The addition of inositol to actively growing yeast cultures causes a rapid increase in the rate of synthesis of phosphatidylinositol and, simultaneously, triggers changes in the expression of hundreds of genes. We now demonstrate that the addition of inositol to yeast cells growing in the presence of choline leads to a dramatic reprogramming of cellular lipid synthesis and turnover. The response to inositol includes a five- to six-fold increase in cellular phosphatidylinositol content within a period of 30 minutes. The increase in phosphatidylinositol content appears to be dependent upon fatty acid synthesis. Phosphatidylcholine turnover increased rapidly following inositol addition, a response which requires participation of Nte1p, an endoplasmic reticulum-localized phospholipase B. Mass spectrometry revealed that the acyl species composition of phosphatidylinositol is relatively constant regardless of supplementation with inositol or choline. Whereas, phosphatidylcholine acyl species composition is influenced by both inositol and choline. In medium containing inositol, but lacking choline, high levels dimyristoylphosphatidylcholine were detected. Within 60 minutes following addition of inositol, dimyristoylphosphatidylcholine levels had decreased from approximately 40% of total phosphatidylcholine to a basal level of less than 5%.

nte1Δ cells grown in the absence of inositol and in the presence of choline exhibited lower levels of dimyristoylphosphatidylcholine than wild type cells grown under these same conditions, but these levels remained largely constant after addition of inositol. These results are discussed in relationship to transcripational regulation known to be linked to lipid metabolism in yeast.

The addition of the phospholipid precursor, inositol, to the growth medium of yeast leads to increased phosphatidylinositol (PI) synthesis and increased consumption of the immediate precursors of PI, phosphatidic acid (PA) and CDP-diacylglycerol (CDP-DAG) (Fig 1) (1,2). Recent genome-wide analyses revealed that growth in the presence of inositol also affects the steady-state transcript abundance of over a hundred genes (3,4). Many of these genes encode enzymes involved in lipid metabolism and contain the conserved UASINO element in their promoters. INO1, which encodes inositol-3-phosphate synthase, is the most highly regulated of the UASINO-containing genes (5). INO1 is repressed some 50-fold if inositol is present and 150-fold, if choline, precursor to phosphatidylcholine (PC) (Fig 1) is present in addition to inositol (4). Activation of INO1 and other UASINO-containing genes requires the Ino2p and Ino4p transcription factors which bind as a heterodimer directly to UASINO (6-8).

In certain genetic backgrounds, increased phospholipase D mediated turnover of PC also results in derepression of the INO1 gene (9,10). Likewise, mutations affecting the synthesis of PC via methylation of phosphatidylethanolamine (PE) (Fig 1) result in abnormal patterns of phospholipid accumulation and misregulation of UASINO-containing genes (11-13). These and related observations led to the hypothesis that a signal, or signals, generated by ongoing lipid metabolism are involved in the transcriptional regulation of UASINO-containing genes (14). Recently, the mechanism by which a signal from lipid metabolism is sensed, and subsequently influences the expression of the INO1 gene was elucidated. The negative regulator, Opi1p, required for repression of the UASINO-containing
genes (15,16), was shown to reside in the endoplasmic reticulum (ER) as a part of a protein complex which also contains the membrane-spanning protein Scs2p (17,18). Opi1p interacts with Scs2p via a domain called FFAT and this interaction is required for retention of Opi1p in the ER (2,18). However, Opi1p also binds PA which accumulates as an intermediate in the absence of inositol (2) and this binding is required in addition to the interaction with Scs2p in order for Opi1p to remain in the ER. Upon the addition of inositol, PA levels in the ER drop as a consequence of increased PI synthesis, resulting in the release of Opi1p from the ER, and its subsequent translocation to the nucleus where it acts to repress INO1 and co-regulated genes (2).

Analysis of the kinetics of the transcriptional responses to inositol genome wide revealed that over 700 genes show a significant change in expression in at least one time point over a two-hour time course following addition of inositol to medium already containing choline. Many of these changes occur in the first 15 to 30 minutes following inositol addition. The transcript abundance of many of these genes is affected only transiently, returning to basal levels within 60 to 120 minutes after inositol addition. This explains, in part, the fact that larger numbers of genes were detected in the kinetic analysis of the response to inositol (19), in comparison to steady state analysis of transcript abundance (3,4). While many of the genes regulated in response to inositol contain the UASINO element, the majority of the genes that respond to inositol and choline fall into other categories, including those that respond to the Unfolded Protein Response (UPR) pathway and the Low Oxygen Response Element (LORE) (4,19).

We now report on an analysis of phospholipid biosynthesis and turnover employing a time course and growth conditions identical that permits direct comparison of changes in potential signaling molecules produced in the course of lipid metabolism to the kinetics of the transcriptional response to inositol reported by Jesch, et al. (19). We report that the addition of inositol to the growth medium induces not only a rapid increase in the rate of synthesis of PI and an almost equally rapid increase in the total cellular content of PI, but also a concurrent decline in PC content brought about by the combined effects of increased turnover and diminished synthesis. We also show that the increased turnover of PC in response to inositol requires Nte1p, a phospholipase B that resides in the ER (20) and has previously shown to respond to the presence of choline and increased growth temperature (21,22). The relationships among these changes in lipid metabolism and the correlated changes in transcription will be discussed.

Experimental Procedures

Strains and media. Saccharomyces cerevisiae strains: “wild type” BY4742 (MATa his3Δ1, leu2Δ0, lys2Δ0, ura3Δ0) and nte1Δ (MATα nte1::KanMX, his3Δ1, leu2Δ0, lys2Δ0, ura3Δ0) are derived from the S288C genetic background (23) and were purchased from Research Genetics. Cultures were maintained on YEPD (1% yeast extract, 2% peptone, 2% glucose, 2% agar) medium plates. All experiments were conducted using cultures grown to mid-logarithmic phase at 30°C on a rotary shaker (New Brunswick Scientific Co., Inc.) at 200 rpm using chemically defined synthetic media, as described by Jesch, et al. (4), containing 1 g of potassium phosphate per liter. Cells were grown in 50 mL batches of complete synthetic media with (I’) or without (I) inositol (75 μM) with (C’) or without (C) choline (1 mM) as indicated.

Materials. All chemicals were reagent grade. Phospholipid standards were purchased from Avanti Polar Lipids, Inc. Neutral lipid standards and cerulenin were purchased from Sigma. Dimyristoyl phosphatidylcholine (DMPC) was purchased from Avanti Polar Lipids, Inc. Radiochemicals were purchased from Amersham, and scintillation-counting supplies were purchased from National Diagnostics. Silica gel-loaded SG81 chromatography paper was purchased from Whatman, Inc., and HPTLC plates from Merck. Growth medium supplies were purchased from Difco Laboratories.

Electrospray ionization mass spectrometry analysis of phospholipids. Electrospray ionization tandem mass spectrometry (ESI-MS/MS) analysis of phospholipid mixtures was carried out utilizing a Bruker Esquire LC Mass Spectrometer (Hewlett-Packard, Inc.) equipped with a standard orthogonal electrospray source and a three-dimensional ion trap. The samples were introduced into the mass spectrometer at a rate of infusion of 120 μL/hr. The
capillary voltage was set at +3500 or –3500 volts for positive or negative modes respectively.

Phospholipid samples for mass spectrometry were isolated from 3L cultures (in duplicate) grown to mid-logarithmic phase in the specified synthetic media (i.e., no inositol or choline, inositol alone, no inositol with choline, and both choline and inositol) at 30°C. In general, cells were harvested by rapid filtration through Durapore® membrane filters (0.65µm DVPP, Millipore, Inc.), and immediately transferred to an Erlenmeyer flask containing a 45 mM aqueous solution of potassium cyanide. The cells were then centrifuged at 5000 rpm at 4°C for 15 min, the cell pellet was washed twice with water and lyzed with 10% trichloroacetic acid (TCA) in an ice bath for 25 min. The cell membranes were spun down and the supernatant discarded. The cell membrane phospholipids were extracted with a solvent mixture consisting of ethanol, water, ethyl ether, pyridine, and ammonium hydroxide (45:45:15:3:0.003, v/v/v/v/v). The extract was centrifuged and the supernatant recovered and partitioned twice with 50 mL of a chloroform and methanol (2:1, v/v) mixture and 5 mL of a saturated aqueous sodium chloride solution in a separatory funnel. The lower organic phase was collected, pooled, and taken to dryness with a gentle stream of N2. Phospholipid extracts isolated from S. cerevisiae cells were retaken in a mixture of chloroform and methanol (1:2, v/v). Samples for analysis in the negative mode were added 2.5% of triethylamine, or left untreated for analysis in the positive mode (chemical species were detected as sodiated ions).

