Depletion of phosphatidylcholine in yeast induces shortening and increased saturation of the lipid acyl chains
Evidence for regulation of intrinsic membrane curvature in a eukaryote

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Abbreviations used: ESI-MS/MS, electrospray ionization tandem mass spectrometry; DAG, diacylglycerol; MME, 2-(methylamino)-ethanol; DME, *N*,*N*-dimethylamino-ethanol; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; SFA, saturated fatty acid; UFA, unsaturated fatty acid.

ABSTRACT

To study the consequences of depleting the major membrane phospholipid phosphatidylcholine (PC), exponentially growing cells of a yeast *cho2opi3* double deletion mutant were transferred from medium containing choline to choline-free medium. Cell growth did not cease until the PC level had dropped below 2 % of total phospholipids after 4-5 generations. Increasing contents of phosphatidylethanolamine (PE) and phosphatidylinositol (PI) made up for the loss of PC. During PC depletion the remaining PC was subject to acyl chain remodeling with monounsaturated species replacing diunsaturated species, as shown by mass spectrometry. The remodeling of PC did not require turnover by the *SPO14*-encoded phospholipase D. The changes in the PC species profile were found to reflect an overall shift in the cellular acyl chain composition that exhibited a 40 % increase in the ratio of C16 over C18 acyl chains, and a 10 % increase in the degree of saturation. The shift was stronger in the phospholipid than in the neutral lipid fraction, and strongest in the species profile of PE. The shortening and increased saturation of the PE acyl chains were shown to decrease the non-bilayer propensity of PE. The results point to a regulatory mechanism in yeast that maintains intrinsic membrane curvature in an optimal range.

INTRODUCTION

Phosphatidylcholine (PC) is an abundant glycerophospholipid present in the membranes of eukaryotic cells. Apart from being a major structural component of all organellar membranes, it serves as a reservoir of signaling molecules (Exton, 1994; Kent and Carman, 1999), and it has been implicated in apoptosis (Cui and Houweling, 2002). In the model eukaryote *Saccharomyces cerevisiae*, mutations in the genes encoding PC biosynthetic enzymes lead to respiratory deficiency (Griac *et al.*, 1996), indicating that PC is important for mitochondrial function. PC was found to interact with Gut2p, the mitochondrial glycerol-3-phosphate dehydrogenase, in a photolabeling study (Janssen *et al.*, 2002). Furthermore, the biosynthesis of PC is involved in the regulation of intracellular vesicle trafficking in yeast (reviewed in (Howe and McMaster, 2001)).

The triple methylation of phosphatidylethanolamine (PE), catalyzed by the methyltransferases Cho2p (Pem1p) and Opi3p (Pem2p), is the primary route for the synthesis of PC in yeast in the absence of exogenous choline (Carman and Henry, 1999). When choline is supplied in the growth medium, the CDP-choline pathway contributes to the net synthesis of PC (Fig. 1). However, also in the absence of choline, the CDP-choline pathway contributes to PC synthesis using (phospho)choline derived from the turnover of PC (McMaster and Bell, 1994). Electrospray ionization tandem mass spectrometry in combination with stable isotope labeling revealed that the two biosynthetic routes produce the PC molecular species, i.e. PC molecules with specific acyl chains, in different ratios (Boumann *et al.*, 2003).

Whereas the biosynthesis of PC and its regulation has been extensively characterized (Carman and Henry, 1999), knowledge on the processes downstream of PC synthesis in yeast is fairly limited. The mechanism(s) responsible for distributing

PC from its site of synthesis in the ER to the other organellar membranes are still obscure (see e.g. (de Kroon *et al.*, 2003)). With respect to the metabolic fates of PC, the turnover of PC by the *SPO14* encoded phospholipase D yields phosphatidic acid, and is essential for sporulation (Waksman *et al.*, 1996; Rudge *et al.*, 1998). PC was shown to be deacylated by Plb1p *in vivo* (Lee *et al.*, 1994), and by Plb2p *in vitro* (Merkel *et al.*, 1999). It can serve as acyl chain donor for the synthesis of triglycerides by Lro1p *in vitro* (Oelkers *et al.*, 2000). In the presence of choline in the culture medium or upon raising the growth temperature to 37^{o}C, PC synthesized by the CDP-choline route is converted to glycerophosphocholine by the enzyme Nte1p encoded by YML059c (Dowd *et al.*, 2001; Zaccheo *et al.*, 2004). Recently, the occurrence of remodeling by acyl chain exchange at the *sn1* position of PC was demonstrated (Boumann *et al.*, 2003).

According to the shape-structure concept of lipid polymorphism (Cullis and de Kruijff, 1979), PC has a cylindrical shape with similar cross-sectional areas for the headgroup and acyl chain parts of the molecule, and prefers to organize as a bilayer, which makes it ideally suited to preserve membrane integrity. In contrast, PE and DAG, the lipid precursors of PC, are so-called type II lipids that have an overall conical molecular shape resulting from the comparatively small cross-sectional area of the polar headgroup. The propensity of isolated type II lipids to organize as reversed non-lamellar structures upon hydration increases with increasing acyl chain length and unsaturation (Koynova and Caffrey, 1994). Type II lipids confer negative curvature stress to biological membranes (Gruner, 1985). Prokaryotes maintain intrinsic membrane curvature in an optimal window by adapting the membrane lipid composition in response to changes in growth conditions (reviewed by (Dowhan, 1997)).

We set out to investigate the consequences of lowering the cellular PC content in yeast as a novel approach to find clues regarding the specific cell biological roles of PC, the mechanism(s) of its intracellular transport, and the metabolism of PC downstream of its synthesis. Moreover, depletion of the bilayer-forming lipid PC affects the balance between bilayer and non-bilayer forming lipids in yeast, enabling us to study how a eukaryotic cell with its intracellular membrane trafficking processes responds to changes in membrane curvature stress. Deletion of the genes encoding Cho2p and Opi3p renders the yeast cell dependent on the supply of choline in the medium for the synthesis of PC (Summers *et al.*, 1988; Kodaki and Yamashita, 1989). By transferring *cho2opi3* cells in the exponential phase of growth to choline-free medium, net synthesis of PC was stopped while growth continued for several generations. This strategy of choline deprivation was used to study the time dependent effects of PC depletion on growth, cell viability, and lipid composition of the *cho2opi3* cells.

It is reported here that yeast cells respond to depletion of the bilayer lipid PC by global changes in lipid acyl chain composition, that of the declining PC pool included. The shortening of the acyl chains and the increased saturation were most pronounced in PE. The results point to a regulatory mechanism in yeast that maintains the balance between bilayer and non-bilayer phospholipids by adjusting the acyl chain composition.

MATERIALS AND METHODS

Yeast strains, Media and Culture Conditions

The parental wild type strain BY4742 (*MATα his∆1 leu2∆0 lys2∆0 ura3∆0*) and the congenic *cho2* strain (*cho2::KanMX*) were obtained from Invitrogen (Carlsbad, CA).

The *OPI3* gene of the *cho2* strain was replaced by *LEU2* using PCR-mediated one step gene replacement to yield the *cho2opi3* strain (*cho2::KanMX opi3::LEU2)* as described (Boumann *et al.*, 2004). The *SPO14* gene in the *cho2opi3* strain (nucleotides –2 to 5052) was replaced with a PCR-generated construct containing the *HIS5* gene of *S. pombe* flanked by coliphage *loxP* sites (kindly provided by J. Holthuis, Utrecht University), to yield the *cho2opi3spo14* strain (*cho2::KanMX opi3::LEU2 spo14::HIS5)*. Correct integration of the *HIS5* marker was verified by PCR. The *cho2opi3* and *cho2opi3spo14* strains were cultured under aerobic conditions at 30^{o}C in complete vitamin-defined synthetic medium (Griac *et al.*, 1996) containing 0.1% glucose and 2% lactate (SD-lactate) or 3% glucose (SD-glucose) as carbon source. SD-lactate medium was adjusted to pH 5.5. SD media contained 75 μM inositol, and were supplemented with 1 mM choline as indicated.

Growth Phenotype

To analyze growth phenotypes, cells were cultured in YPD medium (1% yeast extract, 2% bactopeptone, 2% glucose) to the mid-logarithmic phase of growth. Cells were collected by centrifugation, washed three times with sterile water, and adjusted to OD_{600} = 1.0. Next, 10 μl aliquots of 1:10 serial dilutions were applied to solid SD-glucose medium containing 2% agar (Sigma) with or without 75 μM inositol and the supplements indicated.

Assay for Opi^{-} Phenotype

Overproduction of inositol (Opi^{-}) phenotypes was monitored as described previously (Greenberg *et al.*, 1983). Briefly, yeast strains were patched onto synthetic inositol-free medium. After growth for 3 days at 30 °C, the agar plates were sprayed with a

suspension of the inositol auxotrophic indicator strain AID (MAT α/a *ino1-13*/*ino1-13 ade1*/*ade1*) and incubated for another 3 days at 30°C. Excretion of inositol was detected by a red halo of the AID strain.

