Changes of gangliosides and other lipids in skeletal muscle from rabbits with experimental dystrophy

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Abstract Comparison of the skeletal muscles from vitamin E-deficient and control rabbits showed that the muscles from the deficient animals had lower contents of protein and glycogen but more water and lipid. Increases of individual lipids per unit weight of muscle from deficient animals compared with those from control animals were 2.2-fold for gangliosides, 2.18-fold for cholesterol, 1.74-fold for sulfatides, and 1.45-fold for neutral glycosylceramides. Total phospholipids did not change; this was the result of an increase in sphingomyelin (1.47-fold) and a decrease of phosphatidylcholine to 83% of the control, while the other fractions remained unchanged. When the measurements were referred to total muscle, the contents of cholesterol, gangliosides, sulfatides, neutral glycosylceramides, and sphingomyelin in muscle from vitamin E-deficient rabbits were also above those of the control rabbits, and only the phoshatidylcholine content was decreased. It was not possible to determine whether the alteration of lipid content preceded or followed the onset of signs of muscular dystrophy.

Supplementary key words vitamin E deficiency · protein · glycogen · cholesterol · gangliosides · neutral glycosylceramides · sulfatides · phospholipids · phosphatidylcholine · sphingomyelin

The antioxidant property of vitamin E has attracted considerable attention in the hope that it is connected with the biological function of this vitamin. Since lipds are readily autoxidized, it is a plausible hypothesis that animals deficient in vitamin E may show alterations in the lipid composition of tissues (1). It is known that the total lipid and cholesterol content of the muscles of several species of animals is increased (2–5) when these animals are fed a tocopherol-deficient diet.

Lassaga, Albarracın, and Caputto (6, 7) and Puro, Maury, and Huttunen (8) reported, independently, the presence of gangliosides in muscle. Since they are membrane components and some of the more outstanding changes during tocopherol deficiency have been related to alterations in permeability, it appeared of interest to study the variations in the content of these and other complex lipid fractions in normal and vitamin E-deficient animals. In the present work the contents of cholesterol, triglycerides, phospholipids, neutral glycosylceramides, sulfatides, and gangliosides in skeletal muscle of the vitamin E-deficient rabbit have been compared with the contents of the same lipids in muscle from the normal rabbit. Significant increases in cholesterol and all the sphingolipids were found. Among the phospholipids only phosphatidylcholine decreased.

METHODS

White male rabbits weighing between 380 and 500 g (30 days of age) were fed ad lib. the diet designed by Caputto, McCay, and Carpenter (9). The basal diet (in grams) consisted of casein, 15; sucrose, 39; cornstarch, 36; salt mixture (10), 3; vitamin mixture, 0.225; cod liver oil, 3; and lard, 3. The vitamins (per 100 g of diet) were nicotinamide, 20 mg; pyridoxine HCl, 0.5 mg; thiamine HCl, 0.5 mg; riboflavin, 0.5 mg; calcium pantothenate, 1 mg; folic acid, 0.5 mg; biotin, 0.005 mg; 2-methylnaphthoquinone, 0.025 mg; vitamin B_{12}, 2.4 μg; choline chloride, 0.1 g; and inositol, 0.1 g. Molecular distilled lard was from Distillation Products Industries, Division of Eastman Kodak Co., Rochester, N.Y. The animals were treated identically except that control rabbits received oral supplements of 70 mg of α-tocopherol acetate twice a week. The animals were killed in pairs after symptoms of deficiency appeared. This occurred usually after the experimental diet was fed for 20 days. Animals that could not stand up after being laid down on a flank were considered deficient. All of the muscles of the hind legs and the longissimus dorsi were removed and stored at −15°C until they were analyzed.

Total lipids were quantitatively extracted and purified according to Masoro, Rowell and McDonald (11), and the purified material was divided into four samples. In one

Abbreviations: TLC, thin-layer chromatography.

TABLE 1. Major constituents of skeletal muscle from normal and dystrophic rabbits

	Control Rabbits	Dystrophic Rabbits	Difference
	g/100 g (wet wt)[a]		%
Water (5)[b]	77.00 ± 1.80	84.72 ± 3.60	+11.00[c]
Dry weight (5)	23.00 ± 1.79	15.28 ± 3.80	−33.48[d]
Proteins (5)	17.35 ± 1.02	11.98 ± 1.03	−31.00[d]
Lipids (11)	1.20 ± 0.21	1.50 ± 0.22	+25.00[c]
Glycogen (5)	0.45 ± 0.23	0.38 ± 0.32	−15.56[e]