**Mass spectrometry determination of PI and PC species composition at steady-state, and turnover following inositol addition.** Wild type cultures were grown overnight under four different growth conditions: no inositol or choline (I’C’), inositol alone (I’C”), no inositol with choline (I’C”), and both choline and inositol (I’C”). For steady-state studies, 3L cultures of wild type cells were grown at 30°C for eight generations and rapidly harvested and processed for phospholipid analysis by mass spectrometry as described above. For turnover studies of PI and PC species following inositol addition, 3L cultures of wild type or nte1Δ cells were grown in I’C” medium to mid-logarithmic phase. Twenty-one mL of a 10 mM inositol solution were added, and the cells were allowed to continue their growth for 0, 5, 15, 30, and 60 min before they were harvested and processed for PI and PC species composition analysis by electrospray ionization mass spectrometry as described above.

**Analysis of fatty acid composition.** Total fatty acid analysis was carried out in a Hewlett Packard 5890 gas chromatograph (splitless mode) coupled to an HP 5970 B Mass Selective Detector (DB-1 capillary column, 30 x 0.25 mm ID, 0.25 mm film thickness, J & W Scientific, Folsom, CA). The oven temperature was held at 100°C for 2 min., raised at 10°C/min to 200°C, held for 10 min, and raised at 3°C/min to 300°C.

Fatty acid samples for gas chromatography analysis were isolated from 50 mL yeast cell cultures (in duplicate) grown to mid-logarithmic phase in the specified synthetic media (i.e., I’C”, I’C”, I’C”, I’C”’) at 30°C. The cells were spun down, washed twice with water, and transferred to a 1.5 mL Eppendorf tube and lyzed with 1 mL of TCA (10%) for 25 min in an ice bath. The Eppendorf tubes were centrifuged for 5 min at 7,500 rpm and the supernatant discarded. The cells were washed twice with sterile water and extracted with 1 mL of a solvent mixture consisting of ethanol, water, ethyl ether, pyridine, and ammonium hydroxide (45:45:15:3:0.003, v/v/v/v/v). The extract was centrifuged, and the supernatant transferred to a 15 mL glass test tube and partitioned with 1 mL of chloroform/methanol (2:1, v/v) and three drops of a saturated solution of sodium chloride. The lower phase was collected and taken to dryness with a gentle stream of nitrogen. The residue was extracted twice with a total of 1 mL of boron trifluoride in methanol (14%, Sigma Chemical Co.) and transferred to 1.5 mL glass vials, capped, and heated to 100°C in a sand bath for 30 min. The reaction mixture was allowed to cool down to room temperature and extracted with 1 mL of hexane. The hexane extract was collected and washed with water and taken to dryness under a nitrogen stream. Three drops of hexane were added to the residue for analysis by gas chromatography coupled with mass spectrometry.

**Determination of steady-state phospholipid composition by ³²P labeling.** Wild type cultures were grown overnight under the four different growth conditions: I’C”, I’C”, I’C” and I’C”. Cells were labeled to steady-state by the addition of 20 μCi/mL [³²P]-orthophosphate (specific activity of
isoctope was 2.7 mCi/mmmole phosphate). To analyze the steady-state lipid composition, cells were grown at 30°C for eight generations in the presence of the label. Labeling for additional generations produced no change in the percentage distribution of the label into lipid classes (data not shown), indicating that a steady-state labeling condition was achieved. Five mL samples were mixed with 0.5 mL 50% TCA and allowed to stand on ice for 20 min. The samples were washed twice with distilled water. Labeled lipids were extracted as previously described (24). The individual phospholipids species were resolved by two-dimensional paper chromatography (25). Phospholipid identity was based on the mobility of known standards and quantified on a STORM 860 PhosphorImager (Amersham Biosciences).

Kinetic analysis of changes in phospholipid composition following inositol addition. To determine changes in the composition of the phospholipids over a set time course following the addition of inositol, cells were first grown in I-C+ medium and phospholipids were labeled with 20 μCi/mL [³²P]-orthophosphate to steady-state. When the cells reached the mid-logarithmic phase of growth (A₆₀₀=0.5), inositol (75 μM) was added and aliquots were taken at 2, 5, 15, 30, and 60 min. A control sample (0 min) was taken just prior the addition of inositol. Cells were harvested and lipids were extracted and analyzed as described above.

Analysis of phospholipid turnover following inositol addition. To follow the turnover of phospholipids after the addition of inositol, wild type and nte1Δ cells were grown, as described above, to steady-state in the presence of 20 μCi/mL [³²P]-orthophosphate in I'C medium. When the cells reached A₆₀₀=0.5 they were quickly collected by filtration, washed with pre-warmed I'C medium at 30°C and placed in unlabeled fresh I'C medium pre-warmed to 30°C. Samples were taken at 0, 5, 15, 30, and 60 min and following filtration and transfer to unlabeled medium and lipid extraction and analysis was performed as described above. As a control, cells were also transferred to fresh unlabeled I'C medium and samples were taken over the same time course as those in I'C medium.

Short-term labeling of phospholipids. To analyze new synthesis of glycerophospholipids following addition of inositol, wild type, and nte1Δ cells were pre-grown to A₆₀₀=0.5 in I'C medium and 100 μCi/mL [³²P]-orthophosphate (specific activity of isotope was 13.51 mCi/mmmole phosphate) was added. Samples were taken 10 and 20 min following addition of [³²P]-orthophosphate. At 20 min, inositol was added to a final concentration of 75 μM, and cells were sampled at 10 min intervals until 50 min following ³²P introduction (i.e., 30, 40 and 50 min following ³²P introduction). Lipids were extracted and quantified by two-dimensional chromatography, as described above. The design of this experiment is very similar to that reported previously in Loewen et al. (2), with the exception that choline was present in the current experiment and the cells were labeled in the I'C medium for 20 min prior to the addition of inositol (as opposed to 10 min labeling with ³²P in I'C medium prior to inositol addition in Loewen, et al. (2)).

Assessment of neutral lipid composition by [1-¹⁴C] acetate labeling. Wild type cells were grown in four different conditions, as described above (i.e., I'C, I'C, I'C*, I'C*). Cultures were grown at 30°C in the presence of 1μCi/mL of [1-¹⁴C] acetate (specific activity, 57 mCi/mmol) overnight. The next day, cultures were diluted to A₆₀₀=0.1 maintaining label at 1μCi/mL of [1-¹⁴C] acetate and allowed to grow until mid-logarithmic phase (A₆₀₀=0.5). The cells were then harvested using the same methods described above, for [³²P] labeling experiments. Total lipids were extracted with a mixture of chloroform: methanol 2:1 v/v (26). The chloroform phase was dried and the residue was dissolved in chloroform-methanol 1:1 v/v. Steady-state incorporation of label into major neutral lipid classes was determined by thin layer chromatography. Neutral lipids were separated on Whatman Silica Gel 60A HPTLC plates using the solvent system: hexane-diethyl ether-formic acid (80:20:2, v/v/v) (27). Labeled lipids on the chromatograms were quantified on a STORM 860 PhosphorImager (Amersham Biosciences). Metabolite identity was determined by comparison to the mobility of known standards.

Kinetic analysis of changes in neutral lipid composition following inositol addition. To follow changes in the relative composition of the neutral lipids classes following the addition of inositol, wild type cells were labeled with 1μCi/mL of [1-¹⁴C] acetate to steady-state, as described above, in I'C.
medium. A 5 mL sample, which served as time zero control, was taken prior to addition of inositol and then inositol was added to a final concentration of 75 μM and 5 mL of cells were harvested at 2, 5, 15, 30 and 60 min. Neutral lipids were extracted and analyzed as described above for assessment of steady-state neutral lipid composition.

