds to a decrease in either correlation time, T_c, or the hindered anisotropy, r_∞, of the fluorophore, thereby, combining the concepts of the dynamic and static (lipid order) components of fluidity.

Methods

Membrane Preparation. Venous blood from age- and sex-matched clinically normal and affected dogs (2-year-old) that had been fasted overnight was collected in sterile tubes containing heparin at Washington State University, Pullman, Washington, U.S.A. All dogs were given routine neurologic and electrophysiologic examinations, including needle electromyography and nerve conduction velocity determinations. The blood specimens were transported in insulated containers with ice and processed within 24 hours. Plasma and platelets were removed from erythrocytes by differential centrifugation. The red cells were then dispersed in isotonic phosphate buffer (310 mOsm, pH 7.4) and washed by repeated centrifugations (20 min, 1000 *g*). Erythrocyte ghosts were prepared by hypotonic lysis in 20 mOsm phosphate buffer (pH 7.4) according to the procedure of Dodge et al. (17). Ghosts were washed repeatedly in the phosphate buffer to remove hemoglobin and other cytoplasmic components. Membranes were finally suspended in wash medium to 1.5 to 2 mg protein/ml, as determined by the method of Bradford (18), using bovine serum albumin as standard.

Lipid Extract and Liposomes. Total lipids were extracted from erythrocyte membrane preparations with chloroform/methanol (2:1 v/v) followed by partition according to Folch et al. (19). For preparation of sonicated dispersions of lipid (liposomes), the dried, extracted lipid was suspended in phosphate-buffered saline (20) to final concentration of approximately 0.3-0.5 mg/ml and the mixture was sonicated for 10 min, under N_2 at 4°C. Thereafter, the preparations were centrifuged at 10,000 *g* for 10 min and the resulting supernatant suspensions were used for fluorescence studies.

Chemical Determinations. Total cholesterol and phospholipids were measured by the methods of Zlatkis et al. (21) and Ames and Dubin (22), respectively. The composition of the lipid extract was examined by quantitative thin layer chromatography according to the procedure of Katz et al. (23). Glycolipids were not estimated in these studies, and relative composition of the extracts is given as a percentage of the neutral lipids plus phospholipids. To determine the acyl chain composition, fatty acids of the total lipid extract were derivatized as described by Gartner and Vahouny (24). Fatty acid methyl esters were analysed on a Perkin-Elmer 8420 capillary gas-chromatograph equipped with flame ionization detector and interfaced with Omega analytical workstation, an optimized chromatography integration and data manipulation system. These analyses were performed using a SP-2330 fused silica capillary column (30m × 0.25mm ID, 0.20μm film) (Supelco, Bellefonte, PA). Nitrogen was used as carrier gas. Initial oven temperature was 165°C for the first 6 min. then increased to 220°C at a rate of 5.5°C/min and held at 220°C for another 20 min. The split ratio was 1:100. Authentic acid methyl esters were used to identify retention times (24).

Fluorescence-Polarization Studies. The fluidity of the red cell membrane and liposome suspensions was assessed at 25°C in triplicate by the steady-state fluorescence polarization of the three lipid-soluble fluorophores: 1,6-diphenyl-1,3,5-hexatriene (DPH), DL-2-(9-anthroyl)-stearic acid (2-AS), DL-12(9-anthroyl)-stearic acid (12-AS), using a Perkin-Elmer LS-5B Luminescence spectrometer equipped with polarizers in both excitation and emission beam (C.N. Wood Mfg., Newton, PA). The methods used to load the membranes and liposomes with each of these probes and estimation of the fluorescence of polarization were essentially as described elsewhere (20, 25). Excitation and emission wavelengths were set at 365 and 430 nm with band widths of 5 and 10 mm, respectively. Data were acquired for 5s for each polarizer setting. The polarization value, P, of the fluorescence signal was measured as follows: $P = I_{VV}\text{-}G.I_{VH}/I_{VV} + G.I_{VH}$, where I_{VV} and I_{VH} are the vertical (parallel) and horizontal (perpendicular)

