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In Vitro and In Situ Tracking of Choline-Phospholipid Biogenesis by MALDI TOF-MS

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The quantitative monitoring of newly synthesized species of phosphatidylcholines (PCs) and sphingomyelins (SMs) has been achieved in cultured human lens epithelial cells, both in situ and in vitro, with the use of MALDI TOF-MS. As the cells were cultured with deuterated choline-d_9, new peaks that differed from the hydrogenated species by 9.06 Da appeared in the mass spectra. The initial rates of appearance of all deuterated species of PCs were comparable and 4 times higher than those for SMs. After 12 h, those rates began to decrease for PCs but not for deuterated SMs, whose relative contents continued to increase throughout the 72 h of the experiment. The differences in initial rates are consistent with the reported initial generation of PCs, their subsequent degradation, and transfer of their headgroup, phosphorylcholine, to SMs. To further test the ability of MALDI TOF-MS to quantify changes in phospholipid (PL) metabolic pathways, myriocin, an inhibitor of SM synthesis, was added to the cells. In vitro and in situ results revealed a decrease in SMs and an unexpected increase in some PCs. With the use of other deuterated precursors and in combination with postsource decay or tandem MS/MS, this approach could allow the simultaneous tracking of the biosynthesis of multiple PL classes while providing details on their acyl chains.

Much of our current understanding of phospholipid (PL) metabolism is based on studies performed with the use of radioactive or stable isotopic markers.[1] Once the labeled precursors have been incorporated, lipids are extracted. Their separation into lipid classes is then performed, most often, by chromatography. The levels of incorporated labels are then measured via radiation counting or NMR spectroscopy. While these approaches allow the quantification of the relative contents of each lipid class, further steps of analysis must be performed to obtain information on the acyl chain distribution of the various PL classes. Recently, mass spectrometric studies using electrospray ionization have demonstrated the ability to monitor the synthesis of phosphatidylcholines (PCs) and phosphatidylinositols (PIs) in vitro. This was accomplished by the analysis of lipids extracted from the nuclei and whole cells that were cultured with exogenous addition of deuterated precursors, namely, choline-d_9 and inositol-d_6.[2−4]

The biogenesis of PCs and sphingomyelins (SMs) and the coupling of their metabolic pathways are of special interest to our research because we have observed strong correlations between the growth of mammalian lenses and the relative contents of PCs, SMs, and dihydrosphingomyelins in their membranes.[5,6] Those correlations were established from compositional studies of lens membranes carried out in vitro by ^{31}P NMR spectroscopy and matrix-assisted laser desorption/ionization (MALDI) time-of-flight mass spectrometry (TOF-MS)[6,7] as well as in situ, on lens slices with the use of MALDI TOF-MS.[8] Other research groups have also demonstrated the ability of MALDI to ionize PLs in situ in brain slices,[9,10] zooplankter,[11] and whole cells.[12−16] In this work, we have applied MALDI TOF-MS to monitor quantitatively and simultaneously the synthesis of both PCs and SMs in a primary line of human lens epithelial cells cultured with the exogenous addition of deuterated choline.

Mass spectral studies on amniotic fluid have demonstrated the accuracy in the quantification of dipalmitoylphosphatidylcholine (DPC) when using the deuterium-labeled DPC-d_9 as internal

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standard.[17] Therefore, the approach described herein is expected to provide quantitative information on choline-containing PLs. To demonstrate such potential, the synthesis of sphingolipids was inhibited by the addition of myriocin or ISP-1, an inhibitor of the serine palmitoyltransferase[18,19] that is involved in the first step of the de novo synthesis of sphingolipids.[20−22] By comparison to PL levels in control experiments (without myriocin), the specific species of SMs and PCs affected by the addition of the inhibitor have been identified. In addition, the extent of the effect has been quantified and compared to results obtained by ^{31}P NMR spectroscopy.