[a] Results are means ± SD.
[b] Figures in parentheses indicate numbers of pairs of determinations. P was calculated according to Student's t test for noncorrelated samples.
[c] $P < 0.01$.
[d] $P < 0.001$.
[e] $P < 0.02$.

sample, total lipids were determined after the lipid extract was dried in vacuo to constant weight. In another sample, total lipid phosphorus was determined by the method of Bartlett (12) as modified by Marinetti (13). In a third sample of the purified extract, sulfatides and neutral glycosylceramides were determined. The sample was dried and then saponified overnight at 37°C with 1 N KOH in methanol–water 1:1. Enough chloroform was then added to form a two-phase system, and the lower phase was dried and passed through a column of Florisil (0.4 × 30 cm). The nonpolar lipids were eluted with chloroform–methanol 19:1, and the neutral glycosylceramides and sulfatides were eluted with chloroform–methanol 2:1. In the mixture of neutral glycosylceramides and sulfatides, total hexoses were determined according to Trevelyan and Harrison (14). The sulfatides were determined by the method of Kean (15), and the neutral glycosylceramides were quantified by substracting from the total hexoses the amount corresponding to the sulfatides (method A). In a few cases the neutral glycosylceramides were separated from sulfatides on a column of DEAE-cellulose according to Svennerholm et al. (16) and quantified by the hexose content (method B). In the fourth sample, neutral lipids and phospholipids were separated on a silicic acid column (11). Five fractions were eluted; in one, total cholesterol was determined by the method of Leiboff (17) and triglycerides according to Van Handel (18). The other fractions were analyzed separately by TLC using chloroform–methanol–glacial acetic acid–water 10:2:1.2:0.5 as solvent (19), and the phosphorus content of each spot was determined. The recovery of phosphorus in the fractions eluted from the silicic acid column was compared with the total phosphorus added to the column. The recoveries for both control and dystrophic animals were between 90 and 95%.

Samples obtained from the silicic acid column were used to study the recoveries of phosphorus from TLC procedures; the phosphorus content of the fractions added to the origin of the thin-layer plates was compared with the added phosphorus values found in the spots detected after exposure of the plates to iodine vapor. For each fraction the recoveries were between 89 and 95% for the samples from both control and dystrophic rabbits.

For gangliosides the method of extraction of Trams and Lauter (20) was adopted with some modifications (6). Ganglioside sialic acid was determined according to Aminoff (21) after hydrolysis with 0.1 N H_2SO_4 at 80°C for 2

TABLE 2. Concentration of lipids in skeletal muscle from normal and dystrophic rabbits

	Control Rabbits	Dystrophic Rabbits	Difference
	mg/100 g (wet wt)[a]		%
Total lipids (11)[b]	1200.00 ± 22	1500.00 ± 21	+25[c]
Total phospholipids (9)	745.00 ± 44	760.00 ± 30	+1.02[d]
Triglycerides (7)	250.00 ± 98	298.15 ± 105	+11.52[d]
Cholesterol (9)	79.00 ± 9.90	173.00 ± 11	+118.86[e]
Gangliosides[f] (9)	4.15 ± 0.50	9.15 ± 1.40	+120.48[e]
Neutral glycosylceramides[g]			
Method A (7)	5.03 ± 0.44	7.31 ± 1.05	+45.33[e]
Method B (3)	4.50 ± 0.60	6.80 ± 0.57	+51.11[h]
Sulfatides[g]			
Method A (7)	1.76 ± 0.59	3.07 ± 0.15	+74.43[e]
Method B (3)	1.50 ± 0.57	2.80 ± 0.58	+86.66[h]

[a] Results are means ± SD.
[b] Figures in parentheses indicate numbers of pairs of determinations. P was calculated according to Student's t test for noncorrelated samples.
[c] $P < 0.01$.
[d] Not significant.
[e] $P < 0.001$.
[f] Sialic acid was measured according to Aminoff (21); gangliosides were calculated assuming a value of 26.5% as the average relative amount of sialic acid (24).
[g] Method A: sulfatides and neutral glycosylceramides were determined in a fraction from a Florisil column. Neutral glycosylceramides were determined by subtracting from the total hexoses the amount corresponding to the sulfatides (determined by the method of Kean [15]). For details see Methods. Method B: sulfatides and neutral glycosylceramides were separated on a column of DEAE-cellulose according to Svennerholm and coworkers (16) and measured by the anthrone method (14).
[h] $P < 0.05$.

