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CHANGES IN FATTY ACID DISTRIBUTION AND THERMOTROPIC PROPERTIES OF PHOSPHOLIPIDS FOLLOWING PHOSPHATIDYLCHOLINE DEPLETION IN A CHOLINE-REQUIRING MUTANT OF *NEUROSPORA CRASSA*

CHARLES E. MARTIN and ANNE M. JOHNSTON

Department of Biological Sciences and the Bureau of Biological Research, Rutgers University – DC, New Brunswick, NJ 08903 (U.S.A.)

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Growth of a choline requiring auxotroph of *Neurospora crassa* on medium lacking exogenous choline produces large changes in the levels of phosphatidylethanolamine and phosphatidylcholine. Whole cell fatty acid distributions were found to vary widely between different phospholipid species of normally growing, choline-supplemented cultures with phosphatidylcholine showing the highest levels of unsaturation and anionic phospholipids and cardiolipin having the lowest. In these lipids, choline deprivation produced little change in fatty acid profiles of phosphatidylethanolamine, whereas changes in fatty acids of phosphatidylcholine and acidic phospholipids resulted in increased levels of unsaturation at both growth temperatures. Microsomal phospholipids also showed fatty acid variability with sharp decreases in phosphatidylcholine unsaturates and increases in acidic phospholipid unsaturated fatty acids at low growth temperatures. Fluorescence polarization of 1,6-diphenylhexatriene in vesicles formed from total cellular and microsomal lipids showed that choline deprivation produces changes in thermotropic properties in the lipids in deprived cultures at either growth temperature. The effective differences in fluorescence polarization between choline-deprived and supplemented cultures grown at a given temperature were found to be comparable to those produced by temperature acclimation in normally growing cultures over a temperature range of 22 K.

Introduction

Membranes of eukaryotic microorganisms are composed of heterogeneous collections of lipids which form a fluid environment for numerous cellular functions. Given that phospholipids comprise a wide variety of molecular species with different physical properties generated by different combinations of fatty acid moieties, lipid interactions in membranes of even the simplest organism can assume an enormous degree of complexity. Since these interactions in lipid bilayers exhibit a strong temperature dependence, many organisms respond to changes in environmental temperature by altering the distribution of phospholipids and their fatty acid components to modulate membrane fluidity [1,2]. In a recent study on the effects of temperature acclimation in *Neurospora crassa* we observed large, temperature dependent changes in phospholipid fatty acid distribution [3]. Levels of α-linolenic acid (18 : 3) in particular were found to vary markedly with respect to growth temperature. Since membrane fluidity is strongly influenced by the distribution of phospholipid species in addition to their fatty acid constituents, we subsequently examined the effects produced by altering phospholipid headgroup composition in a mutant defective in phosphatidylcholine biosynthesis [4]. Surprisingly, radical alterations in the levels of phosphatidylcholine and phos-

phatidylethanolamine produced little change in total cellular phospholipid fatty acid compositions at either high or low growth temperatures. Since the distribution of fatty acids among the phospholipid species normally varies widely in most eukaryotic organisms these observations suggested that at least some phospholipid species in *Neurospora* undergo large changes in their fatty acid profiles as a consequence of choline deprivation. In order to determine which phospholipid species were most susceptible to change under these conditions we examined the distribution of fatty acids within major phospholipid species in normally growing and phosphatidylcholine-depleted mutant cultures with respect to growth temperature and the resultant effects depletion produced on phospholipid fluidity as measured by fluorescence polarization methods.

Materials and Methods

Mutant strains, growth media and culture conditions have been previously described [3–5]. Cultures acclimated to 37°C were grown at that temperature for 9 h; cultures acclimated to 15°C were first allowed to germinate at 37°C for 5 h, then shifted to 15°C for 16 h. Cultures grown for microsome isolation on choline supplemented medium were inoculated with 10^6 conida/ml whereas cultures grown on minimal medium were inoculated with 10^7 conidia/ml to compensate for slower growth under those conditions. Microsome isolation, lipid isolation and analytical procedures have also been reported previously [3–5]. Individual phospholipid species were separated for analysis by thin-layer chromatography on Silica gel G plates using a chloroform/methanol/ammonia (65 : 35 : 5, v/v) solvent system. Lipids were located by comparison with standards run in flanking reference lanes. Standard locations were identified with iodine vapors. Phospholipids were extracted from the silica gel with chloroform/methanol (1 : 1, v/v). Phospholipid fatty acids were transmethylated with boron trifluoride and methyl esters were analyzed using gas-liquid chromatography by previously described methods [3].