**Short-time course labeling with [3H] acetate.** To analyze new synthesis of both neutral lipids and selective phospholipids in a single experiment following inositol addition, 1 μCi/mL [3H] acetate was added to cultures of wild type and nte1Δ cells grown to mid-logarithmic phase (A600=0.5) in IC- medium. Samples were taken 10, 20 and 30 min following addition of label. At 60 min following the addition of label, inositol was added to a final concentration of 75 μM and samples were collected at 5, 15, 30, and 60 min following addition of inositol. Lipid extraction and analysis was performed, as described above, for the 32P and 14C steady-state labeling. Phospholipids were separated on Whatman Silica Gel 60A HPTLC plates using the solvent system: chloroform: ethyl acetate: acetone: isopropanol: methanol: water: acetic acid (30:6:6:6:16:28:6:2, v/v/v/v/v/v/v) (28). The neutral lipid classes were analyzed on HPTLC plates using hexane: diethylether: formic acid (80:20:2, v/v/v) (27). Metabolite identity was established based on the mobility of known standards. The amounts of labeled lipids on the chromatograms were quantified on a STORM 860 PhosphorImager (Amersham Biosciences).

**Short-time course labeling in the presence of cerulenin.** In order to measure the effects of inhibition of de novo fatty acid synthesis on phospholipid synthesis, wild type cells were grown in IC+ medium at mid-logarithmic phase (A600=0.5) and were exposed to different concentrations of cerulenin, an inhibitor of de novo fatty acid synthesis (29). Protocols similar to those described above for short-term labeling with either 1 μCi/mL [3H] acetate or 100 μCi/mL [32P]-orthophosphate were carried out.

For experiments involving short-term labeling with [3H] acetate, wild type cells were grown at 30°C to A600=0.5 in IC+ or IC- medium and treated with concentrations of cerulenin ranging from 0.625 μg/mL to 10 μg/mL. Label and cerulenin were added simultaneously and samples were taken at 15, 30 and 45 min following the addition of cerulenin. Cells were harvested and phospholipids and neutral lipid classes were extracted and analyzed as described above for 14C steady-state labeling.

For short-term labeling with [32P]-orthophosphate, the procedure described above for short-term labeling of phospholipids with [32P] of wild type cells was followed. At zero time, both [32P] and 10 μg/mL (4.4 x 10^{-5} M) of cerulenin were added simultaneously. Inositol was added to a final concentration of 75 μM to the cultures 20 min after the addition of the label and cerulenin. Samples were collected at the time points indicated.

**Quantitation of transcripts by Northern blotting.** Wild type and nte1Δ cells were grown in liquid IC+ medium to mid-logarithmic growth phase and inositol was added to a final concentration of 75 μM. Cells were harvested by filtration and immediately frozen on dry ice immediately prior to, and 5, 15, 30, and 60 min following the addition of inositol. Total RNA from each time point was isolated according to manufacturer instructions with the RNeasy mini kit (Qiagen, Inc.). 250 ng of total RNA was fractionated on 1.1% glyoxal agarose gels, transferred to positively-charged nylon membrane using turboblotter (30) and probed for INO1, OLE1, KAR2, and ACT1 transcripts using 32P-labeled antisense riboprobes as described (4). Transcript levels were quantitated by PhosphorImager. Values were expressed as a ratio of INO1, OLE1, and KAR2 mRNA to ACT1 mRNA levels.

To evaluate the expression of the INO1 and OLE1 genes when the de novo synthesis of fatty acids is impaired, wild type cells were grown in IC+ medium to mid-logarithmic growth phase and were treated with a final concentration of 10 μg/mL of cerulenin for 20 min prior to addition of inositol. Samples were harvested at 0, 10 and 20 min following addition of cerulenin and at 10 min intervals for 30 min (i.e., 30, 40 and 50 min) following the addition of inositol. Cells were harvested and RNA isolation and Northern blotting were carried out as described above.

**Results**

*The abundance of PI is dramatically influenced by the presence of inositol whether choline is present or not.* Wild type cells grown in the presence of inositol with or without choline (IC+
or I\(^+\)C\(^-\)) were found to have levels of PI that were significantly higher than those observed in cells grown in the absence of inositol (I\(^-\)C\(^-\); I\(^+\)C\(^-\)) (Fig 2A). Likewise, the addition of choline to the growth medium resulted in higher levels of PC, an effect that was more pronounced in cells grown in the absence of inositol (i.e., I\(^-\)C\(^+\); Fig 2A). However, overall increases in PC content in response to choline were two-fold or less as compared to five- to six-fold increases in PI levels in response to inositol. A greater degree of variability in PC content was also observed, culture to culture, as compared to the levels of PI and other phospholipids (compare error bars for PI and PC, Fig 2A). Other phospholipids that showed marked changes in response to inositol were PA and CDP-DAG. The levels of these phospholipids were higher in cells grown in medium lacking inositol, as previously reported (1,2), whether choline was present or not (Fig 2A). The abundance of other phospholipid species examined, such as phosphatidylserine (PS) and PE, did not vary significantly in response to the different growth conditions analyzed (Fig 2A).

The relative levels of the major classes of neutral lipids assessed by steady-state labeling with \(^{14}\)C acetate are shown in Fig 2B. In general, the various classes of neutral lipids showed much less pronounced change in response to inositol and choline supplementation than the various phospholipids (Fig 2A). However, diacylglycerol (DAG) levels were slightly higher in cells grown in medium lacking inositol, as previously reported (1,2), whether choline was present or not (Fig 2A). The abundance of other phospholipid species examined, such as phosphatidylserine (PS) and PE, did not vary significantly in response to the different growth conditions analyzed (Fig 2A).

Inositol stimulates rapid net synthesis of PI and simultaneous rapid reduction in PC content in wild type cells. Wild type cells were grown in the presence of \(^{32}\)P, as described for the steady-state analysis shown in Fig 2B. After the addition of 75 \(\mu\)M inositol, samples were taken over the same time intervals as described in \(^{32}\)P labeling shown in Fig 3. \(^{14}\)C acetate associated with total neutral lipids decreased from approximately 33% to 25% of total lipid associated label within 15 min following inositol addition. Label associated with total phospholipids conversely rose from 67% to 75% of total lipid associated label within 15 min and the proportion of label associated with phospholipid vs. neutral lipid remained constant thereafter. Most neutral lipid classes did not exhibit dramatic changes over the 120 minute time course in response to inositol supplementation (data not shown). However, DAG content was reduced to about half of its original level within 5 minutes following inositol addition and squalene, which is a precursor in the biosynthesis of sterols, experienced a ten-fold decrease in the first 15 min and was undetectable by 60 min (data not shown). Other neutral lipid classes, including FFA, triacylglycerols (TAG), free sterols (FS) and steryl esters (SE) exhibited smaller changes in content over a longer time frame following inositol addition.

Effect of inositol and choline on the species composition of PI and PC at steady-state analyzed by electrospray ionization mass spectrometry. Consistent with the results obtained in \(^{32}\)P steady-state labeling experiments, mass spectrometry analysis revealed a marked increase in the level of total PI species in cells grown in the presence of...
inositol whether choline was present or not in all four combinations (i.e., I\(^{+}\), I\(^{-}\) vs. I\(^{-}\), I\(^{+}\)). The major PI species detected were 32:1 (containing C16:0 and one C16:1 acyl chain), 34:1 (C16:0, C18:1 or C16:1, C18:0) and 36:1 (C18:0, C18:1), which collectively represented 85-90% of total PI. These species were detected at a fairly constant peak height ratio of approximately 4:5:1 in all four growth conditions (i.e., I\(^{+}\)C\(^{+}\), I\(^{+}\)C\(^{-}\), I\(^{-}\)C\(^{+}\), I\(^{-}\)C\(^{-}\)) (Fig 4).

