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Regulation of Lipid Biosynthesis in *Saccharomyces cerevisiae* by Fumonisin B_1*

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The regulation of lipid biosynthesis in the yeast *Saccharomyces cerevisiae* by fumonisin B_1 was examined. Fumonisin B_1 inhibited the growth of yeast cells. Cells supplemented with fumonisin B_1 accumulated free sphinganine and phytosphingosine in a dose-dependent manner. The cellular concentration of ceramide was reduced in fumonisin B_1-supplemented cells. Ceramide synthase activity was found in yeast cell membranes and was inhibited by fumonisin B_1. Fumonisin B_1 inhibited the synthesis of the inositol-containing sphingolipids inositol phosphorylceramide, mannosylinositol phosphorylceramide, and mannosyldiinositol phosphorylceramide. Fumonisin B_1 also caused a decrease in the synthesis of the major phospholipids synthesized via the CDP-diacylglycerol-dependent pathway and the synthesis of neutral lipids. The effects of fumonisin B_1 and sphingoid bases on the activities of enzymes in the pathways leading to the synthesis of sphingolipids, phospholipids, and neutral lipids were also examined. Other than ceramide synthase, fumonisin B_1 did not affect the activities of any of the enzymes examined. However, sphinganine and phytosphingosine inhibited the activities of inositol phosphorylceramide synthase, phosphatidylserine synthase, and phosphatidate phosphatase. These are key enzymes responsible for the synthesis of lipids in yeast. The data reported here indicated that the biosynthesis of sphingolipids, phospholipids and neutral lipids was coordinately regulated by fumonisin B_1 through the regulation of lipid biosynthetic enzymes by sphingoid bases.

Cell growth is dependent on the membrane structures in the cell which compartmentalize various cellular processes (1, 2). Lipids are major membrane components which play a critical role in the structure and function of membranes. The major membrane lipids found in eucaryotic cells include phospholipids, sphingolipids, and neutral lipids (2). In addition to their role as structural components of membranes, lipids function as cofactors and activators of membrane-associated enzymes (3) and play a major role in cell signaling mechanisms (4–6).

A great deal is known about the synthesis and regulation of phospholipids in the yeast *Saccharomyces cerevisiae* (7–11). Nearly all of the structural genes encoding for the phospholipid biosynthetic enzymes have been cloned and characterized, and many of the enzymes have been purified and characterized (7–11). The enzymes in the pathway are regulated by both genetic and biochemical mechanisms. The gene expression of most of the enzymes responsible for the synthesis of the major membrane phospholipid PC[1] is coordinately regulated by the water-soluble phospholipid precursor inositol (7–13). The biochemical mechanisms affecting the activity of phospholipid biosynthetic enzymes include regulation by inositol (14), nucleotides (15, 16), phosphorylation (17–19), lipids (20–23), and sphingoid bases (24).

In *S. cerevisiae*, the pathways for the synthesis of phospholipids, sphingolipids, and neutral lipids share common lipid intermediates such as DG, CDP-DG, and PI (Fig. 1). Thus, it is reasonable to question whether overall lipid biosynthesis is coordinately regulated. Much attention has been paid to the role sphingoid bases play in lipid metabolism and cell signaling in mammalian cells (6, 25). For example, sphingosine has been suggested to be a regulator of the PC signaling pathway since sphingosine activates phospholipase D (26, 27) and inhibits PA phosphatase (28, 29) and protein kinase C (6, 30–32). Our approach in this work was to elevate the cellular concentration of sphingoid bases in *S. cerevisiae* and examine its effect on lipid biosynthesis. Sphingoid base levels were elevated in *S. cerevisiae* by supplementing cells with fumonisin B_1. Fumonisin B_1 is a neurotoxin (33) and phytotoxin (34) which bears structural similarity to sphingoid bases (35) (Fig. 2). Fumonisin B_1 has been shown to elevate sphingoid base levels in mammalian cells (36) due to the inhibition of ceramide synthase activity (37). The addition of fumonisin B_1 to *S. cerevisiae* cells resulted in a decrease in the synthesis of sphingolipids, phospholipids, and neutral lipids and dramatically affected the overall lipid composition of the cell. The data reported here were consistent with the conclusion that the synthesis of the major lipid classes was coordinately regulated by sphingoid bases. The mechanism of this regulation was due in part to the inhibition of key lipid biosynthetic enzymes including IPC synthase, PS synthase, and PA phosphatase by sphingoid bases.

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This paper is dedicated to Eugene P. Kennedy on the occasion of his retirement.

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[1] The abbreviations used are: PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; CDP-DG, CDP-diacylglycerol; DG, diacylglycerol; PA, phosphatidate; PI, phosphatidylinositol; IPC, inositol phosphorylceramide; MIPC, mannosylinositol phosphorylceramide; $M(IP)_2C$, mannosyldiinositol phosphorylceramide.

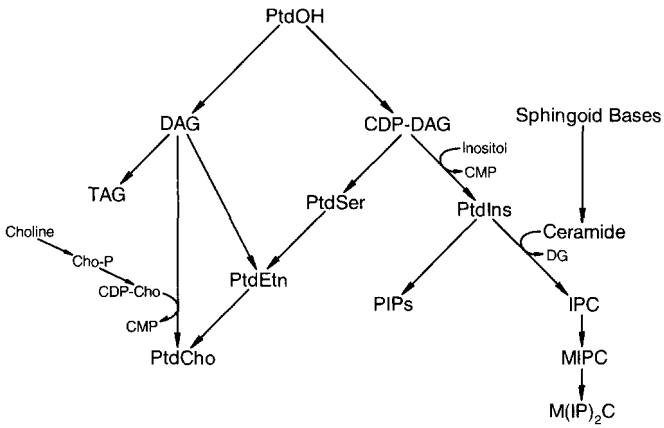
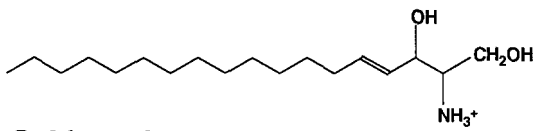
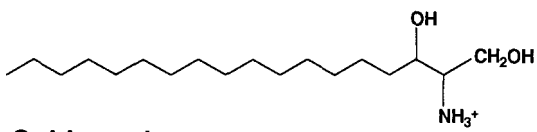