Depletion of Phosphatidylcholine in cho2opi3 Cells and Labeling with (D_{13})-choline

The *cho2opi3* strains were cultured to the mid-logarithmic phase of growth (OD_{600} ~0.5, Hitachi 150-20 spectrophotometer) in SD medium containing 1 mM choline. Cells were collected by filtration, washed thoroughly with choline-free SD medium (30°C) and used to inoculate fresh SD medium with (C^+) or without 1 mM choline (C^-) to an OD_{600} of 0.05 (t = 0), unless indicated otherwise. At the indicated times, OD_{600} values were measured, and samples were taken for further analysis. To determine the molecular species composition of PC newly synthesized after 16 h of growth in C^- medium, the cells were pulsed for 10 min with 0.2 mM (D_{13})-choline (Cambridge Isotope Laboratories, Andover, MA), added to the culture medium. In the control experiment, cells cultured for 16 h in C^+ medium were collected by centrifugation, and transferred to C^- medium containing 0.2 mM (D_{13})-choline. After 10 min of labeling with (D_{13})-choline, cells were inactivated by adding a mixture of KCN, NaF, and NaN_3 to final concentrations of 15 mM each. The cells were pelleted, homogenized, and lipid extracts prepared.

Phospholipid Analysis

The phospholipid class composition of the *cho2opi3* cells was determined by labeling with [^{32}P]orthophosphate. Briefly, cells were precultured as above in the presence of 10 μCi/ml of [^{32}P]orthophosphate, collected by centrifugation, washed, and transferred at t = 0 to C^+ or C^- medium containing 10 μCi/ml of [^{32}P]orthophosphate,

to an OD_{600} of 0.02. Cells corresponding to 2 OD_{600} units were harvested at the times indicated, and treated with trichloroacetic acid. Lipids were extracted (Atkinson *et al.*, 1980), and the phospholipid classes were resolved by two-dimensional paper chromatography (Steiner and Lester, 1972), and quantified using a PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

MS Analysis of Phospholipid Molecular Species

The molecular species compositions of PE, PC, and D_{13}-labeled PC in total lipid extracts were analyzed by ESI-MS/MS, using neutral loss scanning for *m/z* 141 and parent ion scanning for *m/z* 184, and *m/z* 197, respectively, on a Quattro Ultima triple quadrupole MS instrument (Micromass, Manchester, UK) in the positive ion mode. Total lipid extracts were dissolved at 0.5 mM phospholipid phosphorus in chloroform/methanol/water 2:15:3 (v/v/v), with 1% (v/v) formic acid added to reduce the content of $[M + Na]^+$ adducts. The instrument settings, the collection and quantification of the ESI-MS/MS data, including the correction for the inverse relationship between mass and signal response, were as described previously (Boumann *et al.*, 2004).

Fatty Acid Analysis

Total lipid extracts corresponding to 400 nmol phospholipid phosphorus were applied to HPTLC silica 60 plates (Merck) to separate phospholipids from neutral lipids by elution with chloroform/methanol 9:1 (v/v). The phospholipid and neutral lipid fractions were eluted from the silica with chloroform/methanol, 1:1 and chloroform/methanol, 9:1, respectively. The eluates and total lipid extracts were transesterified by heating at 70^{o}C for 2 h in 2.5% (v/v) H_2SO_4 in dry methanol. The

fatty acid methylesters were extracted in hexane, and separated on a Chrompack CP-9001 gas chromatograph (Middelburg, the Netherlands) using a capillary column CP-WAX58 CB at 190^{o}C. Fatty acid methylesters were identified and signal intensities were calibrated using a fatty acid methylester standard (Nu-ChekPrep, Elysian, MN).

Northern blot analysis

The isolation of total yeast RNA and quantitative Northern blot analysis, including the preparation of radiolabeled probes for the *OLE1* mRNA and the internal control *PGK1*, were performed as described previously (Gonzalez and Martin, 1996).

Purification of the phospholipid fraction and PE

Total lipid extracts were prepared from yeast spheroplasts and subjected to silicagel column chromatography using chloroform as eluent to remove neutral lipids. Subsequently, the total phospholipid fraction was eluted with chloroform/methanol 1:1 (v/v). Purification of PE from the total phospholipid fraction involved repeated silicagel column chromatography using the following solvents: (i) chloroform/methanol/water/ammonia 30:70:2:2 (v/v/v/v), (ii) chloroform/methanol/water 65:25:4 (v/v/v), and (iii) chloroform/methanol/water 80:20:2 (v/v/v). The final product did not contain any impurities as judged by HPTLC.

^{31}P-NMR

Samples were prepared by hydrating phospholipid films corresponding to at least 10 μmol of phospholipid phosphorus in 0.2 mL 100 mM NaCl, 10 mM Pipes pH 7.4, followed by 10 freeze-thaw cycles. ^{31}P-NMR spectra were recorded on a Bruker

Avance 500 MHz spectrometer (Bruker Biospin, Karlsruhe, Germany). ^{31}P-NMR measurements were performed at 202.5 MHz, using a single-pulse experiment with a 12.0 μs pulse, 1.2 s relaxation delay time, and broadband proton decoupling. Typically, 4000 scans were acquired. An exponential multiplication, corresponding to a line broadening of 100 Hz, was applied to the FID prior to Fourier transformation. The temperature was increased in steps of 5^{o}C. Samples were allowed to equilibrate for 30 min at each temperature before data acquisition.

Other Methods

To determine cell viability, yeast samples were serially diluted, spread on YPD plates, and colonies were counted after 3 days of incubation at 30^{o}C. For preparing total lipid extracts, cell homogenates were obtained by vortexing yeast cells (~100 OD_{600} units) in the presence of glass beads (Boumann *et al.*, 2003), and subjected to lipid extraction (Bligh and Dyer, 1959), unless indicated otherwise. Phospholipid-to-protein ratios were determined in yeast spheroplasts. Spheroplasts were prepared by zymolyase treatment (Daum *et al.*, 1982), subjected to phospholipid extraction, and the phospholipid phosphate content of the organic phase was quantified (Fiske and Subbarow, 1925) after destruction with perchloric acid. The protein content of yeast samples solubilized with YPER (Pierce) according to the manufacturer's instruction, was determined using the BCA method (Pierce) with 0.1% (w/v) SDS added and BSA as a standard.

RESULTS

Phenotypic Characterization of the cho2opi3 Double Deletion Mutant To characterize the *cho2opi3* double deletion mutant constructed in the BY4742 genetic background,

growth and overproduction of inositol (Opi$^-$ phenotype (Greenberg *et al.*, 1983)) were examined under several conditions (Fig. 2). With choline present in the medium, *cho2opi3* cells grew like wild type, irrespective of the presence of inositol. In the absence of inositol, the double mutant was strictly auxotrophic for choline, as neither MME nor DME, substrates for the synthesis of phosphatidylmonomethylethanolamine (PMME) and phosphatidyldimethylethanolamine (PDME) via the Kennedy pathways, respectively, supported growth. In the presence of inositol, DME could substitute for choline in supporting growth, whereas MME could not (Fig. 2A). The Opi$^-$ phenotype of the *cho2opi3* strain was suppressed by supplementing the medium with choline, DME or MME (Fig. 2B). The phenotype of the *cho2opi3* double deletion mutant is comparable with that reported previously for *cho2opi3* mutants (McGraw and Henry, 1989).

The Effect of Choline Deprivation on Growth and Viability of cho2opi3 Cells The effects of removing choline from the culture medium of *cho2opi3* cells were studied in liquid media containing 75 μM inositol, to preclude any limitation on the ability of the cells to synthesize PI. Mid-log *cho2opi3* cells were transferred within 2 min from C^+ to fresh C^+ or C^- liquid medium by filtration, and incubated at 30^{o}C. Exponential growth continued for about 12 h corresponding to approximately 4.5 generations, irrespective of the presence of choline, and independent of lactate (Fig. 3A) or glucose (data not shown) serving as carbon source. After 12 h, the growth in C^- medium ceased as compared to growth in C^+ medium (Fig. 3A), where the cells reached densities comparable to wild type cells (not shown).

During the first 20 h of choline deprivation over 60 % of the cells maintained viability (Fig. 3B) in agreement with published data (Summers *et al.*, 1988). After 5

days, cell survival in choline-free lactate medium decreased to 25 %, whereas less than 1 % of the cells survived in choline-free glucose medium. Because of the more favorable cell survival, all following experiments were carried out using lactate-based culture media.

The Effect of Choline Deprivation on the Phospholipid Class Composition of cho2opi3 Cells The effect of depriving *cho2opi3* cells from choline on the phospholipid composition was examined by steady state labeling with $^{32}P_i$. The results from lactate-grown cells have been plotted on a logarithmic scale for the major phospholipid classes in Figure 4. In C^+ medium, the phospholipid composition of *cho2opi3* cells remained essentially constant during growth, with about 42 % PC, 27 % PI, 20 % PE, and 9 % PS (Fig. 4, right panel). After removing choline from the medium, the PC content decreased with an apparent rate constant that was similar to but of opposite sign as the rate of cell proliferation (Fig. 4, left panel). As the overall phospholipid-to-protein ratio of the cells was not affected by choline deprivation for up to 20 h (data not shown), this indicates that the PC content of the cells was halved with each generation. Consistent with the above result, growth ceased when the PC level dropped below 2 %. The loss of PC was compensated for by a rapid increase in PE content from 20 % to 55 % during the first 8 h of choline starvation, and a gradual increase in PI from 27 % to 50 % in 50 h (Fig. 4, left panel).