In contrast to the apparent stability of the species for PI ratios observed under the four growth conditions, the relative ratios of PC species were influenced by both inositol and choline (Fig 5A). In cells grown in the absence of inositol and choline (I\(^{-}\)C\(^{-}\)), the two most prominent PC species observed were 32:2 (C16:1, C16:1) and 32:1 in a ratio of approximately 4:5 (Fig 5A). Two additional PC species were detected in cells grown in I\(^{-}\)C\(^{-}\) medium, namely, 34:2 (C16:1, C18:1) and 34:1 in a ratio of 5:4 (Fig 5A). When inositol was present, but not choline (I\(^{+}\)C\(^{-}\)), 32:2 species was favored over the 32:1 species in a 2:1 ratio, while the ratio of 34:2 over 34:1 increased to approximately 4:1. As compared to cells grown in the absence of inositol and choline (I\(^{-}\)C\(^{-}\), cells exposed to inositol alone (I\(^{+}\)C\(^{-}\)), exhibited an overall increase in the degree of unsaturation as well as a shortening of the average length in the acyl chains associated with PC (Fig 5). When cells were grown in the presence of both inositol and choline in the medium (I\(^{+}\)C\(^{+}\)), the predominant PC species present were 32:2 and 32:1 in a 5:3 ratio and 34:2 and 34:1 in a 4:1 ratio (Fig 5A).

In addition to the four PC species described above, a unique species was detected at m/z 701.5 in cells grown in I\(^{+}\)C\(^{+}\) medium (Fig 5A). Based on comparison with the fragmentation pattern of an authentic commercial standard, the fragmentation pattern of this species was consistent with that of 1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC). DMPC constituted between 30% and 40% of the total PC species mixture observed in wild type cells grown in I\(^{+}\)C\(^{+}\) medium (Fig 5A, 5B), but it was present only in small amounts (1-3%) in cells grown in any of the other three conditions (i.e., I\(^{+}\)C\(^{-}\), I\(^{-}\)C\(^{+}\), I\(^{-}\)C\(^{-}\)). The low levels of this species in the other three growth conditions are consistent with the levels of DMPC reported in other studies (31).

Diacylglycerol (DAG) species profiles were also examined by mass spectrometry in wild type cells grown to steady-state in the various media (i.e., I\(^{+}\), I\(^{-}\), I\(^{+}\), I\(^{-}\)). The qualitative and quantitative composition of DAG species at steady-state was relatively constant in the various media tested, with major species being 32:1, 32:2, 34:1, and 34:2; and, the minor 26:0, (C12:0, C14:0) 28:0, 30:1 (C14:0, C16:1 or C14:1, C16:0) and 36:1. Among the DAG species detected, the predicted 1,2-dimyristoyl-sn-glycerol (DMG) precursor of DMPC was present in fairly consistent proportions that did not exceed 5% in any of the four experimental conditions, including I\(^{+}\)C\(^{-}\) medium. The overall fatty acid composition revealed only minor differences in cells grown under the four growth conditions. The percentage of myristic acid (C14:0) ranged from 1.5% to 2.8% of total fatty acid; C16:0 was present at 24-27%; C16:1 at 30-34%; C18:0 at 6-7%; and, C18:1 at 24-31% (supplementary data, Fig S1).

Increased turnover of PC accompanies the addition of inositol and this turnover requires the participation of the lipase encoded by the NTE1 gene. Turnover of phospholipids in wild type and nte1\(^{-}\) cells was measured by labeling to steady-state with \(^{32}\)P in I\(^{+}\)C\(^{-}\) medium, as described above, for the experiments depicted in Fig 2A and Fig 3. However, once the cells had grown to mid-logarithmic phase they were harvested by filtration, washed in unlabeled I\(^{-}\)C\(^{+}\) medium and then re-suspended in unlabeled I\(^{-}\)C\(^{+}\) or I\(^{+}\)C\(^{+}\) medium and aliquots were collected over the same time intervals as in Fig 3. Fig 6A, which shows the pattern of loss or gain of label from specific phospholipids following re-suspension of wild type cells in unlabeled medium without inositol (i.e., I\(^{-}\)C\(^{-}\)) serves as a control for the experiment in Fig 6B in which inositol was present in the unlabeled medium (i.e., I\(^{+}\)C\(^{-}\)). In the control culture, approximately one quarter of the \(^{32}\)P label associated with PC was lost during the first 30 min following the shift to unlabeled I\(^{+}\)C\(^{-}\) medium. In the interval between 30 to 60 min, a slight increase in label associated with PC was observed (Fig 6A), presumably, due to ongoing transfer of \(^{32}\)P into PC from pre-existing, labeled intermediates in the PE methylation and Kennedy pathways (Fig 1). Little or no change in the level of label associated with PI was observed in the culture shifted to unlabeled I\(^{+}\)C\(^{-}\) medium during the entire 60 min time course (Fig 6A).
In the wild type culture transferred to unlabeled medium, which contained inositol (i.e., IC\(^-\); Fig 6B), approximately half of the label from PC was lost in the first 30 min, a decrease approximately twice the magnitude observed in the control culture (Fig 6A). Compared to the control culture, cells transferred to unlabeled medium containing inositol (Fig 6B) exhibited enhanced loss of label from CDP-DAG while label continued to increase in PI (Fig 6B). The loss of label from CDP-DAG appeared to be of approximately the same magnitude as the increase in label in PI (Fig 6B).

When exogenous choline is present in the growth medium, turnover of PC in yeast occurs primarily via deacylation (21), catalyzed by Nte1p, an ER-associated PLB (20). Because the media employed in these experiments contained choline, we examined phospholipid turnover in the nte1Δ strain (Fig 6C and 6D), following the same protocol used in the experiments conducted with wild type cells (Fig 6A and 6B). Quite unlike the pattern observed in wild type cells (Fig 6A), following the transfer to unlabeled IC\(^-\) medium, label associated with PC in nte1Δ cells continued to increase during the first 15 min (Fig 6C), implying continuing transfer of label from metabolic precursors in either of the two pathways for PC biosynthesis (Fig 1). Also in contrast to wild type, the presence of inositol had no effect on the pattern of PC turnover in nte1Δ cells following a shift to unlabeled medium (compare Fig 6D to Fig 6C). Following transfer of nte1Δ cells to unlabeled medium, with or without inositol (i.e., IC\(^+\) or IC\(^-\)) the net transfer of label into PC exceeded its removal by turnover. Thus, inositol does not stimulate increased PC turnover in nte1Δ cells. However, similar to wild type, label associated with PI increased rapidly after introduction of the nte1Δ cells into unlabeled IC\(^+\) medium (Fig 6D).

PI species composition remains constant, while PC and PE acyl chain composition is remodeled, following inositol addition to wild type cells growing in IC\(^-\) medium. Cells grown in IC\(^-\) medium sampled just prior to inositol addition (Fig 7A) showed a PI species profile similar to that found in the steady-state experiment under IC\(^-\) conditions (Fig 4). Following inositol addition, rapid and dramatic increases in the overall levels of all PI species (compare PI peaks to PE peaks in Fig 7A vs. 7B) were observed. The increase was essentially proportional for all PI species as illustrated in Fig 7B, showing the profile of PI species 60 min after addition of inositol to cells growing in IC\(^+\) medium. Under these conditions, the ratios of the three major PI species 32:1, 34:1 and 36:1 remained essentially constant while the overall content of PI increased dramatically in response to inositol addition (Fig 7B).

In contrast to the consistent ratio of PI species, the composition of PC species in wild type cells changed markedly upon the addition of inositol (Fig 8A vs. 8B). Prior to inositol addition the ratio of the PC species were similar to the steady-state experiment shown in Fig 5 in cells grown in IC\(^+\). Within 60 minutes following inositol addition (Fig 5B and 8B), DMPC levels had fallen to approximately 2% (Fig 5B and 8B), close to the basal level observed in the steady-state analysis shown in Fig 5A for cells grown in IC\(^+\) medium. Moreover, the overall PC species composition approached the steady-state composition observed for cultures grown in IC\(^+\) medium within 60 minutes following inositol addition (Fig 5A). PE also exhibited changes in species composition following inositol addition. In cells grown in IC\(^+\) medium, PE contained the species 34:2 and 34:1, in a ratio of 2:1. The species 36:2, 36:1, and 36:0 were also detected in a ratio of 2:1:3 (Fig 7A). Sixty min after inositol addition, the ratio of 34:2 to 34:1 had changed only slightly to 3:2 (Fig 7B). However, the ratio of 36:2:36:1:36:0 had changed to 5:4:3 with 36:2 becoming the most prominent of the three (Fig 7B). The 32:1 PE species at m/z 688.5 (not shown) remained constant (less than 3% of the total mixture) in all media tested. As in the steady-state experiments, the same major and minor DAG species in similar relative amounts were observed throughout the time course following the addition of inositol to cells grown in IC\(^+\) (data not shown).