TABLE 3. Phospholipids in skeletal muscle of normal and dystrophic rabbits

	Control Rabbits	Dystrophic Rabbits	Difference
	mg/100 g (wet wt)[a]		%
Total phospholipids (9)[b]	745 ± 44	760 ± 30	+1.02[c]
	percentage of total lipid P[a]		
Polyglycerophosphatide[d] (9)	4.73 ± 0.43	4.88 ± 0.56	+3.10[c]
Phosphatidylethanolamine (8)	20.13 ± 0.43	20.41 ± 0.52	+1.40[c]
Phosphatidylinositol (8)	8.48 ± 0.47	8.67 ± 0.39	+2.24[c]
Phosphatidylserine (8)	6.20 ± 1.10	5.84 ± 0.46	−5.81[c]
Phosphatidylcholine (8)	49.80 ± 0.50	42.47 ± 0.56	−15.72[e]
Sphingomyelin (9)	10.55 ± 0.55	16.64 ± 0.47	+57.70[e]

[a] Results are means ± SD.
[b] Figures in parentheses indicate numbers of pairs of determinations. P was calculated according to Student's t test for noncorrelated samples.
[c] Not significant.
[d] Mainly cardiolipin and phosphatidylglycerol (11).
[e] $P < 0.001$.

hr. Glycogen was determined by using the anthrone reagent according to Hassid and Abraham (22). The muscle was dissolved in boiling 5 N NaOH for 10 min and then the protein was assayed by the method of Lowry et al. (23). Muscle dry weight was determined after drying the samples in vacuo over P_2O_5 to constant weight.

RESULTS

Changes in the main constituents of muscle

The compositions of the muscles from control and vitamin E-deficient rabbits are shown in Table 1. The dry weight of the muscles from the deficient animals was about 66% of the weight of the muscles from control rabbits. The decreases of protein and glycogen amounted to 31 and 15.5%, respectively (compared with control rabbits), and the lipid content increased.

Lipid variations

Comparison of several lipid fractions showed that most were increased in the muscles of the dystrophic animals; the exception was the phospholipid fraction, which remained unchanged (Table 2). There was a 120% increase in gangliosides, a 118% increase in cholesterol, a 74% increase in sulfatides, and a 45% increase in neutral glycosylceramides. The neutral glycosylceramide fraction was chromatographed by TLC, using chloroform–methanol–concentrated NH_4OH 80:20:0.4 as solvent. It was found that the main component was glucosylceramide but there was also a small amount of lactosylceramide. Triglycerides were found slightly increased (11.5%), but the difference was not statistically significant ($P = 0.6$).

Phospholipid changes

No differences were found between the total phospholipid fractions of control and dystrophic rabbits. However, the analyses of the different components showed that the lack of difference was due to an increase of sphingomyelin that was offset by a decrease of phosphatidylcholine (Table 3).

Variations of lipid content per whole muscle

The increase of lipids per unit of weight of muscle may be a reflection of the decreases of other constituents of the muscle or may be due to an absolute increase of the lipids in the whole muscle. To elucidate this point, determinations were carried out on whole muscles from animals paired according to body weight from the time they were started on the experimental diet.

Table 4 shows the results of determinations in the gastrocnemius and the femoral biceps. In each case the total weight of the dystrophic muscle was about 17% lower than that of the control muscle. The percentage increases of cholesterol and gangliosides were clearly above those expected from increases due to the decrease of the total weight of the muscles. The same was found for sulfatides, neutral glycosylceramides, and sphingomyelin, even though these increases were not as striking as in the cases of cholesterol and gangliosides.

In contrast to the sphingolipids, the glycerophospholipids were all decreased in the dystrophic muscles; however, percentage-wise, the decreases of most of the glycerophospholipids were slightly less than decreases in the total weight of the muscles with the exception of phosphatidylcholine; this was the only lipid that decreased with respect to the decrease of the muscle weight.

Time relationships between the appearance of symptoms and the lipid changes

Nearly all the rabbits (weighing 400–500 g, 30–40 days of age at the time they were started on the experimental diet) showed symptoms of muscular dystrophy between the 3rd and the 5th wk on the diet. Older animals required a longer time to become dystrophic, and some of them did not show signs of the disease even after 1 yr.

Using rabbits 30–40 days old, we attempted to determine whether the changes in the concentrations of choles-