EXPERIMENTAL SECTION

Chemicals and Reagents. Methanol, chloroform, chloroform-*d*, 2,5-dihydroxybenzoic acid, the sodium salt of 1,2-ditetradecanoyl-*sn*-glycero-3-phosphatidic acid, PA(28:0), the ammonium salt of 1,2-dioctadecanoyl-*sn*-glycero-3-phospho-*rac*-[1-glycerol], PG-(36:0), trypsin−ethylenediaminetetracetic acid (EDTA) solution (0.5 g/L of porcine trypsin and 0.2 g/L Na_4EDTA) cell-cultured tested, heat-inactivated fetal bovine serum (FBS), myriocin (from *Mycelia sterilia*), gentamicin sulfate, and medium 199 (M199) (with Earle's salts, L-glutamine, and without sodium bicarbonate) were purchased from Sigma (St. Louis, MO). Cesium chloride, sodium bicarbonate, and *p*-nitroaniline (PNA) were obtained from Aldrich Chemical Co., Inc. (Milwaukee, WI). EDTA was purchased from Fisher Scientific (Fair Lawn, NJ). Choline chloride (trimethyl-d_9, 98%) (choline-d_9) was obtained from Cambridge Isotope Laboratories (Andover, MA). Keratinocyte growth medium (KGM) was bought from Cambrex Bio Science Walkersville, Inc. (Walkersville, MD). Choline chloride (choline-h_9) was purchased from MP Biomedicals, Inc. (Aurora, OH). A primary line of human lens epithelial cells (FHL124 cells) was generated and provided by Dr. J. R. Reddan.[23]

Cell Culture. The cells were incubated in a humidified atmosphere with 5% CO_2 at 37 °C using as the medium solution a 1:4 mixture of KGM and M199 (prepared following the manufacturer's directions) supplemented with 50 μg/mL gentamicin and 10% (or 0.75%) FBS.[23] For mass spectrometric studies, cells were seeded in 25-cm^2 culture flasks for in vitro analysis, and in 35-mm-diameter Petri dishes, each with one or two cover glasses (see Figure 1A), for in situ analysis. Because the coverslips may shift their position during culturing, it is best to have a single coverslip per dish to avoid overlap. For analysis by ^{31}P NMR spectroscopy, cells were cultured in 75-cm^2 flasks. To obtain spectra with acceptable S/N after 3−4 h of acquisition, it was necessary to pool the cells from three of these flasks.

Inhibition of SM Synthesis by Myriocin. FHL124 cells were incubated for 24 h in media with 0.75% FBS. After this period of time, the cells were ~25% confluent. The medium was removed and replaced with fresh medium containing 100 μM choline-d_9 and different concentrations of myriocin (50, 10^2, 10^3, and 10^4 nM). Cells in the control group were grown in the same media without myriocin. Time-dependent studies were conducted in vitro and in situ, as described below. In addition, cells cultured with and without myriocin for 72 h were detached from the flasks and centrifuged. The pelleted cells were weighted prior to PL extraction and analysis.

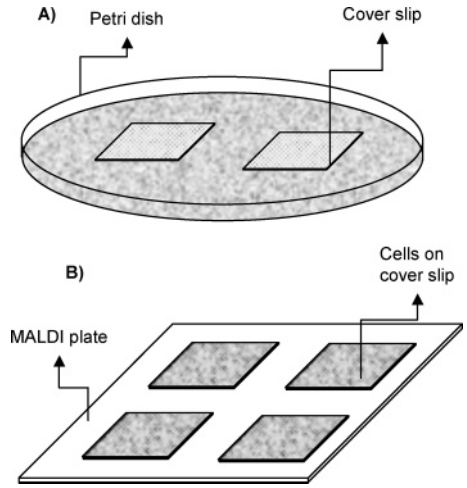

Figure 1. Schematic representation of steps followed for in situ analysis of FHL124 cells by MALDI TOF-MS. (A) Cells cultured on coverslips in a Petri dish. (B) Coverslips with attached cells are taped onto the MALDI plate with double-sided adhesive tape.