In nte1Δ cells at time 0 min (data not shown), the levels of PI were low and the distribution of PI species resembled those of the wild type strain at steady-state in IC\(^+\) medium (Fig 4). In nte1Δ cells grown in IC\(^+\) medium at time 0 min, DMPC represented approximately 12% of PC species (Fig 8C), lower than the proportion observed in wild type cells grown in IC\(^+\) medium (see Fig 8A and Fig 5A). In contrast to wild type at 60 min post-inositol addition, the level of DMPC in the nte1Δ
mutant cells was essentially unchanged (Fig 8D), while proportions of the other PC species showed a significant shift to higher unsaturation (Fig 8D compared to 8C). The DAG species composition of nte1Δ cells was also analyzed by mass spectrometry and found to be essentially identical to that of wild type cells and showed little change in response to inositol (data not shown).

**Short-term labeling with **^{32}P **reveals comparable increases in PI synthesis in wild type and nte1Δ cells.** Cells were grown to mid-logarithmic phase in 1C\({\text{2}}\) medium, ^{32}P was added and samples were taken at 10 and 20 min following addition of label, prior to addition of inositol. Inositol was added at 20 min following the addition of label and further samples were taken at 30, 40 and 50 min (post label addition) as shown in Figs 9A, B, and C. During the period 20 min prior to inositol addition, label in wild type cells accumulated primarily in PA and CDP-DAG. Label associated with PA peaked within 5 min in the absence of inositol (data not shown), while label continued to increase in CDP-DAG, the next intermediate in the pathway, until inositol was added at 20 min. Other phospholipid species such as PS, PE, and PC also began to accumulate label prior to addition of inositol at 20 min. Following the addition of inositol, label associated with both CDP-DAG and PA rapidly declined, and label rapidly accumulated in PI (Fig 9A). The pattern of labeling in nte1Δ cells was similar to that observed in wild type cells (Fig 9A), except that label associated with PI rose even more steeply for the first 20 min. following inositol addition (i.e., the period spanning 20-40 min following addition of label) (Fig 9A), then leveled off abruptly at the 40 min. time point following label addition (i.e., 20 min following inositol addition) (Fig 9B). In addition, less label was incorporated into PC between 30 and 60 min in nte1Δ cells than in wild type cells. Clearly, however, the absence of Nte1p did not prevent the rapid labeling of PI, suggesting that deacylation of PC catalyzed by Nte1p is not the major source of fatty acids required for the synthesis of PI following inositol addition.

**Short-time course labeling with [1-{\text{14}}C] acetate reveals rapid decreases in label associated with DAG and PC in wild type, but not nte1Δ cells.** Short term labeling with ^{32}P does not provide an accurate relative assessment of the overall rate of PC synthesis compared to other phospholipids because the ^{32}P label from PA is lost in the formation of DAG (Fig 1) and, thus, PC synthesis via the CDP-choline pathway is underestimated. To gain a more accurate estimate of the relative rate of synthesis of PC compared to PI and other lipids, we conducted short term labeling with [1-{\text{14}}C] acetate. In this labeling protocol, DAG derived from PA is labeled as efficiently as other products derived from PA, permitting comparison of label accumulation in DAG with that associated with specific phospholipids. PC, which is largely derived from DAG through the CDP-choline pathway during short-term labeling in the presence of choline, is also efficiently labeled with [1-{\text{14}}C] acetate (Fig 10A). In wild type cells, the addition of inositol, 60 min after the introduction of the label, produced an immediate decline in the label associated with PC. Moreover, the amount of label associated with DAG rapidly decreased following inositol addition (Fig 10B). Strikingly, inositol addition did not result in a loss of label associated with PC in nte1Δ cells (Fig 10D), but the rate of accumulation of label into PC slowed, indicating that the presence of inositol affects the overall rate of synthesis of PC (compare Fig 10C and 10D after the 60 min time point). Furthermore, in contrast to wild type cells, inositol addition did not result in a decrease in label associated with DAG in nte1Δ cells. In fact, labeling of DAG appeared, if anything, to increase following inositol addition.

**Blocking de novo fatty acid metabolism with cerulenin substantially affects the magnitude of the burst of PI synthesis that occurs following introduction of inositol.** Cerulenin, an antibiotic known to block de novo fatty acid biosynthesis, has been used in a number of other studies in yeast to block de novo fatty acid biosynthesis (29,32,33). We observed a decrease of approximately 50% in total label from {\text{14}}C acetate incorporated into both phospholipids and neutral lipids during the first 30 min of exposure to cerulenin in wild type cells treated with 10 µg/mL of cerulenin compared to an untreated control. Concentrations of cerulenin above 10 µg/mL did not result in any additional reduction in incorporation of label into phospholipids, DAG or FFA (data not shown). Such continuing low-level labeling in the presence of cerulenin could be due to elongation of pre-existing fatty acids by the fatty acid synthase independent elongation pathway (34) and label is also expected to enter into the glycerol backbone of both DAG and
phospholipids. During the first 30 min after introduction of 10 µg/mL cerulenin, label incorporated into FFA and DAG was reduced two- and four-fold, respectively. In contrast, incorporation of 14C acetate into free sterols increased by about 1.5-fold compared to untreated cultures, indicating uninterrupted synthesis via the mevalonate pathway (data not shown).

Fig 9C illustrates the results obtained by labeling wild type cells with 32P in the presence of 10 µg/mL of cerulenin. During the first 20 min in the presence of cerulenin, prior to the addition of inositol, incorporation of label into total phospholipids was reduced to a significant extent as compared to the untreated control (data not shown). The introduction of inositol after 20 min of exposure to cerulenin, resulted in a burst of PI synthesis that was reduced by about two thirds (Fig 9C), compared to cells not treated with cerulenin (Fig 9A). The proportion of label incorporated into PA, PS, and PE was reduced approximately four-fold while label accumulating into PC was reduced approximately eight-fold in cerulenin treated vs. untreated cells following addition of inositol (Fig 9A). The fact that the magnitude of the burst of PI synthesis in cells exposed to cerulenin is reduced, suggests it is dependent, at least in part, on de novo fatty acid synthesis. It also appears that under conditions of fatty acid limitation in the presence of inositol, PI synthesis is favored over synthesis of PS, PE, and PC, a result consistent with an earlier report (35).

Neither Nte1p mediated hydrolysis of PC nor de novo synthesis of fatty acids is required for the rapid response to inositol by several distinct sets of genes. A number of distinct classes of genes exhibit rapid changes in expression in response to inositol (2,36). Since Nte1p activity appears to be required for PC turnover in response to inositol, we questioned whether the absence of Nte1p would alter the transcriptional response of selected target genes known to respond to inositol. We examined transcript levels of INO1, OLE1, and KAR2, shown by Jesch, et al. (19) to respond to inositol addition by distinct signaling mechanisms. No significant differences in the expression profiles of these three genes were observed in nte1Δ cells as compared to wild type (data not shown). We also examined INO1 and OLE1 mRNA expression profiles in wild type cells treated with 10 µg/mL of cerulenin over a 50 min period of time. Cerulenin was added at time zero and samples were collected at 0, 10 and 20 min prior to the addition of inositol at 20 min. A modest decline in the level of INO1 mRNA occurred prior to the addition of inositol in cells treated with cerulenin in contrast to the untreated control (Fig 11A). However, following the addition of inositol the level of INO1 mRNA in the treated culture decreased in parallel to the observed repression of INO1 expression in the untreated culture (Fig 11A). OLE1 mRNA expression increased in response to inositol in cerulenin treated cells in a fashion comparable to that observed in untreated cells. However, the subsequent decline in OLE1 transcript observed in the untreated culture did not occur in the treated cells suggesting that inhibition of fatty acid synthesis may affect OLE1 expression over the longer term (Fig 11B).