During PC Depletion, the PC Pool Remaining Is Subject to Extensive Acyl Chain Remodeling To investigate whether the existing PC pool was metabolically silent during choline starvation of *cho2opi3* cells, the molecular species composition of PC was monitored by ESI-MS/MS, using parent ion scanning for *m/z* 184, corresponding

to the phosphocholine headgroup. The most abundant PC species in yeast are 32:2, 32:1, 34:2, and 34:1 that predominantly have combinations of C16:0, C16:1, C18:0, and C18:1 acyl chains esterified at the *sn*1 and *sn*2 positions of the glycerol backbone (Wagner and Paltauf, 1994; Schneiter *et al.*, 1999). The *cho2opi3* strain cultured in C^+ lactate medium exhibited a PC species profile similar to that of the parental wild type strain (Boumann *et al.*, 2003), with a slight increase in 34:2 at the expense of 32:1 as the cells progressed into stationary phase (Fig. 5, lower panel). Upon transfer to choline-free medium the PC species profile changed dramatically with strong increases in the proportions of 32:1 and 34:1 at the expense of diunsaturated 32:2 and 34:2 (Fig. 5, upper panel). This result shows that during PC depletion, in the absence of any net biosynthesis of PC, the pre-existing PC pool is metabolized to attain a more saturated species profile.

The change in PC species composition upon PC depletion could be due to PC turnover and recycling of the released (phospho)choline via the CDP-choline route or, alternatively, it could be due to exchange of the acyl chains via deacylation-reacylation, or transacylation reactions. To address the first possibility, the species composition of PC newly synthesized after 16 h of choline deprivation was determined by pulsing the cells for 10 min with (D_{13})-choline. Parent ion scanning at *m/z* 197 revealed that the species profile of the newly synthesized (D_{13})-PC closely resembled that of the PC pool remaining after 16 h of choline deprivation (Fig. 6, upper panel). This result is consistent with the changes in the PC species profile resulting from turnover and re-incorporation of the released (phospho)choline moiety into PC. Comparison of the profiles of newly synthesized PC between cells grown in the presence and absence of choline (black columns in lower and upper panel of Fig. 6, respectively) implies that the species composition of the DAG pool serving as

substrate for the CDP-choline pathway, has been enriched in monounsaturated species in response to PC depletion.

Remodeling of the Remaining PC Pool Does not Require Turnover Mediated by Spo14p The phospholipase D encoded by the *SPO14* gene presented a potential candidate responsible for the turnover of PC (Patton-Vogt *et al.*, 1997; Sreenivas *et al.*, 1998). To test the involvement of Spo14p, a triple *cho2opi3spo14* mutant was constructed. It was verified that deletion of the *SPO14* gene abolished the Ca^{2+} independent conversion of fluorescent NBD-labeled PC to NBD-PA (data not shown; (Waksman *et al.*, 1996)). The effect of 24 h of choline deprivation on the PC species profile of the *cho2opi3spo14* mutant was indistinguishable from that of the *cho2opi3* mutant (compare Figs. 7 and 5), demonstrating that *SPO14* is not required for the remodeling of PC.

Lipid Class-Dependent Changes in Acyl Chain Composition in Response to PC Depletion The PC depletion-induced changes in the species profiles of PC and its lipid precursor DAG may reflect a global change in the cellular fatty acid composition of *cho2opi3* cells. To address this possibility, *cho2opi3* cells were cultured in C^+ or C^- medium and examined for their fatty acid content by gas chromatography of the fatty acid methyl esters (Table 1). The acyl chain composition of *cho2opi3* cells cultured in C^+ medium was comparable to that of wild type yeast (Daum *et al.*, 1999). After 20 h of choline deprivation, the content of unsaturated acyl chains has decreased by some 10 %, whereas that of C16 acyl chains has increased by 7% at the expense of C18 chains in total lipid extracts, compared to cells cultured in C^+ medium. Examination of the acyl chain compositions of the separate phospholipid and neutral lipid fractions

revealed that the former is more strongly affected by PC depletion, with the contents of C16:0 and C18:0 more than doubling at the expense of C18:1 and, to a lesser extent, C16:1 (Table 1). In both phospholipid and neutral lipid fractions similar increases in the content of C16 chains were apparent. The fatty acid compositions (Table 1) are in agreement with neutral lipids and phospholipids contributing more or less equally to the total cellular acyl chain content in the late-log phase of growth (Tung *et al.*, 1991; Sandager *et al.*, 2002).

To further investigate the changes in the phospholipid species, the species profile of PE was monitored by ESI-MS/MS using neutral loss scanning for *m/z* 141. PE rapidly became the most abundant phospholipid class in *cho2opi3* cells during choline deprivation, accounting for more than 50% of the total phospholipids (Fig. 4). The PE species composition of *cho2opi3* cells cultured in the presence of choline is similar to that of the parental wild type, with 34:2 the most prominent PE species, followed by 32:2, and with relatively low levels of monounsaturated 32:1 and 34:1 (Boumann *et al.*, 2003). In C^+ medium the PE profile is hardly affected by the growth phase (Fig. 8, lower panel). In response to choline deprivation, during the first 7 h, the contents of 32:2 and the mono-unsaturated species increased at the expense of 34:2 (Fig. 8, upper panel). Subsequently, the contents of both diunsaturated PE species decreased, while the contents of 32:1 and 34:1 PE strongly increased reaching levels 7 and 2.5 times higher than under C^+ conditions, respectively (Fig. 8).

As summarized in Figure 9, after 20 h of PC depletion the shift towards shorter (C16) acyl chains was stronger in PE than in the total phospholipid or neutral lipid fractions. Furthermore, the increase in saturation degree was largest in PE, and larger in the phospholipid than in the neutral lipid fraction. The combined results indicate lipid class specific adaptations in acyl chain composition in response to PC depletion.

PC depletion is accompanied by a rise in the level of OLE1 mRNA The increase in saturation of total fatty acids during PC depletion (Table 1), suggests that the activity of the only fatty acid Δ-9 desaturase in yeast encoded by the essential *OLE1* gene (Stukey *et al*., 1990), is reduced. Regulation of *OLE1* gene expression by nutrient fatty acids has been extensively investigated, and occurs via transcriptional and mRNA stability controls (McDonough *et al*. 1992; Gonzalez and Martin, 1996; Hoppe *et al.*, 2000; Kandasamy et al., 2004). To find a first clue with regard to the apparent change in Ole1p activity in choline-deprived *cho2opi3* cells, the *OLE1* mRNA levels were examined in Northern blots. Whereas in C^+ medium the *OLE1* mRNA level remained constant during growth, after 12 h of choline deprivation the *OLE1* mRNA level had increased 2.6-fold, and this level was maintained for at least another 20 h (Fig. 10). We conclude from these data that *OLE1* gene expression is regulated under these conditions by one or more mechanisms that act to compensate for the effects produced by PC depletion.

The rise in PE level is compensated for by an increase in the bilayer to non-bilayer transition temperature of PE The increased PE content resulting from PC depletion is expected to enhance the intrinsic membrane negative curvature stress. The accompanying shortening and increased saturation of the acyl chains of PE (Figs. 8 and 9) could serve as a compensatory mechanism reducing membrane negative curvature. The tendency of PE to adopt non-lamellar phase is a measure for its contribution to membrane negative curvature, and can be measured by ^{31}P-NMR, the method of choice for studying aggregate structures of phospholipids in aqueous

dispersion. Therefore the phase behavior of PE purified from *cho2opi3* cells cultured for 16 h in the absence or presence of choline, was compared by ^{31}P-NMR.

As shown in Figure 11, at 10 ^{o}C the ^{31}P-NMR signals of both PE samples showed line shapes with a high field peak and a low field shoulder and a chemical shift anisotropy (csa) around –40 ppm, characteristic for PE in the lamellar liquid crystalline phase (Cullis and de Kruijff, 1978). The spectrum of the PE sample from the cells cultured with choline also revealed a minor superimposed isotropic component. At 20 ^{o}C the isotropic component slightly increased and an additional component appeared in the spectrum, indicating the onset of a phase transition. At 30 ^{o}C the shift to a line shape with a reversed asymmetry and reduced line width, characteristic for the non-lamellar hexagonal H_{II} phase, was complete. The superimposed signal intensity at the isotropic position originates from motional averaging of a minor fraction of the lipids due to the formation of small vesicles or cubic-like phase, which is often observed in systems undergoing a bilayer to hexagonal H_{II} phase transition. The PE sample from the PC-depleted cells maintained lamellar phase up to 45 ^{o}C (not shown). Here, the phase transition to non-lamellar phase was first visible at 50 ^{o}C (Fig. 11), and it was completed at 55 ^{o}C (not shown). The combined results show that the lamellar to non-lamellar phase transition temperature of PE increased by approximately 30 ^{o}C in response to PC depletion.

The phase behavior of the total phospholipid extracts of *cho2opi3* cells cultured in the absence or presence of choline for 16 h was also investigated by ^{31}P-NMR. The dispersions of both phospholipid extracts maintained lamellar liquid crystalline phase up to a temperature of 50 ^{o}C (not shown). The data indicate that yeast counterbalances the rising level of the type II lipid PE by increasing the lamellar to non-lamellar phase transition temperature of PE.

DISCUSSION

The effects of depleting the major membrane phospholipid PC on cell growth, viability, and lipid composition have been studied in yeast. After cessation of *de novo* synthesis of PC upon transferring *cho2opi3* cells to choline-free medium, cell growth remained unaffected for 4-5 generations, providing a window for examining the effects in exponentially growing cells. The main findings were that the PC remaining during choline deprivation is subject to extensive acyl chain remodeling, and that PC depletion induces an overall shift to shorter and more saturated acyl chains that is most pronounced in PE. The implications of the results for PC function, PC turnover, and the regulation of membrane lipid composition in yeast will be discussed.