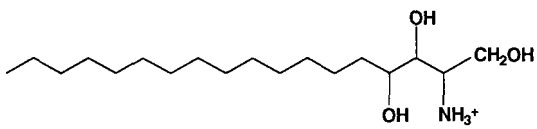
FIG. 1. **Lipid biosynthetic pathways in *S. cerevisiae*.** The pathways shown include the relevant steps discussed in the text. More comprehensive pathways which include lipid and water-soluble intermediates may be found in Refs. 7 and 11. Abbreviations: *PtdCho*, phosphatidylcholine; *PtdEtn*, phosphatidylethanolamine; *PtdSer*, phosphatidylserine; *CDP-DAG*, CDP-diacylglycerol; *DAG*, diacylglycerol; *PtdOH*, phosphatidate; *Cho-P*, choline phosphate; *CDP-Cho*, CDP-choline; *PtdIns*, phosphatidylinositol; *IPC*, inositol phosphorylceramide; *MIPC*, mannosylinositol phosphorylceramide; *M(IP)$_2$C*, mannosyldiinositol phosphorylceramide; *PIPs*, polyphosphoinositides.



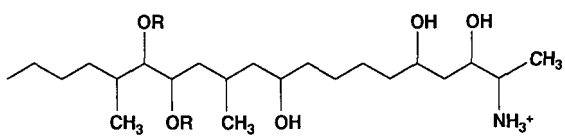
Sphingosine



Sphinganine



Phytosphingosine



R = $COCH_2CH(COOH)CH_2COOH$

Fumonisin B_1

FIG. 2. **Structures of sphingoid bases and fumonisin B_1.**

EXPERIMENTAL PROCEDURES

Materials

All chemicals were reagent grade. Triton X-100, Tergitol (Nonidet P-40), sphingoid bases, ceramide, fumonisin B_1, ATP, CTP, inositol, serine, choline, phosphocholine, CDP-choline, and bovine serum albumin were obtained from Sigma. Phospholipids and neutral lipids were purchased from Avanti Polar Lipids and Sigma. CDP-DG was prepared as described previously (38). Radiochemicals and EN^{3}HANCE were purchased from DuPont NEN, and scintillation counting supplies were from National Diagnostics. Silica gel-loaded SG81 chromatography paper was from Whatman, Inc., and Silica Gel 60 thin layer chromatography plates were from EM Science. *Escherichia coli* DG kinase was obtained from Lipidex Inc. Growth medium supplies were purchased from Difco Laboratories.

Methods

Strain and Growth Conditions—Strain *MAT***a** *ade5* (39), which shows normal regulation of phospholipid metabolism (40–43), was used for analysis of lipids in response to fumonisin B_1 and for the preparation of enzymes. Cultures were maintained on YEPD medium (1% yeast extract, 2% peptone, 2% glucose) plates containing 2% Bacto-agar. Cells were grown in complete synthetic medium (39) containing 0.5% Tergitol in the absence and presence of the indicated concentrations of fumonisin B_1 at 30 °C. Cell numbers were determined by microscopic examination with a hemacytometer. Fumonisin B_1 caused cells to clump. Prior to counting, cell clumps were dispersed by a brief sonication. Viable cells were determined by plate counts on YEPD medium.

Mass Analysis of Sphingoid Bases and Ceramide—Sphingoid bases were extracted from unlabeled cells by the method of Merrill *et al.* (44). *O*-Phthalaldehyde derivatives of the sphingoid bases were prepared and analyzed by high performance liquid chromatography using C20-sphinganine as an internal standard (44). The identity of the sphingoid bases was determined by comparing its elution profile with that of authentic standards. For ceramide analysis, lipids were extracted from unlabeled cells (45) and subjected to mild alkaline hydrolysis (46) to deacylate DG. Ceramide was then quantified by the method of Bell and co-workers (47, 48) using *E. coli* DG kinase (49).

Labeling and Analysis of Sphingolipids, Phospholipids, and Neutral Lipids—Pulse and steady-state labeling of lipids with [2-^{3}H]inositol and [2-^{14}C]acetate were performed as described previously (50–54). Sphingolipids, phospholipids, and neutral lipids were extracted from labeled cells as by Hanson and Lester (55). Sphingolipids were analyzed by one-dimensional chromatography on silica gel thin layer plates (56). Phospholipids were analyzed by the two-dimensional chromatography (57) using Na_2EDTA-treated SG81 paper (58). Neutral lipids were separated by one-dimensional chromatography on silica gel thin layer plates (59). The positions of the labeled lipids on chromatograms were determined by fluorography using EN^{3}HANCE and compared with standard lipids after exposure to iodine vapor. The amount of each labeled lipid was determined by liquid scintillation counting of the corresponding spots on chromatograms.

Preparation of Enzymes—PA phosphatase was purified to homogeneity as described by Lin and Carman (60). IPC synthase was solubilized from microsomal membranes with 1% Triton X-100 as described by Fischl and Carman (61). Cell extracts (61) and total membranes (60) were prepared as described previously and used for the assay of the indicated enzymes.

