Skeletal muscle lipids. II. Changes in phospholipid composition in man from fetal to middle age

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Abstract Phospholipid compositions were determined in samples of gastrocnemius muscles of individuals from fetal stage to the age of 55 yr. The lipids were separated by thin-layer chromatography. In order to enable characterization of the individual phospholipids, a lipid extract was prepared from pooled samples of gastrocnemius muscles from adult males and separated by ion-exchange chromatography on TEAE-cellulose. The individual phosphoglycerides were purified by thin-layer chromatography and then characterized according to their content of fatty acids, aldehydes, phosphorus, and the identity of the bases. The relative amounts of the major phospholipids, choline and ethanolamine phosphoglycerides, changed little with age, and in adult males they constituted 47% and 24%, respectively. Cardiolipin increased from 3% in the fetal stage to 9% by the end of the first year of life. Sphingomyelin and serine phosphoglyceride decreased with increasing age, while inositol phosphoglyceride increased. In adult males, cardiolipin constituted 10% of total lipid phosphorus, inositol phosphoglyceride constituted 9%, serine phosphoglyceride 3%, and sphingomyelin 7%.

Supplementary key words thin-layer chromatography · ion-exchange chromatography · gas–liquid chromatography · nitrogen bases · inositol · fatty acids · aldehydes · lyso phospholipids

If alterations in the phospholipid pattern of a certain organ, due to diseases or to nutritional or metabolic disorders, are to be recognized, it is neccessary first to determine with precision the lipid composition of the organ from a number of normal humans of different ages. Such studies have been performed on only a few human organs, and only scattered recent data are available on the phospholipid composition of normal human skeletal muscles.

The major aim of this study was to determine the phospholipid composition of human skeletal muscle from fetal to middle age. The phospholipids were isolated by two methods: one-dimensional thin-layer chromatography and DEAE- and TEAE-cellulose ion-exchange chromatography. The TLC method was used for quantitative determination of the phospholipid composition and the ion-exchange method for isolation of the individual phospholipids to enable characterization. These phospholipids were used as reference substances. The concentrations of total phospholipids and lecithin and the fatty acid composition of lecithin have been given in a previous report, in which the methods for the collection and extraction of the samples were also described (1).

MATERIALS AND METHODS

Materials

The source of the material and the procedure used for collection have been described earlier (1). The material in this study consisted of muscle samples, obtained at autopsies, from five fetuses, four males less than 1 yr old, three males between 1 and 10 yr of age, five males between 10 and 20 yr of age, one male aged 27, and two males between 50 and 55 yr of age.

The chemicals and solvents used were of analytical grade. The solvents were redistilled before use.

Cardiolipin, inositol phosphoglyceride (IPG), serine phosphoglyceride (SPG), lysolecithin (lyso CPG), lyso ethanolamine phosphoglyceride (lyso EPG), and phosphatidic acid were purchased from Koch-Light Laboratories Ltd., Colnbrook, Buckinghamshire, England. Ethanolamine phosphoglyceride (EPG), choline phosphoglyceride (CPG), and sphingomyelin were prepared in this laboratory by silicic acid chromatography and TLC of brain lipids. Ethanolamine, choline chloride, and serine were obtained from E. Merck A.G., Darmstadt, Germany, and *meso*-inositol from Kebo, Stockholm, Sweden. Whatman DE 22 DEAE-cellulose was obtained from W. & R. Balston Ltd., Maidstone, England; Selectacel TEAE-cellulose from Brown Co., Berlin, N.H.; and silica gels G and H from Fluka A.G., Buchs, Switzerland. 14% BF_3 in methanol in sealed ampoules was bought from Applied Science Laboratories, Inc., State College, Pa.

Abbreviations: CPG, choline phosphoglyceride, lecithin; EPG, ethanolamine phosphoglyceride; IPG, inositol phosphoglyceride; SPG, serine phosphoglyceride; TLC, thin-layer chromatography; GLC, gas–liquid chromatography; DEGS, diethylene glycol succinate polyester; DEAE, diethylaminoethyl; TEAE, triethylaminoethyl.

Methods

Total lipid extracts were prepared from 1–2 g of muscle tissue after homogenization, extraction with chloroform–methanol, and partitioning of the lipid extracts with the methods described earlier (1). Phosphorus was determined in a portion of the lipid extract, equivalent to 8–10 mg wet weight, with a modified Fiske-SubbaRow method (2).