In the absence of choline or a substrate replacing choline, growth of *cho2opi3* cells ceased 4-5 generations after deprivation of choline, as was observed earlier (Kodaki and Yamashita, 1989; Howe *et al.*, 2002). Subsequently, a gradual, time dependent loss of cell viability was observed, which was more severe in medium containing the fermentable carbon source glucose than in medium containing non-fermentable lactate. Possibly, the low PC levels interfere with the diauxic shift, leading to increased cell death. Previously, a virtually complete loss of cell viability within 23 h was reported upon inhibition of PC synthesis in yeast cells cultured on glucose at 37 ^{o}C, whereas cells cultured at 25 ^{o}C stayed viable (Howe *et al.*, 2002). The increased cell death was attributed to the enhanced turnover of PC at 37 ^{o}C via deacylation (Dowd *et al.*, 2001). Even in the presence of choline, *cho2opi3* cells grow very slowly at 37 ^{o}C compared to wild type cells (our unpublished data; (McGraw and Henry, 1989)), most likely due to increased PC turnover.

In the absence of choline, the PC content of the *cho2opi3* cells was halved with each division cycle, while the total phospholipid content of the cells remained constant. Under these conditions, the remaining PC pool was turned over with an apparent $t_{1/2}$ in the order of 10 h to yield PC with a more saturated acyl chain composition (Fig. 5). Compared to the half-life values of PC in yeast at 30 ^{o}C determined in radiolabeling experiments (Patton-Vogt *et al.*, 1997; Dowd *et al.*, 2001), the rate of PC turnover in the *cho2opi3* cells may seem fast. However, it should be realized that the occurrence of acyl chain exchange could not be detected in these studies, as the isotope labels were present in the glycerophosphocholine moiety of PC.

The acyl chain remodeling of PC could be due to several metabolic conversions. The resemblance of the species profiles of newly synthesized and existing PC pools after PC depletion (Fig. 6) suggests recycling of (phospho)choline derived from PC degradation to PC via the CDP-choline pathway. In this scenario, the yeast *SPO14* (*PLD1*) gene product that is known to hydrolyze PC (Waksman *et al.*, 1996) was shown not to be required (Fig. 7). Involvement of the only other, Ca^{2+}-dependent PLD activity in yeast that so far escaped identification at the gene level, is unlikely because this enzyme has a strong substrate preference for PE over PC (Mayr *et al.*, 1996; Waksman *et al.*, 1997). In the absence of any known PC-degrading PLC activity in yeast, this leaves the possibility of PC deacylation by B type phospholipases, followed by degradation of glycerophosphocholine to choline mediated by Gde1p (Fisher *et al.*, 2005; Fernandez-Murray and McMaster, 2005). Candidate PLBs capable of degrading PC include Plb1p (Lee *et al.*, 1994), Plb2p (Merkel *et al.*, 1999), and Nte1p (Zaccheo *et al.*, 2004). In the alternative scenario, PC could be remodeled independently of the CDP-choline pathway by acyl chain exchange via de- and re-

acylation, or transacylation reactions. The occurrence of PC remodeling by acyl chain exchange was recently demonstrated in live yeast cells (Boumann *et al.*, 2003).

The change in the PC species profile was found to reflect a global shift in the composition of the fatty acid content of the cells. To our knowledge this is the first report of an isothermal shift in acyl chain composition in yeast with an intact fatty acid synthesis machinery, and cultured aerobically in the absence of any fatty acid supplements. As the PC content of the cells decreased, the ratios of C16 over C18 acyl chains and that of saturated over unsaturated acyl chains increased. The changes were larger in the phospholipid than in the neutral lipid fraction, and were most pronounced in PE that becomes the most abundant phospholipid during PC depletion.

Changes in acyl chain composition may serve to adjust membrane fluidity, membrane thickness, or intrinsic membrane curvature. The combination of shortening and increased saturation of acyl chains is not compatible with unidirectional changes in membrane fluidity or thickness. However, it is consistent with regulation of intrinsic membrane curvature maintaining the balance between bilayer and non-bilayer lipids. Since bilayer lipids have a different intrinsic radius of curvature as compared to non-bilayer lipids, changes in their relative proportions affect membrane properties, in particular the membrane's intrinsic curvature (Gruner, 1985). Whereas PC is considered a bilayer-forming lipid, PE can form non-bilayer structures under physiological conditions (Cullis and de Kruijff, 1979). The changes in the acyl chain composition of PE were found to increase the bilayer to hexagonal H_{II} phase transition temperature of this type II phospholipid (Fig. 11), reflecting a reduction of the contribution of the PE molecules to negative membrane curvature. The increase in transition temperature is in agreement with physico-chemical studies showing that the transition temperature from liquid crystalline to hexagonal H_{II} phase increases as

the acyl chains of PE become shorter and, more drastically, as the acyl chains become saturated (Koynova and Caffrey, 1994). The results provide evidence for a novel regulatory mechanism enabling yeast to counteract the increased negative membrane curvature stress conferred by increasing PE levels by reducing the propensity of the PE molecules to adopt the H_{II} phase.

Polymorphic regulation of membrane lipid composition in response to changes in growth conditions is well established in prokaryotes (reviewed in (Dowhan, 1997)). Comparably to the response of yeast to rising PE levels, *E.coli* with a PE content of 70 % of total glycerophospholipids, adapts to rising growth temperatures by a shortening and increased saturation of its acyl chains (Morein *et al.*, 1996). The purpose of polymorphic regulation of membrane lipid composition is to maintain the intrinsic membrane curvature within certain limits in order to provide an optimum environment for the functioning of membrane proteins and to facilitate dynamic membrane processes such as fusion and fission (van den Brink-van der Laan *et al.*, 2004). In this context, it should be noted that reduction of the PE content in yeast is deleterious for growth, particularly on non-fermentable carbon sources (Birner *et al.*, 2001).

Interestingly, the results from earlier studies in yeast, in which phospholipid class compositions, the PE/PC ratios in particular, were found to change in response to fatty acid supplements in the culture medium, support the concept of yeast regulating the relative proportions of bilayer and non-bilayer lipids within a certain window. A strong increase in PE content at the expense of PC was observed when wild type cells were cultured in the presence of short chain C14:1 (Schneiter *et al.*, 2000), in line with the H_{II} phase propensity of PE declining with decreasing acyl chain length. A yeast fatty acid auxotroph cultured in the presence of *trans* C16:1 was found to have

an increased PE/PC ratio as compared to the same strain cultured with *cis* C16:1 (Tung *et al.*, 1991), consistent with the bilayer to H_{II} phase transition temperatures for PEs with *trans* unsaturated acyl chains being higher than for PEs with *cis* unsaturated acyl chains. Similarly, polymorphic regulation of membrane lipid composition may explain why yeast cells cultured in the presence of C18:2 have an almost 3-fold lower PE/PC ratio than cells cultured in the presence of C16:0 (Mizoguchi and Hara, 1997).

The model eukaryote *S. cerevisiae* is very tolerant with regard to its membrane lipid composition. Of the major membrane phospholipids, PI (Nikawa *et al.*, 1987) and PE (Birner *et al.*, 2001; Storey *et al.*, 2001) are essential in yeast, whereas PS (Atkinson *et al.*, 1980) and cardiolipin (Jiang *et al.*, 1997) are not. Contrary to the situation in mammals (Waite and Vance, 2004), PC is not essential in yeast either. In *cho2opi3* cells, PC could be replaced by PDME, its precursor in the PE methylation pathway, provided that the culture medium contained inositol, substrate for the synthesis of PI (Fig. 2; (McGraw and Henry, 1989)). In contrast PMME failed to compensate for the loss of PC irrespective of the presence of inositol. These findings lend further support to the importance of balancing bilayer and non-bilayer lipids in yeast membranes, given that the propensities of PMME and PDME to adopt non-bilayer structures are intermediate between those of PE and PC (Gagne *et al.*, 1985), and that PI is a bilayer-forming phospholipid (Nayar *et al.*, 1982). PDME in combination with an increased PI level may compensate for the increased membrane curvature stress resulting from the loss of PC, whereas PDME by itself, or PMME with or without extra PI, do not. A recent study demonstrated that the non-natural, non-bilayer forming phospholipid phosphatidylpropanolamine could substitute for the methylated phospholipids in a *cho2opi3* mutant altogether (Choi *et al.*, 2004). In line with the proposed polymorphic regulation, the lipid composition of cells cultured in

the presence of propanolamine revealed a strongly increased PI level, and a shift to shorter acyl chains in PE as compared to the choline-grown cells, however, the degree of PE saturation was not affected.

How does yeast accomplish the adaptations in acyl chain composition in response to PC depletion? Shortening of the acyl chains could result from an earlier release of newly synthesized fatty acids from the fatty acid synthase, and/or by reduced activity of the elongases, Elo1p and/or Elo2p that reportedly have the ability to elongate C16-acyl-CoA to C18-acyl-CoA (Schneiter *et al.*, 2000; Rossler *et al.*, 2003). The decreased unsaturation must be due to a reduction of the activity of Ole1p, the only fatty acid desaturase in yeast. Interestingly, the reduction in Ole1p activity is accompanied by an increase in the level of *OLE1* mRNA (Fig. 10). This reduced Ole1p enzyme activity could be a consequence of the changing lipid composition and may reflect a mechanism by which the *OLE1* transcript level is upregulated to compensate for the lower desaturase activity. *OLE1* has been previously shown to be regulated by a complex set physiological and nutrient factors (including molecular oxygen, nutrient fatty acids, cobalt, and iron chelators) at the levels of transcription and mRNA stability (Stukey *et al*, 1990; Hoppe *et al*., 2000; Vasconcelles *et al.*, Chellappa *et al*., 2001; Kandasamy *et al*., 2004). While we speculate that the *OLE1* transcript levels are upregulated in phosphatidylcholine deprived cells to compensate for the lower desaturase activity, it is not clear whether the increased mRNA levels result from an increase in the relative rate of *OLE1* transcription or through a combination of transcriptional and mRNA stability controls.