Preparation of Labeled Substrates—[^{32}P]PA was synthesized enzymatically from DG and [γ-^{32}P]ATP using *E. coli* DG kinase (60). [^{3}H]PI was synthesized from CDP-DG and [2-^{3}H]inositol using PI synthase purified from *S. cerevisiae* (61).

Enzyme Assays—All assays were conducted at 30 °C in a total volume of 0.1 ml unless otherwise indicated. Ceramide synthase (acyl-CoA: sphinganine (sphingosine) *N*-acyltransferase, EC 2.3.1.24) was measured at 37 °C with 25 mM potassium phosphate buffer (pH 7.4), 0.5 mM dithiothreitol, 40 μM stearoyl-CoA, 3 μM [^{3}H]sphingosine (prepared as a liposome of PC and sphingosine at a molar ratio of 2:1), and enzyme protein (62). IPC synthase (phosphatidylinositol:ceramide phosphoinositol transferase) was measured with 50 mM Tris-HCl buffer (pH 7.0), 1 mM $MnCl_2$, 5 mM $MgCl_2$, 5 mM Triton X-100, 0.1 mM ceramide, 0.25 mM [^{3}H]PI, and enzyme protein (63). CDP-DG synthase (CTP:phosphatidate cytidylyltransferase, EC 2.7.7.41) was measured with 50 mM Tris-maleate buffer (pH 6.5), 20 mM $MgCl_2$, 15 mM Triton X-100, 0.5 mM phosphatidate, 1.0 mM [5-^{3}H]CTP, and enzyme protein (64). PI synthase (CDPdiacylglycerol:*myo*-inositol 3-phosphatidyltransferase, EC 2.7.8.11) was measured with 50 mM Tris-HCl buffer (pH 8.0), 2 mM $MnCl_2$, 3.2 mM Triton X-100, 0.2 mM CDP-DG, 1 mM [2-^{3}H]inositol, and enzyme protein (38). PS synthase (CDPdiacylglycerol:L-serine 3-*O*-phosphatidyltransferase, EC 2.7.8.8) was measured with 50 mM Tris-HCl buffer (pH 8.0), 0.6 mM $MnCl_2$, 3.2 mM Triton X-100, 0.2 mM CDP-DG, 0.5 mM [3-^{3}H]serine, and enzyme protein (65). PA phosphatase (3-*sn*-phosphatidate phosphohydrolase, EC 3.1.3.4) was measured with 50 mM Tris-maleate buffer (pH 7.0), 10 mM 2-mercaptoethanol, 2 mM $MgCl_2$, 1 mM Triton X-100, 0.1 mM [^{32}P]PA, and enzyme protein (66). Choline kinase (EC 2.7.1.32) was measured with 50 mM glycine-NaOH buffer (pH 9.7), 10 mM $MgSO_4$, 10 mM ATP, 50 μM [*methyl*-^{14}C]choline, and enzyme protein (67). Phosphocholine cytidylyltrans-

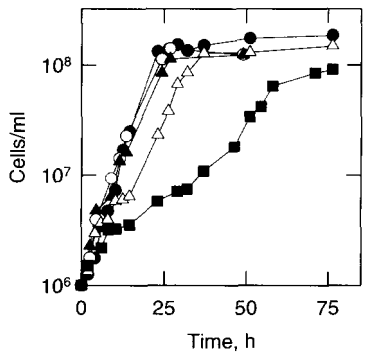


FIG. 3. **Effect of fumonisin B_1 on cell growth.** Cells were grown in the absence and presence of the indicated concentrations of fumonisin B_1. Cell numbers were determined by direct microscopic examination. These values were consistent with the number of viable cells determined by plate counts. The data shown is representative of three independent growth studies. Concentrations of fumonisin (μM): ●, 0; ○, 25; ▲, 50; △, 100; ■, 200.

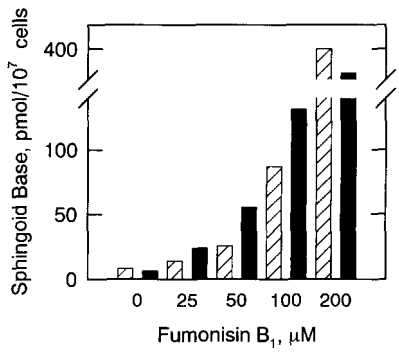


FIG. 4. **Effect of fumonisin B_1 on cellular concentrations of sphingoid bases.** Cells were grown for 30 h in the absence and presence of the indicated concentrations of fumonisin B_1. Sphingoid bases were extracted, and their *O*-phthalaldehyde derivatives were prepared and analyzed by high performance liquid chromatography as described in the text. The reported values were the average of three determinations. ▨, sphinganine; ■, phytosphingosine.

ferase (CTP:choline-phosphate cytidylyltransferase, EC 2.7.7.15) was measured with 50 mM Tris-HCl buffer (pH 8.0), 25 mM $MgCl_2$, 4 mM phosphocholine, 1 mM [α-^{32}P]CTP, and enzyme protein (68). Cholinephosphotransferase (CDPcholine:1,2-diacylglycerol cholinephosphotransferase, EC 2.7.8.2) was measured with 50 mM MOPS-NaOH buffer (pH 7.5), 20 mM $MgCl_2$, 6.5 mM Triton X-100, 1.3 mM DG, 1.3 mM PC, 0.5 mM [*methyl*-^{14}C]CDP-choline, and enzyme protein (22). All assays were linear with time and protein concentration. A unit of enzymatic activity was defined as the amount of enzyme that catalyzed the formation of 1 nmol of product/min unless otherwise indicated. Specific activity was defined as units/mg of protein. Protein concentration was determined by the method of Bradford (69) using bovine serum albumin as the standard.