Sample Preparation. Extraction of Lipids for in Vitro Analysis. FHL124 cells were detached from the flask with the trypsin−EDTA solution (4 mL/25-cm^2 flask). After incubation at 37 °C for 10 min, medium was added to dilute the trypsin. The suspended cells were centrifuged at 2000 rpm for 5 min (Centrific model 225, Fisher Scientific, Fair Lawn, NJ). The pelleted cells (~5 mg of wet weight) were obtained and washed with 200 μL of a 0.1 M CsCl solution to exchange Cs^+ for H^+, Na^+, and K^+. The aqueous suspension was shaken gently for 1 min and centrifuged at 2000 rpm for 5 min. The pellet was obtained and the lipids were extracted by adding 100 μL of methanol. This monophasic extraction precludes the formation of an interfacial layer between the organic and aqueous phases of conventional biphasic extraction protocols. Because lipids may be trapped within that layer, the monophasic extraction with methanol reduces lipid loss.[24] The suspension was sonicated at 40 °C in an ultrasonic bath (model 8890 ultrasonic cleaner, Cole-Palmer Instruments Co., Vernon Hills, IL) for 10 min and centrifuged for 5 min. The supernatant was collected for analysis by MALDI TOF-MS. The same procedure was followed to prepare the sample for ^{31}P NMR analysis, but the volumes of solvents and reactants were scaled up to account for the larger amount of cells present in the pooled flasks.

In Situ Mass Spectral Analysis. The coverslips with the attached cells were removed from the solution in the Petri dishes, washed with deionized water, and dried.

PL Analysis by MALDI TOF-MS. MALDI TOF-MS was used to identify and quantify the acyl chain composition of the various

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PL classes as well as to track the synthesis of SMs and PCs. A Voyager Biospectrometry DE workstation (Applied Biosystems, Foster City, CA) equipped with a 337-nm pulsed nitrogen laser was used to acquire all mass spectra. The extraction voltage was set at 20 kV. First, spectra were acquired by setting the low-mass gate at 70 Da to detect possible fragments and adjust the laser intensity.[7,25] Then the mass range was narrowed (800−1100 Da) to prevent saturation of the detector by ions generated from the matrix.[26,27]

The analysis in vitro was performed on a 400-spot (20 × 20) hydrophobic plate (Teflon plate) from Applied Biosystems. Aliquots of the sample and the matrix (0.3 μL each) were spotted onto the plate. PNA, prepared in chloroform/methanol (2:1) at a concentration of 0.15 M, was used as the matrix. Because the cells were washed with a 0.1 M CsCl solution, Cs adducts were generated, and it was not necessary to add CsCl crystals to the sample, as reported previously.[7,25]

For the analysis in situ, coverslips with the cells were attached onto a stainless steel MALDI plate with double-sided adhesive tape (Figure 1B). To avoid spreading, a 0.3-μL aliquot of the matrix was carefully spotted for every 3−4-mm^2 area of the coverslip. The matrix used to acquire positive-ion spectra was a methanolic solution of 0.15 M PNA with 0.09 M CsCl. CsCl not only reduced spectral complexity but also increased homogeneity in the crystallization and minimized the loss of matrix caused by the high vacuum in the ionization chamber.[8] All samples were allowed to crystallize at ambient conditions, and the spectra were acquired in the reflector mode. For each of the 7−10 spots of a given sample, a mass spectrum was obtained from the average of multiple (50−150) traces acquired at different locations within each spot. Adducts of two synthetic PLs of known masses, [PA-(28:0) + 2Cs + H]$^+$ (m/z = 857.21) and [PG(36:0) + 2Cs]$^+$ (m/z = 1043.38), were used to perform a preliminary mass calibration of the spectra in the positive mode.[7] After the masses and identities of the PLs in the FHL124 cells were determined, subsequent mass calibrations were carried out using PLs present in the samples as references. [SM(16:0) + Cs]$^+$ (m/z = 835.47) and [SM(24:1) + Cs]$^+$ (m/z = 945.58) were chosen to calibrate the spectra. The identification of PL-related peaks was carried out with software developed in our laboratory, as reported previously.[25] The quantification was performed with the areas of the monoisotopic peaks after subtracting the baseline noise. This step was carried out with the Advanced Baseline Correction function incorporated in the Data Explorer software. The parameters used for this correction were peak width 32, flexibility 0.5, and degree 0.1. Each of the 7−10 spectra acquired for a given sample was processed to determine the relative contents of each PL. The results obtained from 4−8 spectra were then averaged.