Superimposed on the changes in fatty acid synthesis, regulatory mechanisms must be in place to afford the lipid class specific adaptations in acyl chain composition. Somehow the changing membrane lipid composition must be conveyed to the fatty

acid and lipid synthesizing machinery. The membrane-associated enzymes involved may directly sense and respond to changes in membrane curvature stress or in the membrane lateral pressure profile (Attard *et al.*, 2000; van den Brink-van der Laan *et al.*, 2004). Alternatively, sensor proteins in the membrane may transmit signals affecting lipid synthesis, as has been proposed for Spt23p (Hoppe *et al.*, 2000; Chellappa *et al.*, 2001). Whether or not Spt23p or its homologue Mga2p, known activators of Ole1 transcription (Hoppe *et al.*, 2000), are involved in sensing and transmitting changes in membrane curvature stress will be subject of future research. It is of interest to consider the effect of varying the growth temperature on the acyl chain composition of yeast. As the growth temperature is decreased from 37 ^{o}C to 10 ^{o}C, the average acyl chain length decreases while the degree of unsaturation is unaffected (Suutari *et al.*, 1990). In response to heat shock, yeast slightly decreases the degree of acyl chain unsaturation (Swan and Watson, 1997). The sensing mechanism(s) and regulatory network(s) underlying these adaptations may in part overlap with those responding to PC depletion.

In conclusion, depletion of PC in yeast unveiled changes in acyl chain composition that are fully consistent with regulation of intrinsic membrane curvature, which so far has only been documented in prokaryotes. Since PC depletion is unlikely to occur in yeast in its natural habitat, we speculate that the polymorphic regulation of membrane lipid composition is a reflection of a versatile mechanism to maintain optimal membrane function in changing environments. Choline deprivation of *cho2opi3* cells provides a promising experimental system for identifying the factors involved in regulating and accomplishing the adaptations in acyl chain composition.

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Legends

Figure 1. The two biosynthetic pathways leading to PC in yeast with the enzymes indicated.

Figure 2. Growth (A) and overproduction of inositol (Opi$^-$) phenotypes (B) of the *cho2opi3* mutant compared to wild type. (A) Cells cultured to mid-log phase in YPD medium were spotted as serial dilutions on SD-glucose plates containing 0 or 75 μM inositol and the supplements indicated at a concentration of 1 mM, and incubated at 30^{o}C for 3 days. (B) Strains were patched on inositol-free SD-glucose agar plates containing the supplements indicated at 1 mM. Excretion of inositol results in growth of the inositol auxotrophic tester strain, as observed by a red halo around the patch.

Figure 3. The effect of choline deprivation on the growth and viability of *cho2opi3* cells cultured in liquid medium at 30^{o}C. (A) Growth of *cho2opi3* upon transfer of mid-log cells from synthetic complete lactate medium containing 1 mM choline to synthetic complete lactate medium with (□) or without 1 mM choline (■). Growth rates were monitored by optical density at 600 nm (OD_{600}). (B) Viability of *cho2opi3* cells after transfer of mid-log cells from lactate- (□) or glucose-based (Δ) synthetic defined medium containing 1 mM choline to the corresponding medium with (open) or without 1 mM choline (solid). Averaged values from 2 independent experiments are shown with the error bars representing the variation. For experimental details see the Methods section.

Figure 4. The PC content of *cho2opi3* cells in choline deficient medium is halved with every doubling of the cells, until growth ceases at a PC content below 2% of total phospholipids. Mid-log *cho2opi3* cells (OD_{600} 0.4) cultured in synthetic complete lactate medium containing 1 mM choline were harvested, washed, and used to inoculate fresh synthetic complete lactate medium with (right panel) or without 1 mM choline (left panel) to an OD_{600} of 0.02 at time zero. At the indicated times, samples were analyzed for phospholipid composition by steady state labeling with [^{32}P] orthophosphate as detailed in the experimental section. The contents of the four most abundant phospholipid classes are shown on a logarithmic scale (left y-axis) as percentages of the total label incorporated into glycerophospholipids, and together account for more than 95% of the incorporated label. Growth rates (Δ, dashed lines) were monitored in parallel by the OD_{600} (right y-axis).

Figure 5. During PC depletion in *cho2opi3* cells, the acyl chain profile of the PC remaining is rearranged. Mid-log *cho2opi3* cells were transferred from choline containing medium to medium with or without 1 mM choline as indicated. At the indicated times, samples were subjected to lipid extraction, and analyzed for PC species composition by ESI-MS/MS in the parent ion scan mode (*m/z* 184). The time dependent changes in the PC acyl chain profile are shown for the four most abundant PC species as percentages of the total PC pool. Under the conditions tested, 32:2, 32:1, 34:2, and 34:1 account for at least 90% of total PC. Data are averaged from 3 independent experiments; error bars represent the standard deviation ($n \geq 3$).

Figure 6. After depriving *cho2opi3* cells of choline for 16 h, the acyl chain profile of newly synthesized PC (black columns) strongly resembles that of the remaining PC

pool (white columns). D_{13}-choline was added to a concentration of 0.2 mM, to *cho2opi3* cells cultured without choline for 16 h. After 10 min cells were harvested, and total lipid extracts prepared. The PC species profiles were analyzed by ESI-MS/MS in the parent ion scan mode at *m/z* 197 for newly synthesized D_{13}-PC, and at *m/z* 184 for unlabeled PC. For comparison, *cho2opi3* cells grown in the presence of 1 mM choline for 16 h were pulse labeled for 10 min with 0.2 mM D_{13}-choline after transfer to choline-free medium and analyzed for PC species profiles (lower panel). The relative abundances of the 4 major PC species are shown as percentages of total PC, averaged from 2 independent experiments with the error bars representing the variation.

Figure 7. PC depletion in *cho2opi3spo14* cells reveals that PC turnover mediated by Spo14p (Pld1p) is not required for the changes in the PC species profile occurring during PC depletion. The distribution of the 4 major PC species of *cho2opi3spo14* cells after 24 hours of culturing in the absence or presence of choline is shown. Experimental conditions were as described in the legend of Fig. 5.

Figure 8. PC depletion in *cho2opi3* cells is accompanied by shortening and increasing saturation of the acyl chains of PE. Experimental conditions were as described in the legend of Fig. 5. The PE species composition was analyzed by ESI-MS/MS in the neutral loss mode (*m/z* 141). The time dependent changes in the PE acyl chain profile are shown for the four most abundant PE species as percentages of the total PE pool. Under the conditions tested, 32:2, 32:1, 34:2, and 34:1 account for at least 94% of total PE. Data are averaged from 3 independent experiments; the error bars represent the standard deviation ($n \geq 3$).

Figure 9. The increase in lipid acyl chain saturation and the shortening of the acyl chains accompanying PC depletion in *cho2opi3* cells are most pronounced in PE. (A) The ratios of unsaturated to saturated acyl chains (UFA/SFA) and (B) the ratios of 16-carbon-atom-chain-length to 18-carbon-atom-chain-length (C16/C18) were calculated for the neutral lipid (NL) and the phospholipid fractions (PL), and for PE, isolated from *cho2opi3* cells after 20 h (NL, PL; data taken from table 1) and 17 h (PE; data from Fig. 8) of growth in the presence or absence of 1 mM choline. Ratios are averaged from 3 independent experiments with the error bars representing the standard deviation.

Figure 10. Northern blot analysis reveals an increased level of *OLE1* mRNA under conditions of PC depletion. A Northern blot of total RNA isolated from the *cho2opi3* strain cultured in the presence or absence of 1 mM choline for the times indicated, was probed with *OLE1* and *PGK1* specific probes. Phosphorimaging data was normalized using the *PGK1* transcript as an internal standard, yielding values of the relative abundance of the *OLE1* transcript that were averaged from 2 independent experiments with the errors representing the variation.

Figure 11. PE extracted from choline-deprived *cho2opi3* cells exhibits a higher bilayer to hexagonal H_{II} phase transition temperature than PE from *cho2opi3* cells cultured in the presence of choline. ^{31}P-NMR spectra of dispersions in buffer of PE purified from *cho2opi3* cells cultured for 16 h in the presence or absence of choline, were recorded at increasing temperatures as indicated, and as detailed in the experimental section.

Table 1: Acyl chain composition (mol%) of a total lipid extract (TLE), and of the phospholipid and neutral lipid fractions of *cho2opi3* cells after 20 h of growth in the presence or absence of 1 mM choline

fatty acid	TLE		phospholipids		neutral lipids	
	+ choline	- choline	+ choline	- choline	+ choline	- choline
C12:0	1.2 ± 0.1	1.0 ± 0.1	1.9 ± 0.4	1.0 ± 0.1	2.6 ± 0.4	1.5 ± 0.3
C14:0	1.1 ± 0.1	1.7 ± 0.1	1.1 ± 0.1	1.4 ± 0.3	2.1 ± 0.6	2.3 ± 0.5
C16:0	10.6 ± 0.3	18.3 ± 0.4	9.6 ± 0.3	24.3 ± 0.6	12.8 ± 0.7	16.1 ± 0.4
C16:1	52.5 ± 0.4	51.8 ± 0.5	51.2 ± 1.1	45.5 ± 1.3	47.4 ± 0.5	52.7 ± 0.8
C18:0	4.0 ± 0.1	5.7 ± 0.2	3.0 ± 0.2	6.4 ± 0.5	5.9 ± 0.3	6.0 ± 0.2
C18:1	30.5 ± 0.3	21.5 ± 0.4	33.2 ± 1.4	21.4 ± 0.2	29.3 ± 1.3	21.4 ± 0.6
% unsaturated	83	73	84	67	77	74
% C16	63	70	61	70	60	69

Values represent means ± standard deviation of 3 independent experiments. For experimental details, see Materials and Methods.