RESULTS

Effect of Fumonisin B_1 on Cell Growth—The effect of fumonisin B_1 on cell growth was examined. In these studies it was necessary to include a low percentage of the detergent Tergitol to the growth medium to facilitate fumonisin B_1 uptake by the cells. Tergitol did not have a significant effect on the growth of cells grown in the absence of fumonisin B_1. The addition of fumonisin B_1 to the growth medium resulted in a dose-dependent inhibition in the growth rate of cells (Fig. 3). Fumonisin B_1 also caused an increase in the incubation time required to reach the stationary phase of growth (Fig. 3). By the time cells reached the stationary phase of growth, the final cell densities of the cultures grown in the presence of fumonisin B_1 approached the final cell density of the culture grown in the absence of fumonisin B_1 (Fig. 3). We questioned whether or not a population of cells was being selected for that was resistant to fumonisin B_1. To address this question, cells were taken from a stationary phase culture grown in the presence of 100 μM fumonisin B_1. These cells were washed in fresh growth medium and inoculated into fresh growth medium with and without fumonisin B_1. These cells responded to fumonisin B_1 as described above. Thus, these cells were not resistant to growth inhibition by fumonisin B_1.

Effect of Fumonisin B_1 on Cellular Concentrations of Sphingoid Bases—We previously demonstrated that free sphinganine and phytosphingosine exist in *S. cerevisiae* (24). We examined the cellular concentrations of these sphingoid bases in cells grown for 30 h in the absence and presence of fumonisin B_1. Fumonisin B_1 caused a dose-dependent increase in the cellular concentrations of sphingoid bases (Fig. 4). Cells grown in the presence of fumonisin B_1 accumulated 11- to 50-fold more sphinganine and 22- to 50-fold more phytosphingosine when compared with control cells. Subsequent growth studies were performed with a fumonisin B_1 concentration of 100 μM.

To determine if the accumulation of sphingoid bases was affected by growth phase, the cellular concentrations of sphinganine and phytosphingosine in exponential phase cells were determined and found to be 6 to 11 pmol/10^7 cells (Fig. 5). There was a 2-fold increase in the cellular concentrations of these sphingoid bases when cells entered the stationary phase of growth (Fig. 5). Thus, the cellular levels of sphingoid bases were related to the growth phase. However, the increase in sphingoid base concentrations due to growth phase regulation was small when compared with the increase due to fumonisin B_1 supplementation. The accumulation of sphinganine (Fig. 5*A*) and to a lesser extent phytosphingosine (Fig. 5*B*) was highest in exponential phase cells when compared with stationary phase cells. Thus, the large accumulation of the sphingoid bases was largely due to fumonisin B_1 and not due to growth phase regulation.

Effect of Fumonisin B_1 on Ceramide Synthase Activity and the Cellular Concentration of Ceramide—Sphinganine is a substrate in the reaction catalyzed by ceramide synthase (62). We questioned whether the mechanism of sphingoid base accumulation in *S. cerevisiae* cells was due to the inhibition of ceramide synthase activity by fumonisin B_1. To examine ceramide synthase activity, we used the assay system developed for the measurement of this enzyme in mammalian cells (62). Ceramide synthase activity was indeed found in the total membrane fraction of *S. cerevisiae* at a specific activity of 16.5 pmol/min/mg. Addition of 100 μM fumonisin B_1 to the assay resulted in a 90% decrease in ceramide synthase activity (Fig. 6*A*).

If the mechanism of sphingoid base accumulation in cells grown with fumonisin B_1 was due to the inhibition of ceramide synthase activity, one would expect that the cellular concentration of ceramide would be reduced. To address this question, cells were grown to the exponential phase of growth in the absence and presence of 100 μM fumonisin B_1. Ceramide was extracted from cells, and the cellular concentration was determined using *E. coli* DG kinase. The amount of ceramide in cells grown in the presence of fumonisin B_1 was 15% of the concentration in the control cells (Fig. 6*B*).

Effect of Fumonisin B_1 on Sphingolipid Synthesis and Composition—Sphingolipids in *S. cerevisiae* differ from those of mammalian cells in that they are structurally less complex and contain phosphoinositol as part of their polar head groups (70). The major sphingolipids in *S. cerevisiae* are IPC, MIPC, and $M(IP)_2C$ (70). These inositol-containing sphingolipids are composed of phytosphingosine, to which a long chain fatty acid is linked via an amide bond (70). IPC, MIPC, and $M(IP)_2C$ are believed to be synthesized via the pathway shown in Fig. 1 (46, 70). Since ceramide is the direct precursor for sphingolipid

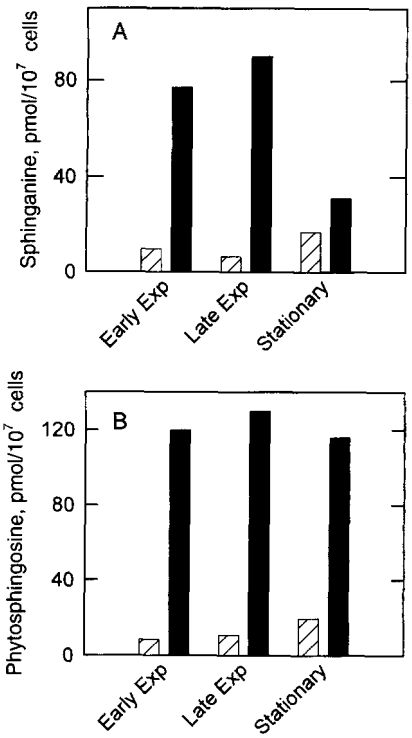


FIG. 5. **Effect of growth phase on cellular concentrations of sphingoid bases.** Cells were grown in the absence and presence of 100 μM fumonisin B_1 as indicated. Cells were harvested in the early exponential (5×10^6 cells/ml), late exponential (5×10^7 cells/ml), and stationary (1.2×10^8 cells/ml) phases of growth. Sphingoid bases were extracted, and their *O*-phthalaldehyde derivatives were prepared and analyzed by high performance liquid chromatography as described in the text. The reported values were the average of three determinations. *Exp*, exponential. ▨, control; ■, fumonisin B_1.