Analysis of PLs by ^{31}P NMR Spectroscopy. ^{31}P NMR spectroscopy was used as a quantitative reference method to evaluate the results obtained by MALDI TOF-MS. To prepare the sample for spectral analysis, the solvent was removed from the lipid extract with a flow of nitrogen. The lipids were redissolved in 500 μL of chloroform-*d*, the solution was transferred to a NMR tube, and 250 μL of a cesium−EDTA (Cs$^+$−EDTA) reagent[28] were then added. The tube was immersed in a temperature-controlled ultrasonic bath for 10 min at 40 °C to homogenize the solution. A Varian INOVA-500 spectrometer was used to record the ^{31}P NMR spectra. The samples were spun at 18 rps. For each sample, spectra were acquired at different temperatures to remove spectral overlap.[6,7,29] Chemical shifts were referenced to internal SMs (δ = −0.09 ppm). Other acquisition parameters were identical to those reported previously.[25] Process and integration of spectral bands were performed with Mestrec NMR software (Mestrelab Research, Santiago de Compostela, Coruña, Spain).

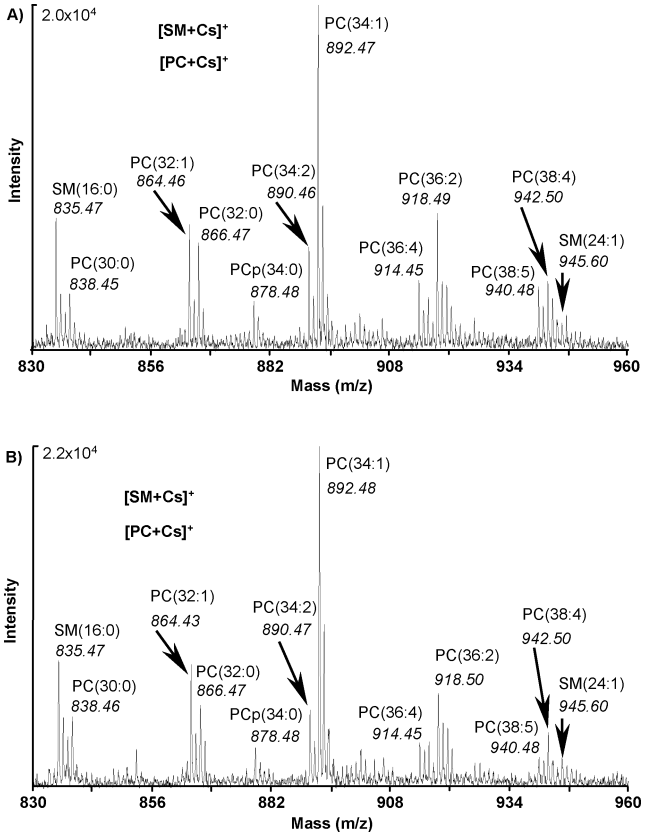

Figure 2. Comparison of positive-ion mass spectra of PLs acquired in vitro and in situ. (A) Spectrum of lipids extracted with methanol from FHL124 cells. (B) Spectrum acquired directly on cells grown on a cover slip. The peaks on the spectra correspond to Cs$^+$ adducts of PCs and SMs.

RESULTS AND DISCUSSION

In Vitro versus in Situ Analysis by MALDI TOF-MS. To test the feasibility of detecting and quantifying PLs in situ in cultured cells, parallel experiments were carried out in vitro and in situ to compare the values and precision of the results. Figure 2 shows the positive-ion spectra acquired in vitro (Figure 2A) and in situ (Figure 2B). The only peaks detected within the shown mass range (m/z = 830−970) correspond to the Cs$^+$ adducts of PCs and SMs. Other PLs, such as phosphatidylserines, phosphatidylethanolamines, and PIs were detected at higher m/z values as they form adducts with 2 Cs$^+$.[7,25] It should be mentioned, however,

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that due to their relatively low content and poor ionization efficiencies, these PL classes produced peaks of low intensities in positive-ion spectra. For this reason, and to eliminate the presence of PCs that make up nearly half of all PLs, noncholine PL classes were analyzed in the negative mode. (The relative levels of each PL class determined by ^{31}P NMR spectroscopy, as well as those corresponding to each species within a PL class evaluated in vitro by MALDI TOF-MS, are included as Supporting Information.) The low S/N of negative-ion spectra acquired in situ did not allow precise quantification. Because the goal of this study was the tracking of PCs and SMs, the other PL classes were not included in the quantitative analyses presented below.