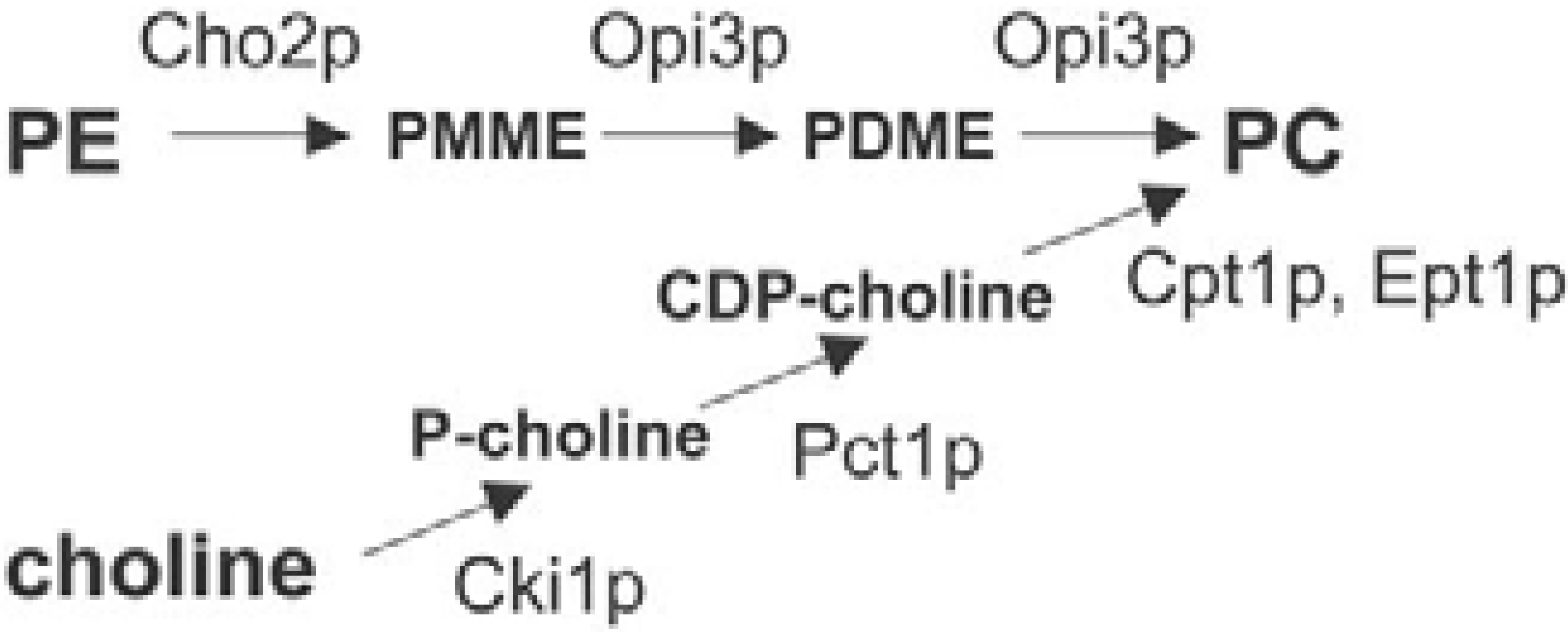
Cho2p
Opi3p
Opi3p
PE
PMME
PDME
PC
CDP-choline
Cpt1p, Ept1p
P-choline
Pct1p
choline
Cki1p

Figure 1

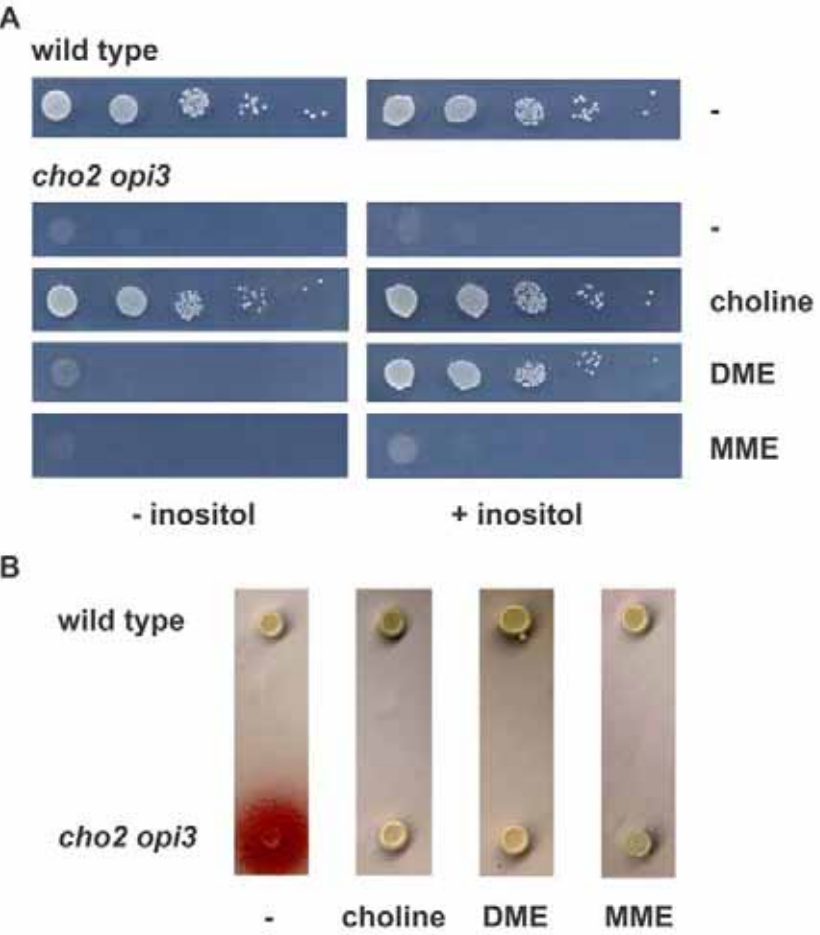
A
wild type
-
cho2 opi3
-
choline
DME
MME
- inositol
+ inositol
B
wild type
cho2 opi3
-
choline
DME
MME

Figure 2

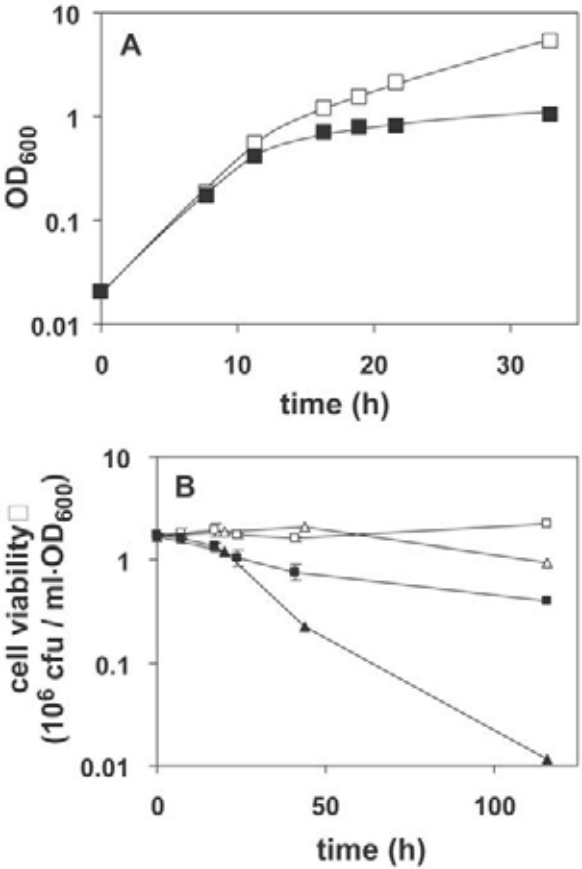
A
OD600
10
1
0.1
0.01
0
10
20
30
time (h)
B
cell viability
(10^6 cfu / ml·OD600)
10
1
0.1
0.01
0
50
100
time (h)