components of the emitted light when excited with vertically polarized light. The factor, G, which serves to correct for emission monochromator polarization effects, is equal to the ratio, I_{HV}/I_{HH}, where I_{HV} and I_{HH} are the vertical and horizontal components of emission from horizontally polarized exciting light (26). To minimize reversible deactivation of the probe, all experiments were conducted in the dark. Fluorescence anisotropy was calculated according to $r = 2P/(3-P)$. Recent time-resolved fluorescence anisotropy decay measurement have demonstrated that the rotation of certain fluorophores, such as DPH, are restricted by molecular packing of biological and model membrane lipids (27, 28). Therefore, the fluorescence anisotropy of such a fluorophore is not adequately described by Perrin equation but rather by a modified relationship (29,30):$r = r_\infty + (r_o - r_\infty)[T_c/(T_c + T_f)]$ where r_o is the maximal limiting anisotropy, taken as 0.390 for DPH and 0.285 for anthroyl probes (20,28), r_∞ is the limiting hindered anisotropy, T_c is the correlation time and T_f is the mean lifetime of the excited state. The r_∞ values of DPH in natural and artificial bilayer membranes are high and largely determine r (31). The r_∞ [$r^\infty = (4r/3)-0.1$] values of DPH can also be used to define an order parameter, S, where $S = (r_\infty/r_o)^{0.5}$ (29,30). The r_∞ and S values of DPH can, therefore, be used to assess the "static component of fluidity" of membranes. Unlike DPH, the anthroyl probes (2-AS and 12-AS) yield relatively low values of r_∞ in bilayer membranes (31). Final molar ratios of each probe/lipid ranged from 0.003 to 0.004 and the anisotropy (r) differences noted in these studies could not be ascribed to differences in the probe concentration of these preparations. Corrections for light-scattering (membrane suspensions minus probe) and for fluorescence in the ambient medium (quantified by pelleting the preparations after each estimation) were routinely performed (20,25). The combined corrections were less than 2% of the fluorescence intensity observed for DPH-loaded preparations and less than 4% of that observed for anthroylstearate-loaded suspensions.

Statistical analysis: All results are expressed as mean values ± standard error. Unpaired Student *t*-test was used for all statistical analysis. $P < 0.05$ was considered significant.

RESULTS

Fluorescence Polarization Studies. The physical state of the lipids of erythrocyte membranes was assessed by the rotational mobilities of the probes DPH, 2-AS, and 12-AS (Tables I and II). These fluorophors differ in structure and shape and localize in different domains of the bilayer (32, 33). In this study, we have presented r, r_∞ and S values as indices of membrane fluidity, an increase in r or r_∞ and S signifying a reduction in lipid fluidity. Both the 'static' and 'dynamic' component of membrane fluidity, as assessed by r_∞ and 'S' values of DPH (Table I) and r values of 2-AS and 12-AS (Table II), respectively, were found to be decreased in the membranes prepared from dystrophic dogs. Differences in fluidity were also detected in liposomes of erythrocyte membranes prepared from affected dogs compared to their control counterparts (Tables I and II).