Fluorescence polarization measurements of 1,6-diphenylhexatriene incorporated into multilamellar lipid vesicles were made in a Perkin-Elmer MPF-3A spectrofluorimeter equipped with a polarization accessory employing polacoat polarizers to polarize excitation and emission beams. Excitation was at 360 nm and emission was measured at 410 nm using an additional 390 nm band pass filter placed in the emission beam path. Fluorescence intensity readings were taken using a 4 digit precision voltmeter (Beckman Instruments) connected across voltmeter output leads on the spectrofluorimeter. Polarization measurements were corrected using the grating correction formula:

$$P = \frac{V_v - L_v(V_h/L_h)}{V_v + L_v(V_h/L_h)}$$

In which the subscripts v and h indicate vertical and horizontal polarizations of the excitation beam and V and L represent vertical and horizontal polarizations, respectively, of the emission beam. Sample temperature was controlled by using a jacketted cuvette holder connected to a refrigerated circulating water bath. Temperature was monitored by a digital voltmeter (Bailey Instruments) through a thermistor placed directly above the excitation beam.

Lipid vesicles were formed with a phospholipid/probe ratio of 500 : 1 using previously published procedures [6]. When sterols and phospholipids were both incorporated into vesicles, the lipids were first fractionated by thin-layer chromatography using a petroleum ether/diethyl ether/acetic acid (70 : 30 : 1, v/v) solvent system to remove carotenoids which might affect the quantum yield of the diphenylhexatriene probe. Extracted sterol and phospholipid fractions were then recombined to form liposomes by the above procedure.

Results

Cultures of the *chol-1;chol-2* mutant grown in the presence of 50 μM or higher levels of choline have phospholipid distributions and growth characteristics similar to those of phenotypically wild type strains at both 37 and 15°C [4]. In cultures grown with this level of choline supplementation, phosphatidylcholine (PC) and phosphatidylethanolamine (PE) comprise between 80 and 90 mol% of

the total cellular phospholipids at both temperatures. PC and PE comprise approx. 50 and 38 mol%, respectively, of the phospholipids at 37°C and exist in approximately equal proportions (42–43%) at 15°C. Analysis of individual phospholipid species from normally growing cultures grown under choline supplemented conditions reveals that fatty acids are not uniformly distributed among the phospholipid classes at either temperature (Table I). PC is the most highly unsaturated phospholipid species at either growth temperature both in terms of number of double bonds per 100 molecules and percentage of unsaturated fatty acyl moieties. At 37°C PC contains appreciably higher levels of unsaturated species 18 : 2 and 18 : 3 than PE, whereas PE contains approximately double the levels of 16 : 0. Anionic phospholipids and cardiolipin (CL) contain twice the levels of saturated fatty acids found in PE and correspondingly lower levels of the unsaturates than the two major phospholipid species. Cardiolipin is the only class of phospholipid to contain significant levels

TABLE I

DISTRIBUTION OF FATTY ACID SPECIES IN PHOSPHOLIPIDS OF *NEUROSPORA chol-1;chol-2* GROWN AT 37 AND 15°C WITH AND WITHOUT CHOLINE SUPPLEMENTATION

Values are weight percent of total fatty acids for each species. Species comprising less than 1 percent are not shown. For comparison, whole cell data from Johnston et al. [3] are given in parentheses. Abbreviations: PE, phosphatidylethanolamine; PC, phosphatidylcholine; CL, cardiolipin; AP, acidic phospholipids (primarily phosphatidylinositol, phosphatidylserine, phosphatidic acid); DB/100, double bonds per 100 molecules. Choline supplemented medium contained 50 μM choline (C suppl. +).