Figure 3

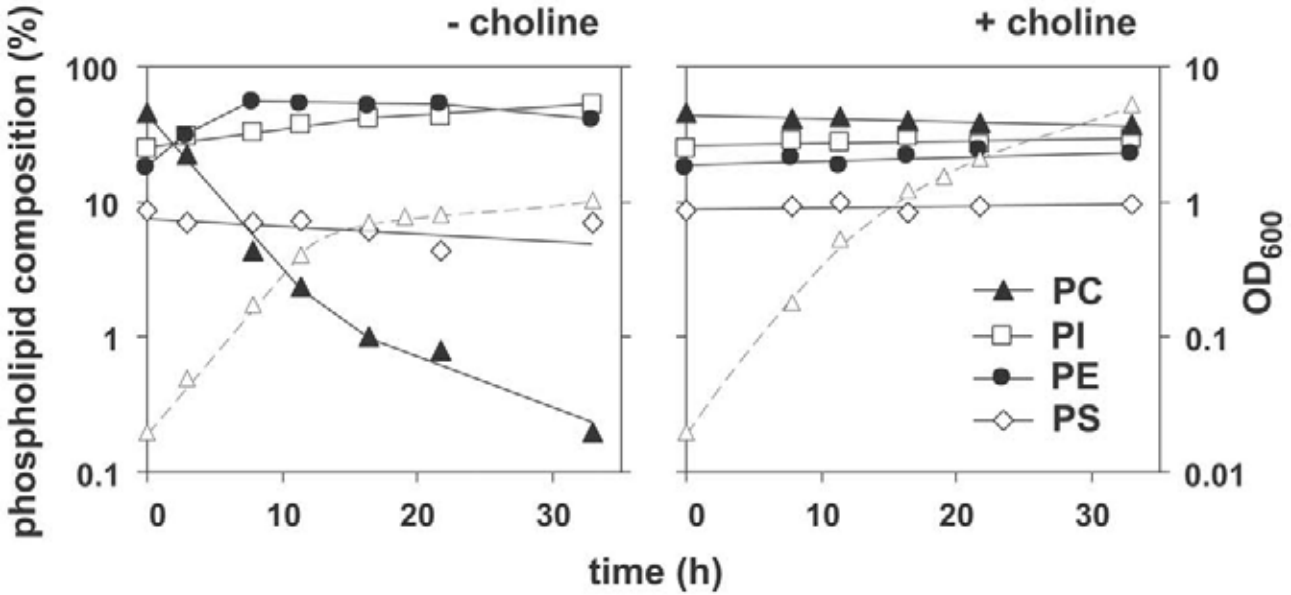
- choline
+ choline
phospholipid composition (%)
100
10
1
0.1
0
10
20
30
time (h)
10
1
0.1
0.01
OD600
PC
PI
PE
PS

Figure 4

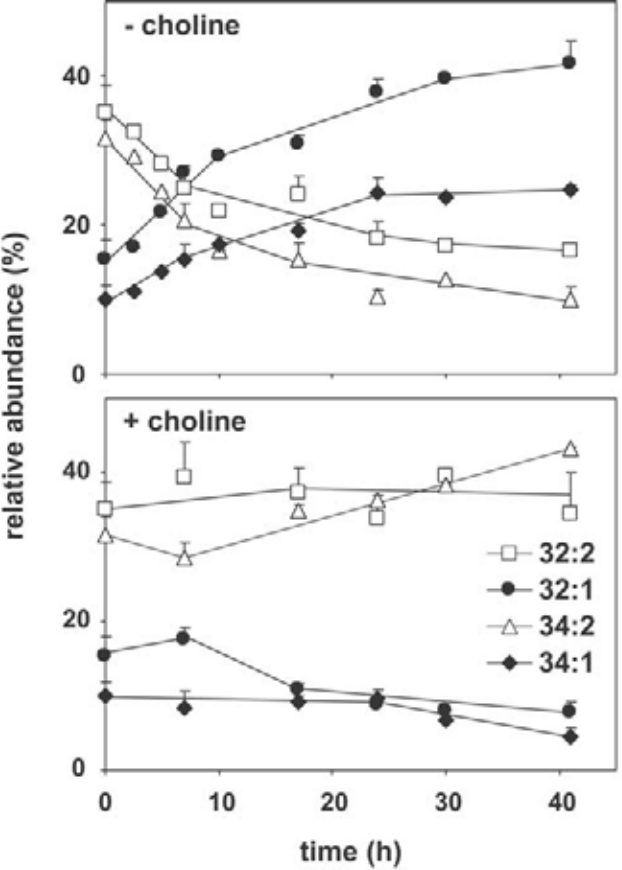
- choline
+ choline
relative abundance (%)
time (h)
32:2
32:1
34:2
34:1
0
20
40
0
10
20
30
40

Figure 5

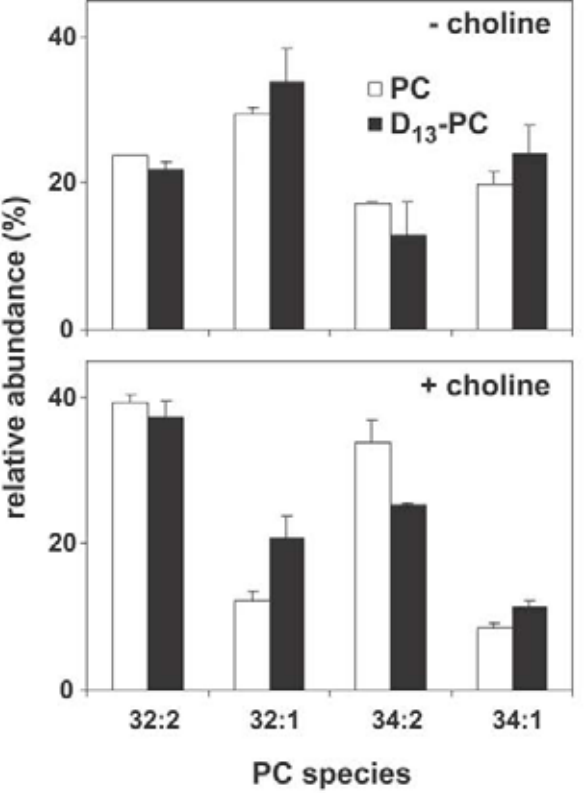
- choline
PC
D13-PC
40
20
0
+ choline
40
20
0
relative abundance (%)
32:2
32:1
34:2
34:1
PC species

Figure 6

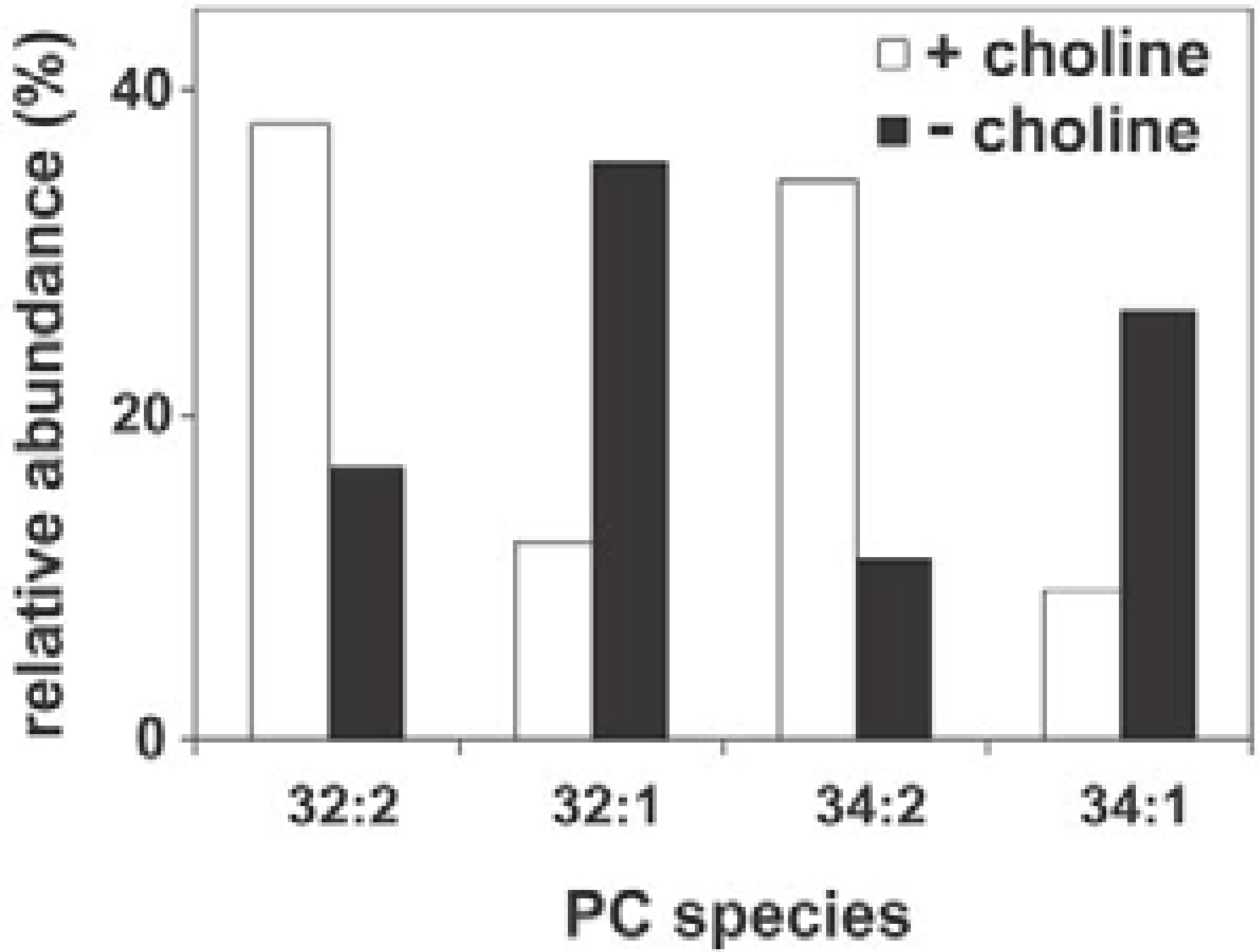
relative abundance (%)
40
20
0
+ choline
- choline
32:2
32:1
34:2
34:1
PC species