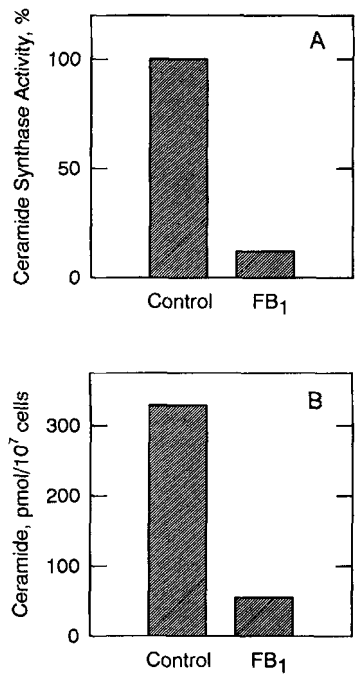


FIG. 6. **Effect of fumonisin B_1 on ceramide synthase activity and the cellular concentration of ceramide.** *A*, ceramide synthase activity was measured in the absence and presence of 100 μM fumonisin B_1 as indicated. Total membranes were used as the source of ceramide synthase. The specific activity of ceramide synthase in total membranes was 16.5 pmol/min/mg. *B*, cells were grown to the late exponential (5×10^7 cells/ml) phase of growth in the absence and presence of 100 μM fumonisin B_1 as indicated. Ceramide was extracted and quantified using *E. coli* DG kinase as described in the text. *FB_1*, fumonisin B_1.

synthesis, we examined the effect of fumonisin B_1 on sphingolipid synthesis and composition. The phosphoinositol head group of yeast sphingolipids is derived from the membrane phospholipid PI (70). Since PI is synthesized from inositol (Fig. 1), sphingolipid synthesis was followed by pulse-labeling cells with [2-^{3}H]inositol. The amount of [2-^{3}H]inositol incorporated into each sphingolipid represented the relative rates of synthesis during the pulse. The addition of fumonisin B_1 to the growth medium resulted in a decreased incorporation of [2-^{3}H]inositol into IPC (5-fold), MIPC (3-fold), and $M(IP)_2C$ (2-fold) when compared with cells grown in the absence of fumonisin B_1 (Fig. 7*A*). The concentration of inositol added to the growth medium was only 0.1 μM, which is too low to affect the synthesis of PI or overall phospholipid synthesis (71).

Cells were labeled with [2-^{3}H]inositol to a steady-state to analyze the effect of fumonisin B_1 on sphingolipid composition. Addition of fumonisin B_1 to the growth medium caused a decrease in the steady-state concentrations of IPC (2.8-fold), MIPC (4.6-fold), and $M(IP)_2C$ (2.4-fold) (Fig. 7*B*).

Effect of Fumonisin B_1 on Phospholipid Synthesis and Composition—When *S. cerevisiae* cells are grown in the absence of choline, the major membrane phospholipid PC is primarily synthesized by a CDP-DG-dependent pathway via the reaction sequence: PA → CDP-DG → PS → PE → PC (7, 8) (Fig. 1). PI is also synthesized from CDP-DG (7, 8) (Fig. 1). The partitioning of CDP-DG between PI and PS is highly regulated in *S. cerevisiae* (7, 14). Since sphingolipid synthesis is dependent on the synthesis of PI (70), we examined the effect of fumonisin B_1 on overall phospholipid synthesis and composition. Phospholipid synthesis was followed by pulse-labeling with [2-^{14}C]acetate of cells grown in the absence and presence of 100 μM fumonisin B_1. The amount of [2-^{14}C]acetate incorporated into each phospholipid represented the relative rates of synthesis during the pulse. The presence of fumonisin B_1 in the growth medium caused a decrease in the incorporation of label into PA (2-fold), CDP-DG (3-fold), PS (2-fold), PE (2-fold), and PC (1.5-fold) when compared with control cells (Fig. 8*A*). The incorporation of the label into PI was not inhibited by fumonisin B_1. Instead, the synthesis of PI increased by 1.4-fold (Fig. 8*A*). As was seen in the labeling experiments using [2-^{3}H]inositol, the synthesis of sphingolipids from [2-^{14}C]acetate was inhibited by fumonisin B_1 (Fig. 8*A*).

The effect of fumonisin B_1 on the steady-state phospholipid composition is shown in Fig. 8*B*. The steady-state concentrations were decreased for PA (2-fold), CDP-DG (1.7-fold), PS (3-fold), PE (2-fold), and PC (1.2-fold) in cells supplemented with fumonisin B_1 when compared with control cells. Fumonisin B_1 did not significantly affect the cellular concentration of PI.

Effect of Fumonisin B_1 on Neutral Lipid Synthesis and Composition—We also examined the effect of fumonisin B_1 on neutral lipid synthesis by growing cells in the absence and presence of 100 μM fumonisin B_1 and pulse-labeling with [2-^{14}C]acetate. Fumonisin B_1 decreased in the synthesis of DG (1.2-fold), monoacylglycerol (2-fold), fatty acids (1.5-fold), fatty alcohols (2-fold), and ergosterol (2.6-fold) when compared to control cells (Fig. 9*A*).

Steady-state labeling of cells with [2-^{14}C]acetate was performed to analyze neutral lipid composition (Fig. 9*B*). Fumonisin B_1 supplementation increased triacylglycerols (1.8-fold), fatty acids (2.6-fold), and ergosterol esters (1.7-fold) and decreased ergosterol (1.2-fold).