Discussion

We have shown that the addition of inositol to logarithmically growing yeast cultures results in major and rapid reprogramming of phospholipid metabolism. The changes detected include rapid adjustments in the overall relative proportions and absolute cellular content of PI and PC. PC acyl chain composition also undergoes extensive remodeling. Previous studies (1,2) demonstrated that the rate of PI synthesis is dramatically increased when inositol is added to the growth medium of wild type cells. We have now demonstrated that this increased synthesis results in a five- to six-fold increase in cellular content of PI within 30 min following inositol addition (Fig 2A), a span of time representing 25% or less of the doubling time of wild type cultures. We have now demonstrated that this increased synthesis results in a five- to six-fold increase in cellular content of PI within 30 min following inositol addition (Fig 2A), a span of time representing 25% or less of the doubling time of wild type cultures growing in synthetic medium. Furthermore, as PI content increases following the addition of inositol, the overall distribution of PI species is remarkably constant (Fig 7A, B), suggesting that the ratio of PI species is tightly regulated by the cell. Under all the growth conditions employed in these experiments, the acyl chain composition of PI consisted of 18 and 16 carbon fatty acids, with predominantly one unsaturated and one saturated chain per PI molecule (Fig 4).

The mechanism by which the acyl chain compositions of PI and PC are regulated is not known. However, the data presented here indicate
that the mechanism maintaining the relatively constant ratio of PI species operates even under conditions involving dramatic changes in the rate of PI synthesis. The fact that the acyl composition of PI does not change markedly, during and following the rapid burst of PI synthesis that occurs upon inositol addition also suggests that little or no remodeling occurs after this burst of PI synthesis.

The burst of PI synthesis in response to inositol requires de novo fatty acid synthesis. Clearly, the observed increase in the absolute content of PI (Fig 3) requires a source of fatty acids. We initially suspected that these fatty acids might, in part, be derived from the pool produced by deacylation of PC catalyzed by Nte1p. Yet, the rapid buildup of PI upon inositol supplementation does not appear to depend upon fatty acids liberated by Nte1p mediated deacylation of PC, since deletion of NTE1 does not prevent or attenuate the burst of PI synthesis (Fig. 9). Rather, to a large extent, the dramatic acceleration of the rate of PI synthesis, that occurs immediately after introduction of inositol, appears to depend on de novo fatty acid biosynthesis (Fig. 11).

The apparent dependence on de novo synthesis of fatty acids for the burst in PI synthesis suggests that the rate of PA, CDP-DAG, and PI synthesis may be coupled with fatty acid biosynthesis, at least during the period of rapid PI synthesis immediately following inositol introduction. Indeed, there is a correlated increase in the level of free fatty acids in cells exposed to inositol (Fig. 2B and data not shown). The rate limiting step in the synthesis of fatty acids is catalyzed by acetyl-CoA-carboxylase (Acc1p) (Fig. 1). The ACC1 gene is subject to transcriptional regulation (37) and its gene product, Acc1p, is subject to post-translational regulation by phosphorylation and other forms of regulation at the enzymatic level (38-41). The ACC1, FAS1 and FAS2 genes encoding the subunits of fatty acid synthase, Fas1p and Fas2p, both contain UASINO elements in their promoters. At steady-state, following growth for 12 generations in the presence of inositol, all three of these genes are repressed less than two-fold compared to cells grown in the absence of inositol (36). However, in the first two hours following inositol addition, the ACC1, FAS1 and FAS2 genes are rapidly repressed to a level some four-fold or greater compared to their expression levels in the absence of inositol (19). Since these three genes exhibit their highest level of repression at the time when the rate of PI synthesis is the greatest, transcriptional regulation of fatty acid biosynthesis clearly cannot be responsible for any increase in de novo synthesis of fatty acids during the first few minutes following inositol addition. Most likely, therefore any increase in fatty acid biosynthesis upon inositol addition must be regulated at a posttranslational or enzymatic level, a prediction which awaits further investigation.

The effect of inositol on PC metabolism. We have shown that inositol supplementation triggers rapid turnover of PC (Fig 3, 6A and 6B) and that this turnover is dependent on the phospholipase B encoded by the NTE1 gene (Fig 6C and 6D) localized in the ER (20). Previously, Nte1p was shown to be responsible for turnover of PC in yeast when choline is present in the medium and/or when cells are grown at an elevated temperature (37°C) (20,21). The data presented here indicates that addition of inositol to medium already containing choline triggers additional PC hydrolysis (Fig 6A and 6B) and that this inositol-dependent turnover is also dependent on Nte1p (Fig 6C and 6D).

The species composition of PC is also influenced by the availability of both inositol and choline (Fig 5 and 8). Boumann, et al. (31) showed that different PC species distributions are produced by the two different routes for PC biosynthesis, namely the PE methylation pathway vs. the CDP-choline (Kennedy) pathway (Fig 1) and, in general, that the CDP-choline pathway contributes the greatest degree of molecular diversity. The conditions we employed for kinetic analysis of changes in phospholipid composition included constant provision of choline, a circumstance which ensures considerable flux through the CDP-choline pathway (20,21). Thus, changes in PC species diversity in response to provision of choline are not unexpected. However, the effect of inositol on PC acyl composition (Fig 5 and 8) has not previously been reported. The change in the species composition of PC in cells grown in IC⁺ medium was most unusual. Under these conditions, DMPC was observed at proportions ten-fold or greater compared to cells grown under any of the other three conditions, an enrichment of ten-fold or greater compared to the proportion of myristic acid in the cellular acyl composition. In cells grown in IC⁺
medium, the proportion of myristic acid in the fatty acid composition was approximately 2.8%, a level slightly higher than the other three growth conditions examined (Fig S1). The level of DMG, the precursor of DMPC in the CDP-choline pathway, was approximately 5% of total DAG species under all of the growth conditions examined (data not shown), an enrichment of about two-fold in C14:0 in DAG pools compared to cellular fatty acid composition. However, it is clear that the high levels of accumulation of DMPC cannot be explained by a major increase in DMG or by the proportion of myristic acid in cellular fatty acids.

Increased turnover of PC catalyzed by Nte1p, which occurs in the presence of choline in wild type cells, is known to increase the rate of synthesis of PC via the Kennedy pathway (20,21). The resulting increased rate of synthesis of PC occurring in the presence of choline might lead to a higher concentration of newly-synthesized acyl chains drawn from cellular acyl-CoA pools, which are not expected to be captured by our extraction procedures. Gaigg, et al. (42) reported that C14:0 represents 10% of the total acyl-CoA pool in wild type cells, a level substantially higher than the level of C14:0 in DAG or in the overall fatty acid composition. However, regardless of the composition of the acyl-CoA pools, to achieve the relative levels of DMPC observed here would still require either selective use of DMG by the CDP-choline pathway and/or extensive remodeling of PC. Selective deacylation of species other than DMPC could also account for its relative accumulation. DMPC levels were observed to be somewhat lower in nte1Δ cells grown in IC’ medium as compared to wild type, suggesting that Nte1p may play a role in DMPC accumulation as well as its turnover (Fig 8C). Interestingly, Johnson, et al. (43) reported that turnover of PC catalyzed by Plb1p could suppress the temperature-sensitive myristic acid auxotrophy of the nmt1-181 mutant defective in protein myristoylation, suggesting mobilization of a cellular pool of myristic acid from PC. Their results also suggested that this endogenous pool of myristic acid generated from membrane phospholipids can also be activated by overexpression of Faa2p, an acyl-CoA synthetase. Likewise, Ashrafi, et al. (44) reported that overexpression of both Plb1p and Tg1p, a triglyceride lipase, rescued the loss of viability of nmt1-451Dfaa4Δ cells on entry into stationary phase. These observations all point to mobilization of cellular pools of myristic acid by specific lipases, and/or acyl synthases. We have shown that DMPC is selectively lost upon addition of inositol in wild type cells, a process that appears to depend, at least in part, on Nte1p (Fig 5B) a phospholipase B located in the ER (20). However, the kinetics of disappearance of DMPC in wild type cells following inositol addition, (Fig 8D) were not as rapid as the overall decrease in bulk PC content (Fig 3) or the rate of loss of label from PC in the turnover experiments depicted in Fig 6B. Thus, it would appear that processes in addition to Nte1p deacylation must be involved in achieving the steady-state composition of PC species after addition of inositol.