Figure 7

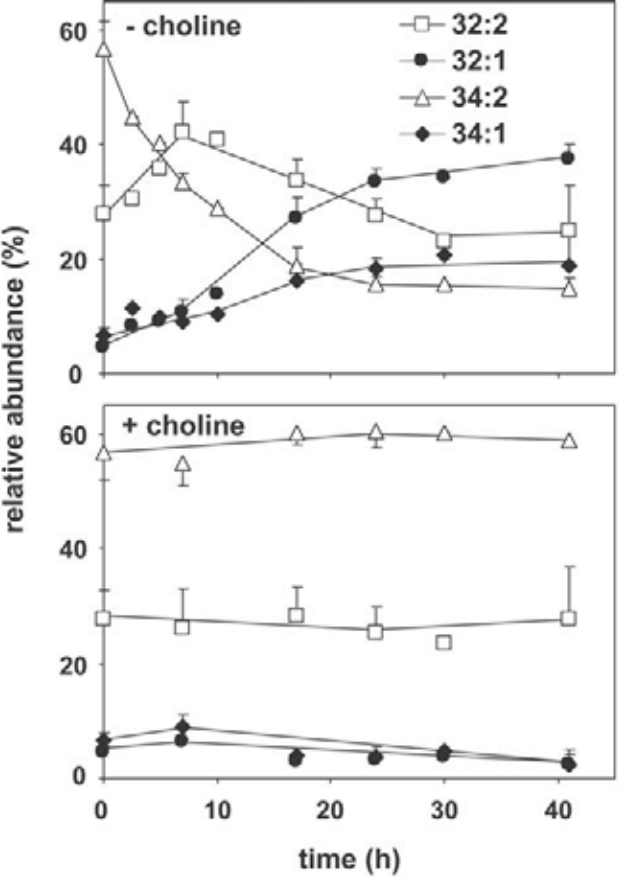
- choline
32:2
32:1
34:2
34:1
+ choline
relative abundance (%)
time (h)
0
20
40
60
0
10
20
30
40

Figure 8

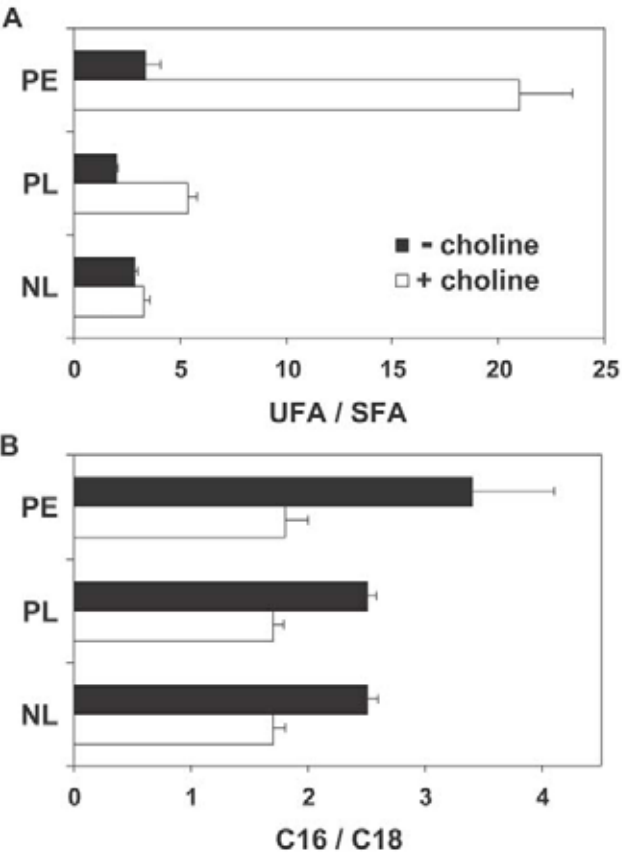
A
PE
PL
NL
- choline
+ choline
0
5
10
15
20
25
UFA / SFA
B
PE
PL
NL
0
1
2
3
4
C16 / C18

Figure 9

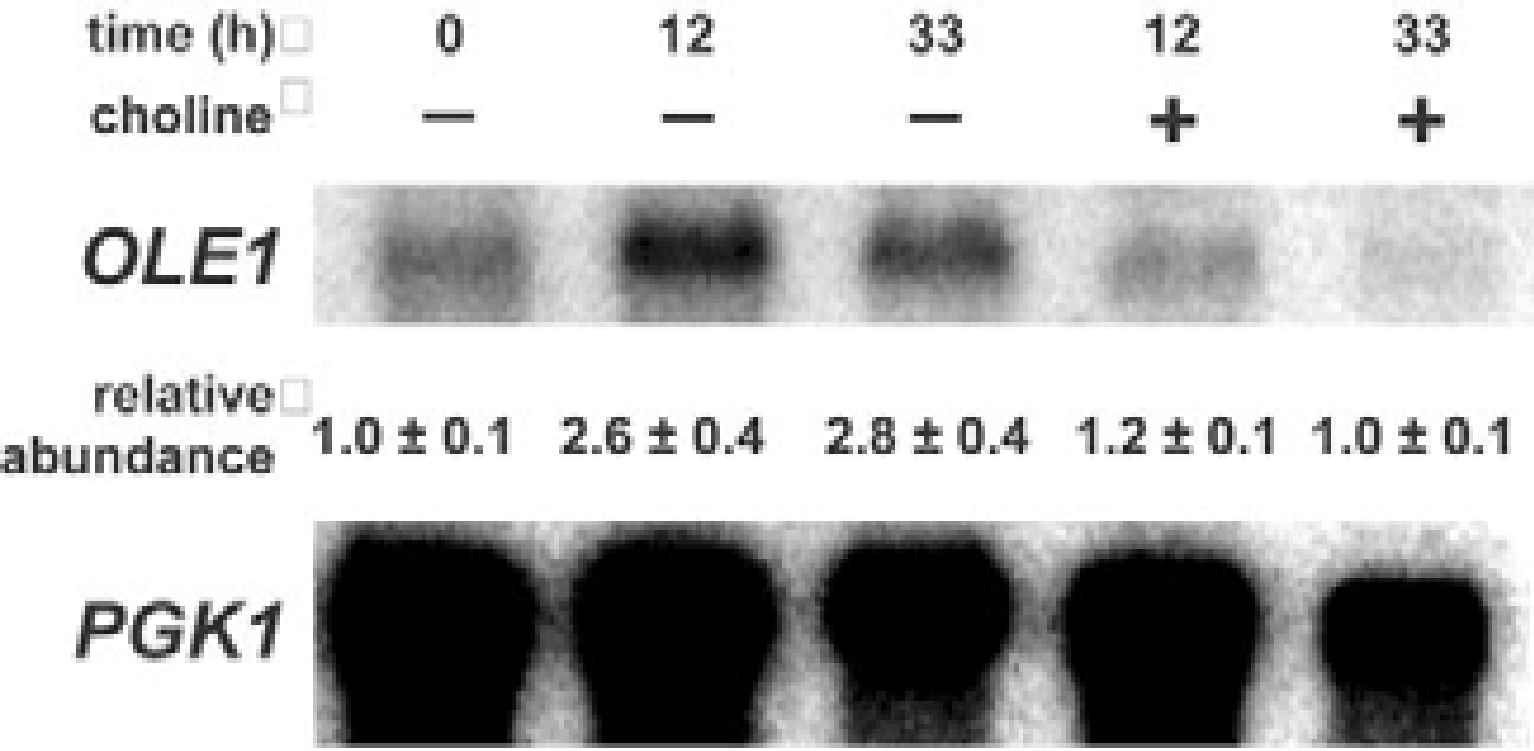
time (h)
0
12
33
12
33
choline
—
—
—
+
+
OLE1
relative abundance
1.0 ± 0.1
2.6 ± 0.4
2.8 ± 0.4
1.2 ± 0.1
1.0 ± 0.1
PGK1

Figure 10

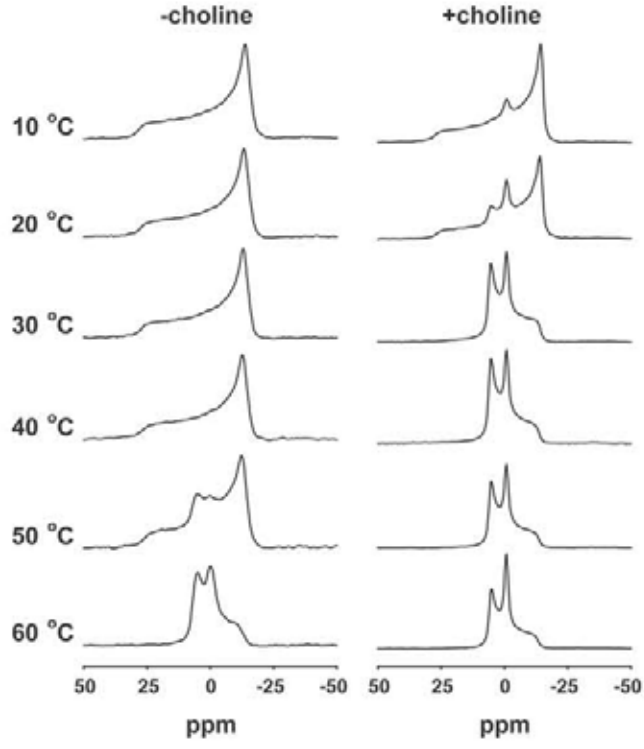

Figure 11