Quantitative determination of individual phospholipids

Lipid extracts, equivalent to 10–12 μg of phosphorus, were applied as bands 3–4 cm broad on a silica gel G TLC plate, which was then developed in chloroform–methanol–12 N ammonia 70:30:5 (v/v). The phospholipids were visualized by spraying the plate with bromothymol blue reagent (3). In those areas containing cardiolipin + EPG, CPG, sphingomyelin, IPG, and SPG, the layers were scraped off. Duplicate samples were transferred to centrifuge tubes, and the phosphorus content of the gels was assayed with standards containing gel from the same plate.

When the isolation of sphingomyelin on TLC was performed under ordinary environmental conditions (18–20° C and a rather low humidity), it contained some phosphoglycerides, mainly CPG and IPG. It was shown that this admixture decreased with increasing humidity and temperature. As the fatty acid composition of sphingomyelin was not to be investigated, no further efforts were made to improve the separation of this lipid.

For the separation of cardiolipin from EPG, total lipid extracts containing about 15 μg of lipid phosphorus were applied on a silica gel H plate, and the plate was developed in chloroform–methanol–water 80:20:2 (v/v) for 10 cm. The TLC plate was air-dried at room temperature for 15 min, after which it was developed in light petroleum–diethyl ether–acetic acid 87:13:1 (v/v) up to the edge of the plate.

Identification of individual phospholipids

Identification by TLC. For identifying cardiolipin, EPG, CPG, IPG and SPG, lyso EPG, and lyso CPG, the lipids were extracted from the gel and spotted on TLC plates together with reference samples. The TLC separations were carried out by a number of methods: silica gel G plates developed in chloroform–methanol–water 65:25:4 (v/v); by the procedure by Skipski, Peterson, and Barclay (4), with Na_2CO_3 in the gel and with the solvent system chloroform–methanol–acetic acid–water 50:30:8:4 (v/v); and by the procedure by Gonzalez-Sastre and Folch-Pi (5), with 1% potassium oxalate in the gel and the solvent system chloroform–methanol–12 N ammonia 9:7:2 (v/v). For the indication of EPG and SPG, the plates were sprayed with ninhydrin reagent. Sphingomyelin was indicated with anisaldehyde reagent (6).

Identification and isolation by ion-exchange chromatography. In order to obtain sufficient amounts of acid and less abundant phospholipids by a separation procedure other than TLC, phospholipids were separated by DEAE- and TEAE-cellulose ion-exchange chromatography, as described by Rouser and coworkers (7, 8). However, the final purification of the individual phospholipids was performed by TLC.

Separation of phospholipids on DEAE-cellulose. Several separations of lipids were performed on DEAE-cellulose in acetate form with essentially the same results. In a typical separation, lipids from gastrocnemius muscles of three males 15–18 yr old, containing 0.40 g of phospholipids, were applied to a column (15 g of dry cellulose, inner diameter of tube 2 cm, final height 30 cm). The phospholipids were recovered in the different eluates according to the procedures of Rouser et al. (7). The recovery of lipid phosphorus was 85%. Lyso phospholipids were formed from CPG, EPG, and cardiolipin, and about 27% of CPG and 16% of EPG were recovered as lyso compounds.

Separation of phospholipids on TEAE-cellulose. In order to find out whether an increase in the elution rate and in the amount of phospholipids applied to the ion-exchange column could decrease the formation of lyso compounds, TEAE-cellulose in OH^- form was used (8). Lipid extract, prepared from the gastrocnemius muscles of five males 21–47 yr old, containing 0.63 g of phospholipids, was applied to a column containing 12 g of dry cellulose (inner diameter 3 cm, final height 15 cm). The recovery of lipid phosphorus was 105%. The separation of SPG from the other acid phospholipids was not achieved and these lipids were, therefore, separated on DEAE-cellulose. Lyso compounds were formed from CPG, EPG, and cardiolipin by separation on TEAE-cellulose, and 7% of CPG and 16% of EPG were recovered as lyso phospholipids.

Determination of fatty acids and aldehydes. Fatty acids were determined by the method of Lauwerys (9). The phospholipids were hydrolyzed with 0.5 N KOH, and the fatty acids were extracted with light petroleum after acidification.