Lipid Composition Studies. Membrane composition was examined in order to determine the factors responsible for the foregoing differences in membrane fluidity. Prior studies in model bilayer and natural membranes have correlated differences in lipid fluidity with variations in their lipid and protein composition (34, 35). It was, therefore, of interest to examine these compositional parameters in these membranes. The results of these studies are summarized in Tables III, IV and V. The relative percentages of individual lipid species as analyzed by thin layer chromatography remain unchanged in erythrocyte membrane preparations from control and dystrophic dogs (Table III). The erythrocyte membranes from control and dystrophic dogs showed similar cholesterol/phospholipid (mol/mol), sphingomyelin/phosphatidylcholine (mol/mol), cholesterol/protein (wt/wt), and phospholipid/protein (wt/wt) ratios (Table IV). Membranes prepared from dystrophic dogs, however, were found to possess a significantly higher saturation index and a lower double-bond index (Table IV). Analysis of total fatty acids of erythrocyte membranes revealed that preparations from dystrophic animals possessed lower percentages of linoleic acid (18:2) and arachidonic acid (20:4) and higher percentages of palmitic acid (16:0) and stearic acid (18:0) than did control membranes (Table V). These differences in fatty acids appeared to be responsible for higher saturation index and lower double bond index seen in the membranes of dystrophic animals.

DISCUSSION

The maintenance of membrane fluidity within narrow limits is presumably a prerequisite for proper functioning of a cell. Lipids play a key role in determining the membrane fluidity, and changes in lipid composition have been reported to alter important cellular functions (36, 37). The concept of fluidity, applied to phospholipidic bilayer refers to orientational and dynamic properties of the lipid hydrocarbon chains in the bilayer matrix, i.e., respectively the limiting rotational amplitude of motion, quantitatively expressed by the lipidic order parameter, and rate of rotational motion related to local microviscosity (38). The dynamic properties of the membrane lipid matrix have been extensively studied using such techniques as nuclear magnetic resonance, electron spin resonance, and fluorescence polarization (39, 40). Of these, steady-state fluorescence polarization using fluorescent probes is a rapid, most convenient and powerful routine technique to determine the overall lipid structural order in membranes. A high structural order implies a high degree of packing or high mutual affinity among the lipids (41).

Table I. Fluorescence Anisotropy of Diphenylhexatriene in Erythrocyte Membranes and Liposomes Prepared from Normal and Dystrophic Dogs

Preparation	Fluorescence anisotropy at 25° C	Limiting hindered anisotropy at 25° C	Order Parameter
	r	r_∞	S
Normal Dogs (5)			
Erythrocyte membranes	0.166 ± 0.033	0.122 ± 0.004	0.561 ± 0.006
Liposomes	0.149 ± 0.003	0.099 ± 0.005	0.502 ± 0.006
Dystrophic Dogs (5)			
Erythrocyte membranes	0.184 ± 0.005*	0.145 ± 0.006*	0.609 ± 0.006**
Liposomes	0.165 ± 0.004*	0.120 ± 0.004*	0.556 ± 0.005**

Values represent mean ± standard error. Number of animals used are shown in parenthesis.
* $P<0.01$ and ** $P<0.001$ compared to control vaues.

Table II. Fluorescence Anisotropy of Anthroyloxy Fatty Acids in Erythrocyte Membranes and Liposomes Prepared from Normal and Dystrophic Dogs

Preparation	2-AS Fluorescence anisotropy at 25°C	12-AS Fluorescence anisotropy at 25°C
	r	r
Normal Dogs (5)		
Erythrocyte membranes	0.212 ± 0.002	0.144 ± 0.001
Liposomes	0.199 ± 0.002	0.131 ± 0.003
Dystrophic Dogs (5)		
Erythrocyte membranes	0.223 ± 0.002**	0.155 ± 0.002**
Liposomes	0.209 ± 0.002**	0.142 ± 0.002*

Values represent means ± standard error. Number of animals used are shown in parenthesis. *$P<0.01$ and **$P<0.005$ compared to control values.

Table III. Lipid Composition of Erythrocyte Membranes Prepared from Normal and Dystrophic Dogs

Component	% by weight of neutral plus phospholipids	
	Normal dogs (5)	Dystrophic dogs (5)
Cholesterol	24.86 ± 0.68	24.43 ± 0.40
Cholesterol esters	1.15 ± 0.15	1.14 ± 0.15
Triacylglycerol	6.59 ± 0.49	7.76 ± 0.42
Fatty acids	5.08 ± 0.40	5.40 ± 0.26
Phosphatidylcholine	29.59 ± 0.99	32.71 ± 1.32
Lysophosphatidylcholine	8.67 ± 1.32	5.45 ± 1.08
Sphingomyelin	12.38 ± 0.49	13.59 ± 0.48
Phosphatidylethanolamine	11.69 ± 0.69	9.62 ± 0.88

Values represent mean ± standard error. Lipid composition is given in per cent by weight of total neutral plus phospholipids. Number of animals used are shown in parentheses.