TABLE 4. Lipid content per whole muscle

	Gastrocnemius			Femoral Biceps		
	Rabbits		Difference	Rabbits		Difference
	Control	Dystrophic	Percentage ± SED[a]	Control	Dystrophic	Percentage ± SED[a]
Wet weight of muscle (g)	5.19	4.31	−16.9 ± 1.61[b]	5.65	4.67	−17.3 ± 4.0[c]
			mg/muscle			
Total lipids	61.33	63.33	+3.5 ± 0.90[d]			
Triglycerides	12.96	12.88	−0.6 ± 1.20			
Cholesterol	3.80	7.70	+102.4 ± 10.2[b]	3.76	6.99	+85.8 ± 11.4[b]
Gangliosides[e]	0.21	0.41	+95.2 ± 9.5[b]	0.21	0.43	+108.5 ± 14.3[b]
Neutral glycosylceramides[f]	0.24	0.31	+27.0 ± 4.1[b]	0.25	0.34	+32.6 ± 8.0[b]
Sulfatides[f]	0.097	0.14	+43.6 ± 10.3[d]	0.098	0.14	+43.6 ± 9.8[d]
			μmoles P/muscle			
Lipid P	44.56	38.10	−15.0 ± 1.7[b]			
Polyglycerophosphatide[g]	2.10	1.85	−12.8 ± 3.3[d]			
Phosphatidylethanolamine	9.03	7.74	−14.2 ± 0.7[h]			
Phosphatidylserine	2.47	2.17	−11.8 ± 4.1[d]			
Phosphatidylinositol	3.76	3.20	−14.8 ± 1.5[b]			
Phosphatidylcholine	22.31	16.50	−26.1 ± 1.4[h]			
Sphingomyelin	4.47	6.87	+44.2 ± 8.9[c]			

Each value is the average from four animals. *P* values were calculated according to Student's *t* test for correlated samples.
[a] Standard error of the difference.
[b] $P < 0.01$.
[c] $P < 0.02$.
[d] $P < 0.05$.
[e] Sialic acid was measured according to Aminoff (21); gangliosides were calculated assuming a value of 26.5% as the average relative amount of sialic acid (24).
[f] Determined by method A; for details see Methods.
[g] Mainly cardiolipin and phosphatidylglycerol (11).
[h] $P < 0.001$.

terol, gangliosides, neutral glycosylceramides, or sulfatides preceded the appearance of the symptoms of dystrophy. Results in Table 5 show that the differences in the lipid concentrations in the animals about to become dystrophic (without signs of dystrophy) were very small or nonexistent and that the changes appeared rather rapidly at the time the signs appeared or immediately afterward.

DISCUSSION

Of the main constituents of skeletal muscle, only water and total lipids were found increased on a per gram of muscle basis. When determined on the basis of the weight of a whole muscle (gastrocnemius or biceps) the analyses showed three different situations in relation to the lipid content in experimental muscular dystrophy. There were lipids that showed an absolute decrease, lipids that showed an apparent decrease, and lipids that showed an absolute increase in relation to the wet weight of the muscle. While there was an absolute decrease of phosphatidylcholine, there were only relative decreases of the glycerophospholipids. The increase of cholesterol that occurred suddenly at the onset of dystrophy may be a compensatory mechanism for the lack of vitamin E, which, according to Lucy and

TABLE 5. Lipid content of skeletal muscle from rabbits fed vitamin E-supplemented and vitamin E-deficient diets for different periods of time

Weeks on Diet	Cholesterol		Gangliosides[a]		Neutral Glycosyl-ceramides		Sulfatides	
			mg/100 g (wet wt)[b]					
Without signs of dystrophy	C[c]	D[d]	C	D	C	D	C	D
3 (3)	70.00	77.66	3.90	4.10	5.20	5.00	1.60	1.73
4 (4)	73.30	75.33	4.15	4.50	5.30	4.60	1.80	1.90
5 (5)	79.25	83.20	4.00	4.30	4.90	5.30	1.75	2.00
With signs of dystrophy								
4 and 5 (3)	74.50	183.00	4.20	8.80	5.10	7.20	1.57	3.00

[a] Sialic acid was measured according to Aminoff (21); gangliosides were calculated assuming a value of 26.5% as the average relative amount of sialic acid (24).
[b] Values are averages of the numbers of pairs of determinations (in parentheses).
[c] Control rabbits.
[d] Rabbits on vitamin E-deficient diet.

coworkers (25, 26), serves to stabilize the exposed hydrocarbon chains of the membrane lipids.

The interpretation of the increase of the gangliosides in the dystrophic muscle is hampered by the scanty information on the distribution of these lipids in muscle. Since the concentration of total gangliosides in muscle is less than one-tenth of the concentration of total gangliosides present in nervous tissue, one cannot discard the possibility that most of the gangliosides analyzed in muscle come from the nervous tissue present in muscle. If this were the case (and in agreement with their localization in nervous tissue) most of the gangliosides would be present in the nerve terminal of the muscles. No explanation can be offered at present for the absolute increase of gangliosides, but in connection with this observation it may be of interest to recall that in muscles whose nerves had been severed, Waser (27) found an increase of motor end plates, and in the same situation Max, Nelson, and Brady (28) found an increase of gangliosides.

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