Inositol availability is known to influence PC metabolism in yeast by a number of mechanisms (5,45). For example, inositol is a non-competitive inhibitor of PS synthase (1) which competes with PI synthase for the precursor, CDP-DAG, at a major branchpoint in phospholipid biosynthesis (Fig 1). Thus, when inositol is present, PI synthesis is favored over PS synthesis (1). This is expected to affect not only PS production, but also PE and PC produced by PE methylation (Fig 1), potentially altering the proportion of PC produced by PE methylation vs. the CDP-choline pathway. In addition, the presence of both exogenous inositol and choline leads to the repression of genes involved in PC biosynthesis by both routes (i.e., via the CDP-choline pathway and via the CDP-DAG pathway through PS to PE to PC) (5,36,45). Thus, there are likely to be multiple mechanisms affecting PC metabolism in response to both inositol and choline, a subject of ongoing investigation in our laboratory.

Relationship between lipid metabolism and gene expression. In a related study employing identical growth conditions and time course (19), we have shown that inositol addition triggers changes in transcript abundance of over 700 genes. Significantly, changes in transcript abundance of major categories of genes; namely, the UASINO-containing genes (such as INO1), as well as genes (such as KAR2) under the control of the unfolded protein response pathway (UPR) and genes (such as OLE1) containing the low oxygen response element (LORE) element in their promoters, also occur within the same time frame as the major changes in PI content and PC turnover and remodeling reported...
here (19). The signals responsible for initiating the three independent transcription regulatory responses all reside in the ER (2, 19), a major site of phospholipid metabolism (46). The ER is also the compartment to which the Nte1p phospholipase is localized (20).

Changes in lipid metabolism in the ER induced by inositol addition known to be responsible for the repression of UASINO-containing genes such as INO1 but, as yet, the signals responsible for the transient activation of OLE1 and other LORE genes or the repression of the UPR genes, such as KAR2, in response to inositol, are not known. Since the pattern of PC turnover and remodeling was altered in nte1Δ cells as compared to wild type, we questioned whether deletion of NTE1 would also alter any of the known transcriptional responses to inositol. However, the overexpression patterns of INO1, KAR2, and OLE1 were similar in nte1Δ cells and wild type cells following inositol addition (data not shown). Thus, the turnover of PC mediated by Nte1p is not involved in any of the mechanisms controlling the regulatory responses of these three classes of genes to inositol. Furthermore, the UASINO, UPR, and LORE regulated genes mentioned above were also regulated in response to inositol in cells treated with cerulenin, indicating that their regulation in response to inositol addition is not influenced dramatically by inhibition of fatty acid biosynthesis (Fig 11).

A number of other classes of genes, in addition to the UASINO, UPR, and LORE genes, were identified by statistical analysis by Jesch, et al. (19) as responding to addition of inositol. The signals, which result from inositol and/or lipid metabolism and control the responses of genes other than those containing the UASINO element (2), remain to be elucidated. In yeast, PI also serves as a precursor to the inositol-containing sphingolipids which have also been shown to undergo changes in metabolism following inositol addition (47). In yeast, PI also serves as precursor to phosphoinositides, as well as a variety of inositol phosphates, many of which play roles in transcriptional regulation signaling processes and membrane trafficking (36). Changes in transcript abundance following inositol addition could result from signals produced by any of these downstream products. A thorough investigation of this prediction will require analysis of transcriptional regulation, genome-wide, in response to inositol in mutants defective in key steps in the metabolism of these inositol-containing components. Future studies comparing the kinetics of alterations in the pools of any of these inositol-containing compounds to changes in transcription patterns genome-wide, may also provide insights into signaling relevant to this regulation.
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Footnotes

1 The abbreviations used are: PA, phosphatidic acid; ER, endoplasmic reticulum; CDP-DAG, CDP-diaacylglycerol, PI, phosphatidylinositol; DAG, 1,2-diacylglycerols; PS, phosphatidylserine; PE, phosphatidylethanolamine; PC, phosphatidylcholine; DMPC, 1,2-dimyristoyl-sn-glycero-3-phosphocholine. DMG, 1,2-dimyristoyl-sn-glycerol; UPR, unfolded protein response pathway; LORE, low oxygen response element; I, inositol; C, choline; FS, free sterol; FFA, free fatty acids; TAG, triacylglycerols; SE steryl esters; GroPC, glycerophosphocholine; ESI-MS/MS, electrospray ionization tandem mass spectrometry; TCA, trichloroacetic acid; LysoPL, lysophospholipids; PL, phospholipids.

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Figure Legends

**Fig. 1. Biosynthesis of PI, PC, PA and DAG in S. cerevisiae.** The metabolic pathways shown in bold are discussed in detail in this report. Phosphatidylinositol (PI) is synthesized from CDP-diaacylglycerol (CDP-DAG) and inositol. Phosphatidylcholine (PC) is synthesized from diacylglycerol (DAG) and free choline via the CDP-choline (Kennedy) pathway or from phosphatidylethanolamine (PE) via the methylation pathway. Phosphatidic acid (PA) is generated de novo from acyl-CoA and glycerol 3-P or from turnover of PC via *PLD1*. Diacylglycerol (DAG) is derived from dephosphorylation of PA or deacylation of triacylglycerol (TAG). The positions of PI, PC, PA and DAG within the metabolic network are circled and the positions at which inositol and choline enter in the metabolic pathway are
boxed. *Arrows* represent routes of metabolic conversion. The names of the structural genes for enzymes catalyzing specific metabolic conversions are shown adjacent to the *arrows*. FFA, free fatty acids; GroPC, glycerophosphocholine; Lyso-PL, lysophospholipid; PL, phospholipid; CoA, coenzyme A; acyl CoA, acyl coenzyme A.

**Fig. 2.** Steady-state phospholipid and neutral lipid profiles of wild type cells grown in different synthetic media. (A) Phospholipid composition of wild type cells grown until mid-logarithmic phase in the presence of $^{32}$P as described in “Experimental Procedures” in medium lacking inositol and choline (I⁻C⁻), (grey bars); with inositol and no choline (I⁻C⁺), (striped bars); only with choline (I⁺C⁻), (white bars); with both inositol and choline (I⁺C⁺), (black bars). PA, phosphatidic acid; CDP-DAG, CDP-diacylglycerol; PI, phosphatidylinositol; PS, phosphatidylserine; PE, phosphatidylethanolamine; PC, phosphatidylcholine. (B) Composition of the main neutral lipid classes of wild type cells grown in the presence of [1-$^{14}$C] acetate in the same conditions described in panel A. 1,2 DAG, 1,2-diacylglycerol; FS, free sterol; FFA, free fatty acid; TAG, triacylglycerol; SE, steryl esters; and Squalene.

**Fig. 3.** Changes in phospholipid composition following inositol addition. Cells were grown in I⁻C⁺ medium and phospholipids were labeled with 20 $\mu$Ci/mL $[^{32}$P]-orthophosphate to steady-state. At the mid-logarithmic phase of growth (A₆₀₀=0.5), inositol (75 $\mu$M) was added (arrow) and aliquots were taken at the indicated times. A time 0 control was also taken just prior the addition of inositol. Data are expressed as counts of radiolabel $^{32}$P incorporated into total phospholipids per OD units in the cell culture. Data depicted represent the average of six experiments. The magnitude of the error is similar to the data in Fig 2, but errors bars were omitted for clarity of the presentation. PI, (■); PC, (●); PS, (△); PE, (○); PA, (▲); CDP-DAG, (□).