Table IV. Compositional Parameters of Erythrocyte Membranes Prepared from Normal and Dystrophic Dogs

Component	Normal Dogs (5)	Dystrophic Dogs (5)
Cholesterol/Phospholipid (mol/mol)	0.93 ± 0.02	0.96 ± 0.02
Spingomyelin/lecithin[f] (mol/mol)	0.42 ± 0.04	0.39 ± 0.03
Cholesterol/Protein (wt/wt)	0.14 ± 0.01	0.16 ± 0.01
Phospholipid/Protein (wt/wt)	0.28 ± 0.02	0.32 ± 0.02
Saturation Index§	0.38 ± 0.03	0.56 ± 0.05*
Double Bond Index†	1.42 ± 0.07	1.06 ± 0.09*

Values represent mean ± standard error. * $P<0.01$ compared to control value. § Saturation index was calculated as (the total number of saturated residues)/(the sum of the number of each type of unsaturated residue multiplied by the number of double bonds in that residue). † Double bond index was calculated as (sum of each unsaturated chain multiplied by number of double bonds/100).
[f] Lecithin = phosphatidylcholine.

Table V. Compositional Analysis of Total Fatty Acids of Erythrocyte Membranes from Normal and Dystrophic Dogs

Fatty Acids	Normal dogs (5)	Dystrophic Dogs (5)
	% by mass	% by mass
16:0	16.48 ± 0.30	19.94 ± 0.67*
16:1	0.55 ± 0.06	0.58 ± 0.06
18:0	35.20 ± 0.99	40.04 ± 0.91*
18:1	11.40 ± 0.55	12.73 ± 0.58
18:2	7.80 ± 0.23	6.55 ± 0.21*
20:4	28.29 ± 1.40	20.16 ± 0.97*

Values represent mean ± standard error. * $P<0.005$ compared to control values.

In the foregoing studies, the erythrocyte membranes and their liposomes, prepared from Labrador Retrievers with hereditary muscular dystrophy, displayed greater quantitative differences in fluidity than their control counterparts. Both the 'static' component of membrane

fluidity i.e. the degree of order of lipid, as well as 'dynamic' component of fluidity in dystrophic dog preparations were decreased, as assessed by r_∞ and S values of DPH and r values of 2-AS and 12-AS, respectively. The three fluorophors used in this study also provide information about different regions of the membrane. While DPH is localized generally in the hydrophobic core of the membrane (35), the membrane localization of the anthroyl fluorophore is more specific; 2-AS monitors the polar head group region and 12-AS the less polar interior of each hemileaflet (33). The fluorescence polarization data obtained with these probes in erythrocyte membranes from dystrophic dogs, suggests a significant alteration in the membrane structure. These findings are in agreement with the previous investigations in fluidity of erythrocyte membranes in human patients with Duchenne muscular dystrophy (42-44), myotonic dystrophy (45), and avian muscular dystrophy (46), as assessed by both electron spin resonance and steady-state fluorescence polarization techniques. However, other studies have failed to confirm the changes in membrane fluidity in myotonic and Duchenne muscular dystrophy (13, 47, 48). These findings probably resulted from variation in the methodologies employed to assess fluidity and membrane preparation or alteration in the rheological behavior of membranes with increasing age of patients (13).