Effect of Fumonisin B_1 and Sphingoid Bases on Lipid Biosynthetic Enzyme Activities—The pulse- and steady-state labeling experiments showed that fumonisin B_1 altered the synthesis and composition of sphingolipids, phospholipids, and

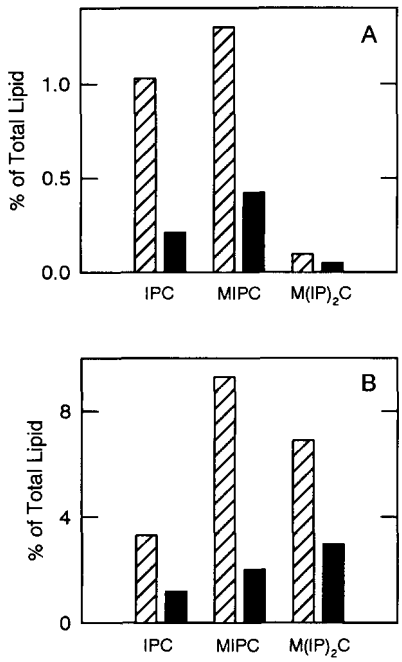


FIG. 7. **Effect of fumonisin B_1 on pulse-labeling of sphingolipids and sphingolipid composition.** *A*, cells were grown to the exponential (2×10^7 cells/ml) phase of growth in the absence and presence of 100 μM fumonisin B_1 as indicated. Cells were then incubated with [2-^{3}H]inositol (4 μCi/ml) for 30 min to pulse-label sphingolipids. The incorporation of [2-^{3}H]inositol into sphingolipids during the pulse was 2,000–4,500 cpm/10^7 cells. *B*, cells were grown to the exponential (2×10^7 cells/ml) phase of growth in the absence and presence of 100 μM fumonisin B_1 as indicated. The steady-state sphingolipid composition was determined by labeling cells for five to six generations with [2-^{3}H]inositol (2 μCi/ml). The incorporation of [2-^{3}H]inositol into sphingolipids during the steady-state labeling was 3,000–8,500 cpm/10^7 cells. The sphingolipid composition of the cells was determined as described in the text. The percentages shown for sphingolipids were normalized to the total lipid composition of cells labeled with [2-^{14}C]acetate. ▨, control; ■, fumonisin B_1.

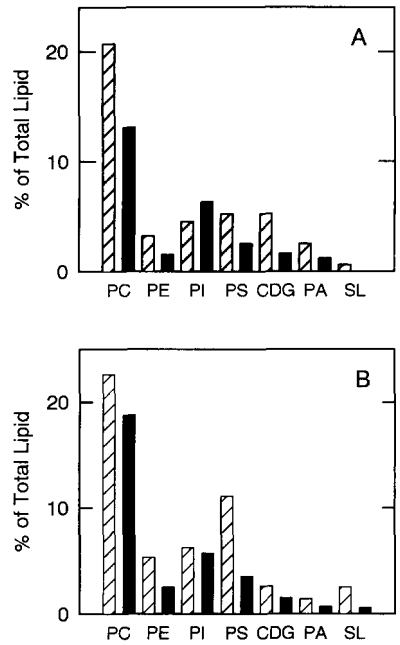


FIG. 8. **Effect of fumonisin B_1 on pulse-labeling of phospholipids and phospholipid composition.** *A*, cells were grown to the exponential (2×10^7 cells/ml) phase of growth in the absence and presence of 100 μM fumonisin B_1 as indicated. Cells were then incubated with [2-^{14}C]acetate (12 μCi/ml) for 30 min to pulse-label phospholipids. The incorporation of [2-^{14}C]acetate into phospholipids during the pulse was 15,000–16,000 cpm/10^7 cells. *B*, cells were grown to the exponential (2×10^7 cells/ml) phase of growth in the absence and presence of 100 μM fumonisin B_1 as indicated. The steady-state phospholipid composition was determined by labeling cells for five to six generations with [2-^{14}C]acetate (2 μCi/ml). The incorporation of [2-^{14}C]acetate into phospholipids during steady-state labeling was 22,000–26,000 cpm/10^7 cells. The phospholipid composition of the cells was determined as described in the text. The percentages shown for phospholipids were normalized to the total lipid composition of cells labeled with [2-^{14}C]acetate. *SL*, sphingolipids. ▨, control; ■, fumonisin B_1.

neutral lipids. We questioned if the expression of several key lipid biosynthetic enzyme activities were affected in cells grown with fumonisin B_1. These enzyme activities included those responsible for sphingolipid synthesis (IPC synthase), phospholipid synthesis via the CDP-DG-dependent pathway (CDP-DG synthase, PI synthase, PS synthase) and CDP-choline-dependent pathway (choline kinase, phosphocholine cytidylyltransferase, cholinephosphotransferase), and neutral lipid synthesis (PA phosphatase). We examined enzymes in the CDP-choline-dependent pathway because this pathway (Fig. 1) contributes to PC synthesis even when cells are cultured in growth medium lacking choline (72, 73). The choline required for the CDP-choline-dependent pathway is presumably derived from the turnover of PC synthesized by the CDP-DG-dependent pathway (72, 73). Cells were grown in the absence and presence of 100 μM fumonisin B_1, cells were harvested in the exponential phase of growth, cell extracts were prepared, and the activities of the enzymes were measured. These enzyme activities were not affected by the addition of fumonisin B_1 to the cells.

We also examined whether fumonisin B_1 and sphingoid bases had a direct effect on the activities of these key lipid biosynthetic enzymes. The activity of each enzyme was measured in the absence and presence of 100 μM fumonisin B_1, 100 μM sphinganine, and 100 μM phytosphingosine. None of the enzymes examined was affected directly by fumonisin B_1. On the other hand, IPC synthase, PS synthase, and PA phosphatase activities were inhibited by sphingoid bases (as will be described below), but the other enzymes were not affected.