Fatty acid	Phospholipids									
	PC		PE		AP		CL		Whole cell	
C suppl.:	+	–	+	–	+	–	+	–	+	–
A. 37°C										
14:0	–	–	–	–	–	–	11.1	–	(–)	(–)
16:0	12.0	6.5	25.1	23.6	43.1	39.6	24.8	5.1	(20.8)	(20.3)
16:1	1.0	–	–	–	–	–	–	–	(1.2)	(1.2)
18:0	1.9	1.7	–	1.8	–	–	–	6.9	(1.9)	(1.1)
18:1	11.8	9.9	11.7	13.7	8.7	5.9	30.3	31.5	(8.5)	(10.6)
18:2	60.6	71.0	54.3	55.0	41.9	50.5	21.9	54.0	(57.5)	(56.1)
18:3	11.3	10.8	8.2	5.6	3.7	3.6	11.8	1.8	(9.8)	(8.8)
DB/100	168	185	146	141	104	118	110	145	(149)	(150)
mol percent phospholipids	(51)	(10)	(37)	(68)	(8)	(17)	(3)	(4)		
B. 15°C										
14:0	–	–		–	1.4	–	4.1	–	(–)	(–)
16:0	2.8	3.0	15.9	11.0	24.9	21.9	15.1	20.0	(14.2)	(12.9)
16:1	–	–	–	–	–	–	–	–	(1.9)	(2.1)
18:0	–	–	–	–	1.7	–	3.5	–	(1.2)	(1.1)
18:1	3.3	3.1	6.2	5.6	4.1	–	18.6	7.4	(8.2)	(7.3)
18:2	24.1	20.1	35.9	44.3	27.0	27.1	22.4	68.0	(27.7)	(37.5)
18:3	67.9	72.3	39.8	38.1	38.7	50.9	33.6	3.0	(46.8)	(38.6)
DB/100	256	261	198	208	175	207	165	153	(205)	(200)
mol percent phospholipids	(43)	(28)	(42)	(57)	(13)	(8)	(2)	(4)		

of 14 : 0 at either growth temperature.

In 15°C choline-supplemented cultures large differences occur in unsaturated fatty acid levels between PC and PE, particularly with regard to 18 : 3, which shows a difference of 28 percentage points between the two species. In terms of relative levels of unsaturation determined by the number of double bonds found in fatty acids at both temperatures phospholipids are ranked in the order PC > PE > CL (anionic phospholipids).

In choline deprived cultures grown under the above conditions, PE levels are almost doubled and PC is reduced to less than one-fifth the normal levels at 37°C whereas at 15°C PE is increased 36% with a corresponding decrease in PC. Fatty acids were also non-randomly distributed between phospholipids under these conditions, but in a manner which differed from that found in normally growing cultures. PE showed the least change in spite of the large increase in the level of this species. The only notable differences were an increase in the levels of 18 : 2 and a decrease in the levels of 16 : 0 at 15°C. By contrast, PC showed similar changes in the same species at the higher growth temperature. The most significant changes in fatty acids of anionic phospholipids involved increases in levels of 18 : 2 at 37°C and in 18 : 3 at 15°C leading to an increase in the level of unsaturation under deprivation conditions at both temperatures. The most radical changes in fatty acid distribution occurred, however, in cardiolipin. These changes resulted in a large increase in levels of unsaturation at high temperatures and a decrease in unsaturation at the low temperatures. In both cases this was due to drastic reductions in 18 : 3 and concurrent large increases in levels of 18 : 2. A further consequence of choline deprivation was the reduction of the normally high levels of 14 : 0 in this species to less than 1 percent of the total fatty acids.

Effect of choline depletion on properties of phospholipids

Previous studies by other investigators using synthetic phospholipids with defined fatty acid composition have shown that there are large differences in the thermotropic behavior of choline and ethanolamine glycerophosphatides [2,7]. In light of the changes found in phospholipids and their fatty acid components reported here it is possible that fatty acyl rearrangements which occur under choline deprivation act to compensate for changes in altered fluidity. In order to assess the effects of choline deprivation on phospholipid thermotropic properties we examined artificial bilayers constructed from phospholipids isolated from whole cells and microsomal membranes of supplemented and deprived cultures grown under the same conditions shown above using fluorescence polarization of 1,6-diphenylhexatriene as a probe of relative lipid mobilities.

Polarization values for total mycelial phospholipids are given in Table II. In order to compare relative mobilities of lipids from cultures grown under both temperature and nutritional conditions, measurements were made on all samples at both 37°C and 15°C. At both measurement temperatures, phospholipids from 37°C cultures exhibited higher polarization values than those from 15°C cells which is consistent with the increased levels of unsaturated fatty acids found in phospholipids at the lower growth temperatures. Readdition of sterols resulted in higher polarization values for both 37°C and 15°C lipid fractions which is consistent with the ordering effects produced by sterols on phospholipid bilayers [2,7,8].