Proteins in a biological membrane perturb the lipid environment and, depending on their nature and concentration, influence membrane fluidity (30, 49). The erythrocyte membrane protein content appeared normal in our dystrophic dogs, as indicated by an unchanged phospholipid/protein ratio. The significant reduction in fluidity of liposomes from erythrocyte membranes of dystrophic dogs suggests that the decreased membrane fluidity was predominantly a consequence of altered lipid composition rather than protein abnormalities. Nevertheless, a contribution to decreased membrane fluidity mediated via changes in protein constituents cannot be completely excluded.

Membrane fluidity is determined by the interaction of membrane constituents, with the lipids producing major effects (35). Cholesterol plays a key role since it appears to maintain the bilayer matrix in an "intermediate fluid state" (50) by regulating mobility of phospholipid fatty acyl chains. Studies in model bilayers and natural membranes have correlated a high lipid fluidity with low molar ratios of cholesterol/phospholipid and sphingomyelin/phosphatidylcholine (35, 51). A low ratio of phospholipid/protein (w/w) (52) as well as less saturated or shorter fatty acyl chains in phospholipids (35) have also been associated with a higher membrane lipid fluidity. In the present study, cholesterol and phospholipid contents and their molar ratio was found to be normal in erythrocyte membranes of dystrophic dogs. Our findings of normal cholesterol and phospholipid contents are in accordance with prior studies using erythrocyte membranes from patients with Duchenne muscular dystrophy and myotonic dystrophy (53-55). However, erythrocyte membranes prepared from our dystrophic dogs were characterized by substantial alterations in the composition of certain fatty acids, thereby resulting in an increased saturation index and decreased double-bond index. In this regard, prior studies in erythrocyte membranes prepared from patients with myotonic dystrophy (56, 57) and paramyotonia congenita (58) are of interest. Compared to normal control human subjects, Antoku et al (56) found a lower content of linoleic (18:2) acid, but a higher palmitic (16:0) acid content in erythrocyte membrane total lipids, derived from patients with myotonic dystrophy. Furthermore, the content of palmitic (16:0) acid was found to be increased in phosphatidylcholine and phosphatidylethanolamine fractions, and that of stearic (18:0) and linoleic (18:2) acids were decreased in phosphatidylcholine fraction of erythrocyte membranes from patients with myotonic dystrophy (57). Using erythrocyte membranes derived from patients with paramyotonia congenita, Marx et al (58) also found an increase in palmitic (16:0) acid and a decrease in linoleic (18:2) and arachidonic (20:4) acids of the membrane phospholipids. Thus, these earlier studies in people and present data in dystrophic dogs, strongly suggest that the fatty acid composition of erythrocyte membranes is abnormal and might be responsible for the reduction of membrane fluidity. Changes in fluidity have been observed to accompany significant alterations in the content of unsaturated acyl chains of a number of membrane types (59, 60). In this regard, an earlier report of reductions in membrane lipid fluidity and significant decreases in membrane arachidonic acid content and double-bond index during calcium treatment in rat hepatocyte plasma membranes is of interest (60). In these studies (60), calcium appears to influence the fluidity of hepatocyte plasma membranes by stimulating endogenous phospholipase A_2. A similar mechanism can also account for the results of the present study, since significantly increased calcium content was observed in most muscles of our dystrophic dogs (8). The precise nature of mechanism(s) responsible for these membrane fatty acid differences in dystrophic dogs are presently unclear, however, altered substrate selectivity or affinity of acyl CoA synthetases (61), activity of the fatty acid desaturase system, and phospholipase A_2 activity are currently under investigation in our laboratory.

Alterations in lipid fluidity and composition of cell plasma membranes have also been reported to affect antigenic expression (62, 63), receptor and protein exposure (64), cation permeability (65), and transmembrane transport processes as well as enzymatic activities (25). The alterations in fluidity and composition found in erythrocyte membranes of this canine model of muscular dystrophy, therefore, are likely to have functional significance and warrant further studies to elucidate the underlying mechanism(s).

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