Dimethyl acetals were prepared from the aldehydes and identified as outlined by Morrison and Smith (10). Lipid extracts, containing 8–12 μg of phosphorus, were methylated with 14% BF_3 in methanol. The combined methyl esters and dimethyl acetals were extracted with light petroleum and analyzed on a Hewlett-Packard gas–liquid chromatograph, model 402 (glass column with 15% DEGS on Diatoport S, 80–100 mesh, 173°C, carrier gas argon, 25 ml/min) equipped with a Hewlett-Packard electronic integrator 3370A. With this column and at this temperature the dimethyl acetals 16:0, 18:0, and 18:1 were resolved from the fatty acid methyl esters 16:0, 16:1, and 18:0. The amount of dimethyl acetals was calculated as weight percent of the sum of the fatty acid esters and the dimethyl acetals.

Identification of nitrogen bases and inositol. CPG and lyso CPG were hydrolyzed with 1 N NaOH for 24 hr at 37°C, and the base in the hydrolyzate was identified as choline by the phosphomolybdic acid method (11) or by paper chromatography (12).

Ethanolamine and serine were estimated after acid hydrolysis of EPG, SPG, and lyso EPG. The fatty acids were removed and ethanolamine and serine were separated and identified on paper (13) and by ion-exchange resin chromatography (JEOL model 5AH automatic amino acid analyzer).

Inositol was determined in IPG after hydrolysis with 6 N HCl for 48 hr at 100°C. The fatty acids were extracted with light petroleum, and the hydrochloric acid was then evaporated in a vacuum desiccator over KOH. Phosphate was removed in a manner similar to the removal of borate as methyl borates (14). The residue was dissolved in 0.5% HCl in absolute methanol and was then evaporated to dryness at 60°C with nitrogen. This procedure was repeated four times. The inositol was acetylated (15) and quantitatively determined on GLC with mannitol as internal standard.

RESULTS

Identification and characterization of individual phospholipids

The phospholipids, isolated by TEAE-cellulose ion-exchange chromatography and then purified on TLC, were used for characterization. The following criteria were used for the identification of the individual phosphoglycerides. *(a)* They were eluted from the ion-exchange column as described by Rouser, Kritchevsky, and Yamamoto (8). *(b)* When separated in the different TLC solvents described, they had the same R_F values as authentic reference compounds prepared in this laboratory from brain or obtained from commercial sources. Unless otherwise stated, the same phospholipid obtained by the two different separation methods had the same R_F value and both were found to be chromatographically homogeneous. *(c)* They had the theoretical compositions of the phosphoglycerides as described below.

Cardiolipin had a fatty acid:phosphorus ratio of 2.08:1. Only traces of aldehydes were found.

EPG contained 19% aldehydes and 81% fatty acids. The ratio fatty acids + aldehydes:phosphorus was 2.13:1. The free base had the same R_F value as ethanolamine on paper chromatography and the same elution time on ion-exchange chromatography. EPG obtained by separation on TLC with the standard solvent chloroform–methanol–12 N ammonia 70:30:5 (v/v) contained cardiolipin.

CPG contained 7% aldehydes and 93% fatty acids. The ratio fatty acids + aldehydes:phosphorus was 1.96:1. After hydrolysis the base was shown to be choline by paper chromatography and by reaction with the phosphomolybdate reagent.

IPG contained only traces of aldehydes. The ratio fatty acids:phosphorus:inositol was 1.98:1:0.92. By GLC the compound was shown to have the same retention time as *meso*-inositol.

SPG had a fatty acid:phosphorus ratio of 1.69:1. Only traces of aldehydes were found. The hydrolyzed compound had the same R_F value as serine on paper chromatography and the same elution time on ion-exchange chromatography. The SPG obtained by separation on TEAE-cellulose contained some impurities, most of which were ninhydrin positive.

Sphingomyelin gave a characteristic blue color with the anisaldehyde reagent.

Phosphatidic acid and glycerol phosphoglyceride. After separation of muscle lipid extracts by DEAE- and TEAE-cellulose, two small fractions (both containing less than 1% of total phospholipids) of acid phospholipids were found; these migrated as phosphatidic acid and glycerol phosphoglyceride on TLC (16, 17). Neither of these phosphoglycerides could be identified by separation of total lipid extracts on TLC and were, therefore, not analyzed further.