Measurements on lipids from choline deprived cultures show a similar pattern in that 37°C lipids

TABLE II

DIPHENYLHEXATRIENE POLARIZATION IN TOTAL CELLULAR LIPIDS FROM *chol-1;chol-2* GROWN UNDER CHOLINE SUPPLEMENTED AND DEPRIVED CONDITIONS

Lipids were isolated from cultures grown at 37 or 15°C in medium containing either 50 μM choline (+) or no choline supplement (−) as previously described.

Lipid fraction	Polarization at 15°C		Polarization at 37°C	
	+	−	+	−
37°C Phospholipids	0.191	0.240	0.128	0.203
15°C Phospholipids	0.170	0.199	0.116	0.150
37°C Phospholipids + sterols	0.194	0.288	0.145	0.254
15°C Phospholipids + sterols	0.174	0.190	0.122	0.150

exhibit higher polarization values than their 15°C counterparts. Addition of sterols resulted in an apparent increased ordering of 37°C lipids when measured at either 37°C or 15°C. The effect on 15°C lipids was negligible when measured at 37°C and appeared to be slightly fluidizing when measured at the lower temperature.

The most striking differences are observed when polarization values of lipids from choline supplemented and choline deprived cultures are compared with each other. Lipids from choline deprived cultures exhibit consistently higher polarization values than their normally growing counterparts, pointing to markedly higher ordering and decreased fluidity as a consequence of the phosphatidylcholine depletion.

Effects of choline deprivation on microsomal membranes

In order to assess the effects of choline deprivation on a specific membrane fraction, microsomal lipids were isolated from 50 μM supplemented and choline-deprived cultures grown under the previously described conditions. Phospholipid fatty acid compositions of microsomes from cultures grown at high and low temperatures under both nutritional conditions are given in Table III. The total membrane phospholipid fractions exhibit similar fatty acid profiles to those found for whole cells, with high levels of 18 : 2 and low levels of 18 : 3 at 37°C and with both species occurring in similar amounts at the low temperature. Unlike our previously reported findings in a phenotypically wild-

TABLE III

DISTRIBUTION OF PHOSPHOLIPID FATTY ACIDS IN MICROSOMAL FRACTIONS OF *chol-1;chol-2* GROWN WITH AND WITHOUT CHOLINE SUPPLEMENTATION

Values are weight percent of total fatty acids for each species. Species comprising less that one percent of the total are not shown. See Table I for abbreviations.

Fatty acid	Microsomal phospholipids							
	PC		PE		AP		Total phospholipid	
C suppl.:	+	–	+	–	+	–	+	–
A. 37°C								
16:0	16.9	10.3	20.9	23.4	34.9	34.7	19.0	25.7
16:1	1.3	–	3.4	1.6	1.0	3.4	2.2	1.4
18:0	1.3	5.2	3.4	2.1	7.2	9.4	1.5	1.6
18:1	24.7	16.0	21.4	15.1	15.2	11.2	19.3	14.4
18:2	50.9	63.2	47.7	54.7	37.2	39.4	52.6	52.9
18:3	4.8	4.4	3.4	2.9	4.2	1.7	5.2	3.7
DB/100	142	156	130	135	103	98	142	131
mol percent phospholipids	51	6	33	68	15	24		
B. 15°C								
16:0	4.6	13.9	17.1	26.6	35.5	3.2	11.4	15.7
16:1	–	1.9	–	–	–	2.0	1.0	2.6
18:0	–	–	–	–	1.9	–	–	–
18:1	3.9	8.3	6.5	2.3	2.7	3.2	3.8	6.1
18:2	30.4	42.8	38.9	26.4	29.9	23.1	33.3	38.8
18:3	60.0	32.6	37.0	43.3	31.8	67.5	49.9	35.8
DB/100	249	194	195	185	161	254	221	191
mol percent phospholipids	52	7	35	75	13	18		

type strain, there were some significant differences between whole cell and membrane fatty acid profiles in mutant cultures. Furthermore, there were notable differences between microsomal fatty acid distributions of supplemented and non-supplemented cultures which led to a general decrease in levels of double bonds as a consequence of choline deprivation. This was due primarily to increases in 16 : 0 and decreases in the polyunsaturates at the high temperature and to increases in 18 : 2 and a decrease in 18 : 3 at the lower temperature. In terms of number of double bonds per molecule the differences appear to be independent of growth temperature with PC depleted microsomes showing 15% fewer double bonds per 100 molecules at both temperatures. Fatty acids in microsomal phospholipids also differed from whole cell compositions, most notably at the lower growth temperatures where acidic phospholipids showed unusually high levels of of unsaturated fatty acids due to a doubling of 18 : 3 levels and a decrease in 16 : 0 to one-tenth the supplemented value. Phosphatidylcholine unsaturated fatty acid levels were sharply decreased under these conditions with 18 : 3 levels lowered to half the normal values. Phospholipid distributions in this membrane fraction reflect the same trends seen in whole cell lipids with large decreases in the levels of phosphatidylcholine and corresponding increases in phosphatidylethanolamine and acidic phospholipids.