IPC synthase (63) and PS synthase (65) activities were assayed with their lipid substrates as part of uniform Triton X-100/lipid-mixed micelles. Since sphingoid bases also form uniform mixed micelles with Triton X-100 and lipids (30), the concentrations of sphingoid bases were expressed as surface concentrations in mol % (24). Sphinganine and phytosphingosine inhibited IPC synthase activity in a dose-dependent manner with IC_{50} values of 3 mol % and 4.3 mol %, respectively (Fig. 10). PS synthase was also inhibited by sphinganine and phytosphingosine in a dose-dependent manner with IC_{50} values of 1.2 mol % and 1.9 mol %, respectively (Fig. 11). IC_{50} values were calculated from plots of the log of the activity values from Figs. 10 and 11 *versus* the inhibitor concentrations. Of the two sphingoid base inhibitors, sphinganine was the more potent inhibitor of IPC synthase and PS synthase activities. Similar results have been previously reported for PA phosphatase (24). Of these three enzymes, PS synthase activity was the most sensitive to inhibition by sphingoid bases.

DISCUSSION

The goal of this work was to examine the overall regulation of lipid biosynthesis in *S. cerevisiae* by sphingoid bases. Our rationale was to elevate sphingoid base levels with fumonisin B_1. Supplementation of *S. cerevisiae* cells with fumonisin B_1 resulted in accumulations in the cellular levels of free sphinganine and phytosphingosine. It is known that fumonisin B_1 inhibits ceramide synthase activity in mammalian cells (37). Ceramide synthase activity in *S. cerevisiae* was identified and shown to be inhibited by fumonisin B_1. Moreover, we showed

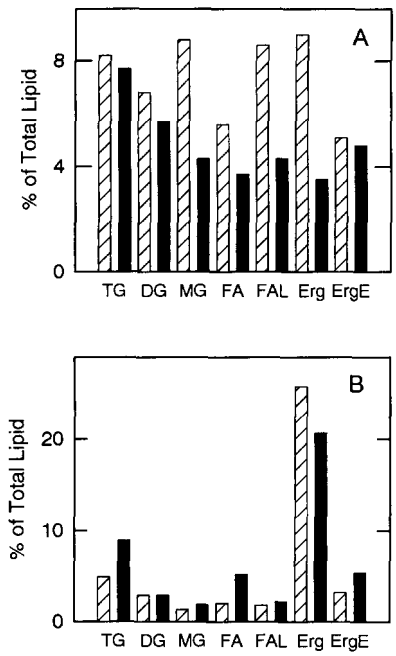


FIG. 9. **Effect of fumonisin B_1 on pulse-labeling of neutral lipids and neutral lipid composition.** *A*, cells were grown to the exponential (2×10^7 cells/ml) phase of growth in the absence and presence of 100 μM fumonisin B_1 as indicated. Cells were then incubated with [2-^{14}C]acetate (12 μCi/ml) for 30 min to pulse-label neutral lipids. The incorporation of [2-^{14}C]acetate into neutral lipids during the pulse was 9,000–16,000 cpm/10^7 cells. *B*, cells were grown to the exponential (2×10^7 cells/ml) phase of growth in the absence and presence of 100 μM fumonisin B_1 as indicated. The steady-state neutral lipid composition was determined by labeling cells for five to six generations with [2-^{14}C]acetate (2 μCi/ml). The incorporation of [2-^{14}C]acetate into neutral lipids during steady-state labeling was 16,000–23,000 cpm/10^7 cells. The neutral lipid composition of the cells was determined as described in the text. The percentages shown for neutral lipids were normalized to the total lipid composition of cells labeled with [2-^{14}C]acetate. *TG*, triacylglycerol; *MG*, monoacylglycerol; *FA*, fatty acid; *FAL*, fatty alcohol; *Erg*, ergosterol; *ErgE*, ergosterol ester. ▨, control; ■, fumonisin B_1.

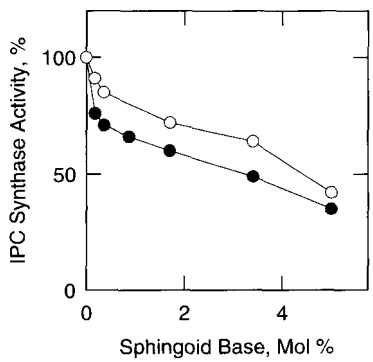


FIG. 10. **Effect of sphingoid bases on IPC synthase activity.** IPC synthase activity was measured in the absence and presence of the indicated surface concentrations of sphinganine (●) and phytosphingosine (○). A Triton X-100 extract of microsomal membranes was used as the source of IPC synthase. The specific activity of IPC synthase in the Triton X-100 extract was 0.5 nmol/min/mg.

here that fumonisin B_1 caused a decrease in the cellular concentration of ceramide. Cells supplemented with fumonisin B_1 also showed decreases in the synthesis and steady-state levels of IPC, MIPC, and $M(IP)_2C$. Taken together, these data indicated that the reduction in the cellular concentration of ceramide, brought about by the inhibition of ceramide synthase activity by fumonisin B_1, resulted in a decrease in sphingolipid synthesis and composition. Inositol-containing sphingolipids

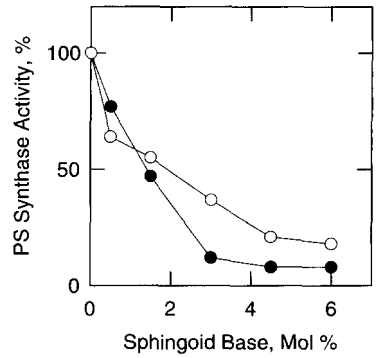


FIG. 11. **Effect of sphingoid bases on PS synthase activity.** PS synthase activity was measured in the absence and presence of the indicated surface concentrations of sphinganine (●) and phytosphingosine (○). A cell-free extract was used as the source of PS synthase. The specific activity of PS synthase in the cell extract was 0.45 nmol/min/mg.

play an essential role in cell growth (74, 75). Thus, the inhibition of cell growth by fumonisin B_1 must be due in part to the decrease in sphingolipid synthesis.