Lyso phosphoglycerides. Lyso CPG contained 8% aldehydes and 92% fatty acids, and the ratio fatty acids + aldehydes:phosphorus was 0.94:1. The base was identified as choline with the phosphomolybdate reagent. When intact lipid extracts were separated on TLC, minute amounts of lyso CPG were occasionally found in the IPG fraction.

Lyso EPG contained 30% aldehydes and 70% fatty acids. The ratio fatty acids + aldehydes:phosphorus was 1.09:1. The hydrolyzed base was identified as ethanolamine by ion-exchange chromatography. Lyso EPG was obtained only after DEAE- and TEAE-cellulose chromatography, and when intact lipid extracts were separated on TLC, lyso EPG was never detected with the ninhydrin spray reagent or found when the isolated phospholipids were rechromatographed.

In the fraction obtained by TEAE-cellulose chromatography and containing the acid phosphoglycerides, two phospholipids with fatty acid:phosphorus ratios of about 1.5:1 and 1:1 were found. These lipids were ninhydrin negative and had no R_F value in common with any of the other phospholipids studied. The fatty acid composition was similar to that of cardiolipin. These lipids were, therefore, tentatively identified as lyso compounds derived from cardiolipin.

Quantitative determination of individual phospholipids

The phospholipid composition of the skeletal muscles from 15 individuals of different ages is shown in Table 1. The variations within the percentages found for the fetuses

TABLE 1. Phospholipid composition in human skeletal muscle[a]

	Age					
	Fetal–Full Term (5)[b]	1 mo (2)	2 mo (1)	10–18 mo (2)	5–55 yr (5)	5–55 yr (10)
	% of total lipid phoshorus					
Cardiolipin	2.5 (0.5)[c]	5.6	6.2	8.8	8.9 (1.3)[c]	9.5 (1.1)[c]
Ethanolamine phosphoglyceride	26.2 (1.3)	25.5	27.1	26.8	24.9 (1.7)	24.1 (1.6)
Choline phosphoglyceride	45.9 (2.3)	44.5	44.5	48.3	45.2 (2.1)	47.4 (1.9)
Inositol phosphoglyceride	4.8 (0.3)	6.0	5.7	4.0	9.7 (1.3)	8.9 (1.4)
Serine phosphoglyceride	8.7 (0.9)	8.2	7.2	4.8	3.5 (0.8)	3.3 (0.3)
Sphingomyelin	11.8 (0.9)[d]	10.1[d]	9.3[d]	7.4[d]	7.9 (0.8)[d]	6.8 (0.7)

[a] Determined by TLC.
[b] Number of individuals in parentheses.
[c] Mean (SD).
[d] In these separations, up to 20% of the sphingomyelin fraction consisted of other phospholipids, mainly CPG; see text.

and for males 5 yr and older were rather small. The values for each of these two age periods are therefore given as means and standard deviations.

The relative amounts of the main phospholipids, CPG and EPG, were constant during the entire period studied, and together they constituted between 72 and 78% of the total phospholipids. A great variation with age was found for cardiolipin, which increased from 3% during the fetal stage to 9% at the end of the first year of life. SPG showed a continuous decrease during gestation and the first year of life, and IPG increased mainly after the age of 1 yr. The relative amount of sphingomyelin decreased moderately towards the end of the fetal period and in the first year of extrauterine life.

DISCUSSION

The aims of this study of the phospholipids in human skeletal muscle were *(a)* to identify the naturally occurring phosphoglycerides of human skeletal muscle, *(b)* to test a simple and reliable routine method by which small amounts (100–500 mg) of muscular tissue can be analyzed for phospholipid content, and *(c)* to analyze muscle samples from individuals of various ages and to elucidate the variation of the phospholipid composition with age.