The effects of temperature acclimation and choline deprivation on microsomal phospholipid thermotropic properties are given in Table IV. The sterol content of these membranes are very low, making accurate reconsititution in liposomes difficult. Consequently, we only examined the phospholipid fraction of these membranes. Microsomal lipids gave lower polarization values than whole cell phospholipids at both measurement temperatures and under all growth and nutritional conditions reported here. The effect of choline deprivation again is to increase polarization values, indicating restricted lipid mobilities due to the alterations in phospholipid and fatty acid compositions.

TABLE IV

DIPHENYLHEXATRIENE POLARIZATION IN MICROSOMAL PHOSPHOLIPIDS OF *chol-1;chol-2* CULTURES GROWN AT 37°C AND 15°C UNDER CHOLINE SUPPLEMENTED AND DEPRIVED CONDITIONS

Growth temperature	Polarization at 15°C		Polarization at 37°C	
	50 μM choline	No choline	50 μM choline	No choline
37°C	0.180	0.212	0.121	0.136
15°C	0.151	0.174	0.108	0.118

Discussion

A useful approach to the study of membrane lipid function has been to modify specific lipid components of living cells in order to observe the effects of such changes on cellular and membrane functions. In a previous publication we reported on the use of a choline requiring mutant of *Neurospora* which was defective in steps involving the methylation of phosphatidylethanolamine to produce phosphatidylcholine [4]. Growth of this mutant on medium lacking choline produced membrane lipid compositions in which phosphatidylcholine levels were severely reduced with a concomitant increase in ethanolamine glycerophosphatides. As reported previously [4], choline deprivation produces surprisingly small changes in levels of unsaturation in the whole cell phospholipid fraction. Data presented here shows that each phospholipid class contains, in fact, a unique fatty acid distribution. Choline deprivation results in a redistribution of the fatty acids which are varies widely between the different phospholipid species. Phosphatidylcholine, which is reduced to one-fifth of its normal cellular levels shows some significant changes in at least two fatty acid species at both growth temperatures; phosphatidylethanolamine, whose levels are almost doubled under deprivation conditions shows little change. The most major changes in fatty acids in fact involve cardiolipin, which at the high growth temperatures undergoes a 32% increase in double bonds per 100 molecules whereas at the low temperature the level of desaturation is decreased with large changes in levels of polyunsaturates.

Microsomal membranes show a somewhat different pattern of changes than that found with whole cell lipids. Here the net effect of the choline deprivation is to reduce the levels of phospholipid

unsaturates in terms of the total number of double bonds at both growth temperatures. Particularly large changes were found in the fatty acyl compositions of phosphatidylcholine and acidic phospholipids from cultures grown at low growth temperatures. The fatty acyl rearrangements produced as a consequence of choline deprivation could conceivably be viewed as part of mechanism used to maintain membrane fluidity in response to nutritional perturbation. This seems unlikely since choline deprivation in the mutant also produces pronounced changes in the thermotropic properties of membrane lipids. The magnitude of these changes are large enough to suggest that choline deprivation will provide a useful system for the study of membrane fluidity related functions in this organism. Useful comparisons of polarization measurements presented here can be made in assessing relative mobilities of the probe between different membrane fractions by comparing changes in polarization produced by mutational alteration with those produced by temperature acclimation in normally growing cultures. The differences in polarization found between lipids from normally growing 37°C and 15°C cultures measured at a given temperature provides a useful benchmark, since the range demarcated by these values should represent a measure of normal fluidity acclimation to a temperature range of 22 K. Comparative examination of polarization values of lipids from supplemented and deprived cultures grown at the same temperature reveals that the effects of phosphatidylcholine depletion tend to exert an ordering influence on membrane lipids which is greater than that normally observed by varying growth temperature over that particular temperature range.

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