One mechanism for the decrease in sphingolipid synthesis could be attributed to the regulation of IPC synthase activity. IPC synthase, which catalyzes the committed step in sphingolipid synthesis from ceramide and PI (46), was inhibited *in vitro* by sphinganine and phytosphingosine. Thus, the elevation of sphingoid bases *in vivo* caused by fumonisin B_1 supplementation may have led to a decrease in IPC synthase activity. Another mechanism that may account for the decrease in sphingolipid synthesis may be the reduction in the cellular concentration of ceramide as available substrate for the IPC synthase reaction.

Fumonisin B_1 caused a decrease in the synthesis and composition of phospholipids primarily synthesized by the CDP-DG-dependent pathway. The mechanism of the inhibition of phospholipid synthesis was likely to be very complex. At least one aspect of this complex mechanism may be the regulation of PS synthase activity, which was inhibited *in vitro* by sphingoid bases. This inhibition was consistent with decreased PS synthesis and composition. Fumonisin B_1 also caused decreased PA synthesis and composition. PA is a potent activator of PS synthase activity (20). Thus, the decrease in PA levels may have also contributed to the decreased synthesis of PS. The decreased synthesis in PS may in turn be responsible for the decreased synthesis and composition of PE and PC. These phospholipids are derived from PS in the CDP-DG-dependent pathway (7). Enzymes responsible for PC synthesis via the CDP-choline-dependent pathway were not affected in cells supplemented with fumonisin B_1 nor were their activities directly affected by fumonisin B_1 or sphingoid bases.

The only major phospholipid whose synthesis and composition was not decreased by fumonisin B_1 was PI. In fact there was a modest increase in PI synthesis. This was not due to an increase in the expression of PI synthase activity in fumonisin B_1-supplemented cells or an activation of activity by sphingoid bases. The increase in PI synthesis may be attributed to the lack of its utilization as a precursor for the synthesis of sphingolipids. Furthermore, the increased synthesis in PI may be attributed to the inhibition of PS synthase activity by sphingoid bases. PS synthase (76) and PI synthase (61) use CDP-DG as a substrate. Previous work has shown that the partitioning of CDP-DG between PS and PI is regulated through the inhibition of PS synthase expression (41, 42, 77) and activity (14) by inositol. This inhibition leads to an increase in PI synthesis at the expense of PS synthesis (14). In a similar manner, the inhibition of PS synthase activity by sphingoid bases may have

contributed to the increase in PI synthesis.

The addition of fumonisin B_1 to cells also caused a decrease in the synthesis of neutral lipids. The effect of fumonisin B_1 on DG synthesis was relatively small, but was consistent with the inhibition of PA phosphatase activity by sphingoid bases (24). The steady-state level of DG was not affected by fumonisin B_1. It should be noted that DG is both a substrate and product of many reactions in lipid metabolism (3, 7, 11), and, thus, cellular levels of DG arise from a balance of both synthetic and degradation reactions. The increase in the steady-state composition of TG in the fumonisin B_1-supplemented cells was just the opposite of what one would expect if TG levels were only due to the regulation of PA phosphatase activity by sphingoid bases. Thus, the regulation of TG synthesis and composition by fumonisin B_1 are not explained by any of these analyses.

Fumonisin B_1 caused a decrease in ergosterol synthesis and composition. The mechanism for these changes was not addressed here. Ergosterol is known to stimulate glycerophosphate acyltransferase and PE methyltransferase activities in *S. cerevisiae* (78). These enzymes are responsible for PA synthesis and PC synthesis via the CDP-DG-dependent pathway, respectively (7). Thus, the decrease in ergosterol synthesis in fumonisin B_1-supplemented cells may have contributed to the decreased synthesis of PA and PC through the regulation of glycerophosphate acyltransferase and PE methyltransferase activities.

When *S. cerevisiae* cells enter the stationary phase, TG is elevated relative to phospholipids (79), and ergosterol esters are elevated relative to ergosterol (80). Similar changes in the lipid composition were observed here when cells were grown in the presence of fumonisin B_1. If sphingoid bases are cellular signals of growth phase, the fumonisin B_1 may have affected yeast growth by simulating the sphingoid base concentrations of stationary phase cells. This would cause the yeast to enter a stationary phase-like stage prematurely. This notion was consistent with the observations in both lipid composition and growth rate of fumonisin B_1-supplemented cells.

Fumonisin B_1 was a useful tool to examine the effect of sphingoid bases on lipid synthesis in *S. cerevisiae*. The data reported here showed that the synthesis of the major lipid classes was coordinately regulated by sphingoid bases. The mechanism of this regulation involved the direct inhibition of IPC synthase, PS synthase, and PA phosphatase activities by sphingoid bases. These three enzymes catalyze reactions which commit to the synthesis of sphingolipids, phospholipids, and TG (Fig. 1). Thus, these enzyme activities play an important role in the regulation of overall lipid synthesis. In addition, the expression of IPC synthase (63), PS synthase (41, 42, 77), and PA phosphatase (53, 81) are coordinately regulated by inositol, which plays a major role in lipid synthesis in *S. cerevisiae* (7, 11). The studies reported here underscore the complexity of the mechanisms which regulate lipid synthesis in *S. cerevisiae*.

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