In an attempt to find a suitable method for separating muscle lipids, several trials were made with DEAE-cellulose in acetate form; however, this method resulted in the production of rather large amounts of lyso phospholipids. Therefore, TEAE-cellulose in OH^- form was tried, although this cellulose has strongly basic groups, which might hydrolyze the ester linkages. The TEAE-cellulose is capable of completely separating CPG from EPG (8) and has a high capacity. Although the preparation of the column and the separation of the lipids was performed according to the procedure described by Rouser et al. (8), this latter ion-exchange procedure also produced lyso phospholipids. The formation of lyso phospholipids by DEAE-cellulose ion-exchange chromatography has previously been reported by Ansell and Spanner (18). Although lyso CPG has been claimed to have been found in muscle lipid extracts (19), it has not been shown that this lipid occurs in living muscle tissue. When the lipid extract used for the TEAE-cellulose chromatography in this study was separated by TLC only, neither lyso EPG nor lyso CPG were demonstrable. The main advantage of the DEAE- and TEAE-cellulose ion-exchange chromatography for the preparation of muscle lipids was the possibility to concentrate the acid phosphoglycerides and to obtain these lipids free from the neutral phospholipids. SPG was obtained completely free from other lipids, but the other acid phospholipids had to be separated by another method, and for this purpose TLC was used.

TLC with chloroform–methanol–12 N ammonia 70:30:5 (v/v) has been used in our laboratory for separating phospholipids (20). When testing the isolated phospholipids, the method was found to separate CPG, IPG, and SPG well, while EPG and cardiolipin were not separated, and sphingomyelin contained other lipids, mainly CPG and IPG. Lipid extracts of muscle from males over 5 yr of age were separated under conditions that afforded better separation of sphingomyelin. The results are shown in a separate column in Table 1. Silica gel H plates developed in chloroform–methanol–water 80:20:2 (v/v) showed very distinct spots of cardiolipin and EPG. The other phospholipids remained close to the origin. The plate was afterwards developed with light petroleum–diethyl ether–acetic acid 87:13:1 (v/v) in order to remove free fatty acids and cholesterol from the cardiolipin area.

The phospholipid composition in skeletal muscles from males of different ages has been studied. In an earlier report (1) it was shown that the concentrations of total phospholipids and lecithin were rather constant, apart from a slight increase in early childhood. In the present study it was shown that the relative amounts of CPG and EPG, expressed as percentages of the total phospholipids, was very constant. Together these two major phosphoglycerides constituted three-fourths of the total amount of phospholipids. The relative amounts of the minor phospholipids varied during the first years of life. From the fetal stage to the age of 1 yr, the relative amount of car-

diolipin increased threefold. The relative amount of IPG doubled between the ages of 2 and 5 yr, while SPG was reduced by 50% between the fetal stage and the age of 2 yr. The relative amount of sphingomyelin was reduced by one-third during the first year of postnatal life.

The variation of the phospholipid composition in different human organs with age has been studied in brain (2, 20), aorta (21), the lens of the eye (22), and muscle (23). The study by Hughes (23) was performed on gastrocnemius muscles from individuals of different ages, but although five samples were obtained from fetuses 10–20 wk old, there were only two samples from individuals between this age and 5 yr after birth. No variation in the relative amounts of IPG and SPG was found. A general feature in the phospholipid composition of aorta and lens at different ages was that sphingomyelin increased steadily with age, whereas most other phospholipids decreased. In brain there was also an increase in the relative amount of sphingomyelin, in both the cerebral cortex and gray matter. At the same time there was a steady decrease in the amount of CPG, while the amount of EPG remained rather constant. These changes were in contrast to those in muscle, in which tissue the relative amount of sphingomyelin decreased during the first year of life, whereafter it remained rather constant.

When different human organs were compared, the greatest relative amount of cardiolipin was found in heart and skeletal muscle (24, 25).

In most mammalian organs, cardiolipin seems to be exclusively confined to the mitochondria (26). An important exception to this rule is the heart muscle, in which large amounts of cardiolipin have been found in the endoplasmic reticulum (26) and microsomes (27). These findings make it likely that cardiolipin can occur in different subcellular particles in skeletal muscles.

An increase in the relative amount of cardiolipin with age has previously been found by Hughes (23) in human muscles and by Jakovcic et al. (28) in rat liver. In the latter study it was shown that the content of mitochondria in the rat liver cells doubled during the immediate postnatal period, while the concentration of cardiolipin in the mitochondria remained constant. Besides this, studies on the effect of exercise on human muscles have revealed that the number of mitochondria increases with prolonged physical training (29). The findings in the studies mentioned make it rather likely that the increase in the amount of cardiolipin during the first year of life, found in this investigation, was due mainly to an increase in the number of mitochondria. However, other explanations cannot be ruled out before determinations have been made on the cardiolipin content of skeletal muscle mitochondria and other subcellular particles at different ages.

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