**Fig. 4.** Major steady-state PI species acyl chain composition profile of wild type cells grown in different synthetic media. PI species acyl composition (total number of carbons:total number of double bonds) 32:1 (striped bars), 34:1 (vertical bars), and 36:1 (horizontal bars) in cells grown to mid-logarithmic phase in medium lacking inositol and choline (I⁻C⁻), with inositol and no choline (I⁻C⁺), only with choline (I⁺C⁻), and with both inositol and choline (I⁺C⁺). The abundance of three major PI species are shown as relative percentages. Data are averages from four independent experiments and the error bars represent the standard deviation (n=4).

**Fig. 5.** PC species acyl chain composition profiles of wild type cells at steady-state and changes following inositol addition. (A) PC species acyl chain composition profile at steady-state (total number of carbons:total number of double bonds) 28:0 (black bars), 32:1 (striped bars), 32:2 (grey bars), 34:1 (vertical bars), and 34:2 (white bars) in cells grown to mid-logarithmic phase in various media (i.e., I⁻C⁻, I⁻C⁺, I⁺C⁻, and I⁺C⁺). (B) PC species acyl chain composition profile at various times (i.e., 0, 15, 30, and 60 min) following inositol addition. The relative abundance of five major PC species are shown as percentages of total PC. Data are averages from four independent experiments and the error bars represent the standard deviation (n=4).

**Fig. 6.** Analysis of phospholipid turnover following inositol addition. Cells were grown as described above to steady-state in the presence of 20 $\mu$Ci/mL $[^{32}$P]-orthophosphate in I⁺C⁺ medium and were quickly collected by filtration, washed with I⁻C⁺ medium, placed in unlabeled fresh medium pre-warmed to 30°C and samples were taken at the indicated times. A. Wild type cells shifted to I⁻C⁺ medium. B. Wild type cells shifted to I⁺C⁻ medium. C. nte1Δ cells shifted to I⁻C⁺ medium. D. nte1Δ cells shifted to I⁺C⁺ medium. Data is representative of two independent experiments. Symbols refer to PI, (■); PC, (●); PA, (▲); CDP-DAG, (□). (A) wild type, I⁻C⁺; (B) wild type, I⁺C⁻; (C) nte1Δ, I⁻C⁺; (D) nte1Δ I⁺C⁺.

**Fig. 7.** Electrospray ionization mass spectra of phospholipids showing PI species in wild type cells and acyl chain composition. (A) Negative mode mass spectrum [M-H]⁻ of phospholipids in wild type cells grown to mid-logarithmic phase in I⁺C⁺ medium showing the PI species present (i.e., 32:1, 34:1, and...
36:1) at time 0. (B) Mass spectrum of phospholipids at 60 min following inositol addition in wild type cells. PI species profile at 60 min following inositol addition remains essentially identical to time 0.

**Fig. 8. Electrospray ionization mass spectra of phospholipids showing PC species in wild type and nte1Δ cells and acyl chain composition.** (A) Positive mode mass spectrum of phospholipids in wild type cells grown to mid-logarithmic phase in I−C+ medium showing [M+Na]+ PC species and their acyl chain composition profile at time 0 (note the prominent peak at m/z 701.5). (B) Mass spectrum of phospholipids showing the change in PC species composition including the drastic decrease in the relative proportion of m/z 701.5 at 60 min following inositol addition. (C) Positive mode mass spectrum of phospholipids in nte1Δ cells grown to mid-logarithmic phase in I−C+ medium showing [M+Na]+ PC species acyl chain composition at time 0 (note the decrease in the formation of m/z 701.5). (D) Mass spectrum of phospholipids at time 60 min following inositol addition showing that the relative proportion of m/z 701.5 does not decline.

**Fig. 9. Short-term labeling of phospholipids.** Newly-synthesized glycerophospholipids were quantified 10, 20 min after the addition of 100 µCi/mL [32P]-orthophosphate to cells growing in mid-logarithmic phase in the absence of inositol. At 20 min, inositol (75 µM) was added (arrow). Cells were sampled at 10, 20 and 30 min following addition of inositol. Data is representative of three independent experiments. The phospholipids indicated are PI, (■); PC, (●); PS, (▲); PE, (○); PA, (▲); CDP-DAG, (▲). (A) wild type (B) nte1Δ (C) wild type + 10 µl/mL cerulenin. Cerulenin was added at time 0.

**Fig. 10. Short time course labeling with [1-14C] acetate.** [1-14C] acetate (1 µCi/mL) was added to wild type and nte1Δ cultures grown to mid-logarithmic phase in I−C+ medium at time 0. Sixty minutes after the addition of label, 75 µM inositol was added (arrow) and samples were collected at 5, 15, 30, and 60 min following inositol addition. Samples were also taken 10, 20 and 30 min after the addition of label, but before the addition of inositol. The lipids indicated are PI, (■); PC, (●); PS, (▲); PE, (○); DAG, (▲). (A) wild type cells, I−C+ medium; (B) wild type cells grown to mid-logarithmic phase in I−C+ medium with inositol addition at the time indicated by the arrow; (C) nte1Δ cells grown to mid-logarithmic phase in I−C+ medium; (D) nte1Δ cells I−C+ label added at time 0, inositol addition at the time indicated by the arrow.

**Fig. 11. Effect of de novo synthesis of fatty acids on the expression profiles of INO1, and OLE1.** Wild type cells growing at mid-logarithmic phase in I−C+ medium were treated with 10µg/mL of cerulenin over a 50 min period of time. Samples were collected at 0, 10 and 20 min prior to the addition of inositol. Cerulenin was added at time zero and inositol was added at 20 min (indicated by the arrow). Following the addition of inositol, samples were collected 10 min intervals for another 30 min (i.e., at 20, 40, and 50 min following cerulenin addition). Total RNA was analyzed by Northern blotting as described above. Wild type cells treated with cerulenin (▲) control cells (●). (A) INO1 mRNA; (B) OLE1 mRNA.
Figure 3

+ INOSITOL

Cpm/ODU X 10^3

Time (min)
Figure 4

Relative %

I^−C^−  I^+C^−  I^−C^+  I^+C^+
Figure 5

A

B
Figure 7

A

WT t = 0 min

PE 34:2
714.6

PE 36:0
746.6

PI 32:1
807.4

PI 34:1
835.6

PI 36:1
863.5

B

WT t = 60 min

PE 34:2
714.6

PE 36:2
742.7

PI 32:1
807.4

PI 34:1
835.6

PI 36:1
863.5
Inositol induces a profound alteration in the pattern and rate of synthesis and
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Inositol induces a profound alteration in the pattern and rate of synthesis and turnover of membrane lipids in Saccharomyces cerevisiae.

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In this article, we reported detection of abnormally high levels of a phosphatidylcholine (PC) species containing two myristoyl (14:0) acyl chains (dimyristoylphosphatidylcholine (DMPC)) in wild-type and nte1Δ cells grown in synthetic medium containing choline but lacking inositol (ΔC− medium). However, in repeated growth studies since publication of this work, we have detected levels of DMPC averaging ~2% of the total PC species, similar to the levels we reported for the other growth conditions we studied. However, retesting of frozen samples of the lipid extracts from the original experiments confirmed the presence of the originally detected high levels of DMPC. We currently have no explanation for the occurrence of the high levels of DMPC in these samples. We tested several scenarios involving inadvertent fluctuation of temperature and/or inclusion of 10-fold more choline in the growth medium during the experiments. None of these interventions caused significant increases in DMPC levels above 2%.

The revised proportions of PC species in wild-type cells grown in ΔC− medium are essentially as we reported, with the exception of DMPC levels at an average of 2%. The major PC species detected in wild-type cells grown in ΔC− medium are 32:1 and 32:2 in a ratio of 3:2, with the less abundant 34:1 and 34:2 in a ratio of 4:5. The proportion of 32:1 to 32:2 and that of 34:1 to 34:2 in nte1Δ cells grown in ΔC− medium are also as we reported, with DMPC levels, as in wild-type cells, not exceeding 2%. All other results presented in the original article stand as reported, supporting the major conclusion of the study that the addition of inositol causes profound changes in the synthesis and turnover of membrane lipids in yeast.