The quantification of species of PCs and SMs revealed no significant differences between results obtained in vitro and in situ. For example, the levels of PC(34:1), the most abundant PC in the cells, were 30.4 ± 3.3% and 29.6 ± 0.9% ($n = 8$) of all PCs and SMs when measured in vitro and in situ, respectively. The values obtained for SM(16:0) in vitro and in situ were 11.4 ± 0.7% and 13.0 ± 1.6%, respectively. (All other values are listed in Table 1 of the Supporting Information). Regardless of the acquisition approach, the precision decreased for PLs present in smaller levels. For example, for those PLs making up less than 4% of all SMs and PCs, relative standard deviations (RSDs) between 20 and 45% were seen. This is a consequence of the increase in the relative contribution of background noise to the signals.

Because of the agreement observed between values obtained in vitro and in situ, both approaches were used to analyze the cell PLs in the experiments to be discussed next. It is pertinent to mention that there was a decrease in the S/N when performing the in situ analysis of cells that were less than 50% confluent. This is an expected consequence of the smaller number of cells available for analysis. This limitation could be alleviated by increasing the diameter of the laser so that more cells can be probed with each laser shot or by using more sensitive matrixes. For in vitro analysis, the reduction in the amount of extracted lipids from fewer cells was easily corrected by concentrating the extracts.

Phospholipid Incorporation of Choline-d_9 in FHL124 Cells. The concentration of choline in the culture medium was estimated to be 3 μM based on the specifications of the M199. It was therefore possible that the increase of choline in the medium to 100 μM could affect the lipid composition and cell growth. To test this possibility, control samples derived from cells fed with 100 μM choline-h_9 (Figure 3A) and without addition of exogenous choline-h_9 (spectra not shown) were analyzed. No significant differences were detected for those samples either by MALDI TOF-MS or by ^{31}P NMR spectroscopy. Although a choline-free medium would be a better choice[15] when tracking the biogenesis of deuterated PCs and SMs, the contribution of the protium form of choline was less that 3% after addition of the deuterated choline (100 μM). Therefore, no significant changes would be expected in the trends reported below.

When feeding the cells with choline-d_9, and as shown in Figure 3B, peaks not present for the control sample (Figure 3A) appeared and differed from the nondeuterated species by $\Delta m/z = 9.06$. The broken lines are aligned with peaks corresponding to nondeuterated species of PCs and SMs whereas the arrows point to those peaks that appeared after incorporation of choline-d_9. In addition

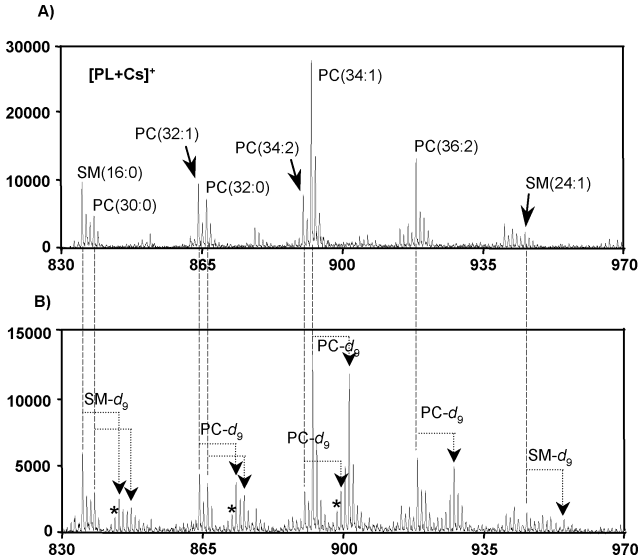

Figure 3. Positive-ion mass spectra of PLs from FHL124 cells incubated for 48 h with (A) 100 μM choline-h_9 and (B) 100 μM choline-d_9. The broken lines indicate the position of the peaks of nondeuterated PCs and SMs and the arrows in (B) point to newly synthesized PCs and SMs after the addition of choline-d_9. The asterisks indicate peaks with eight deuterons.

to those peaks, others that differed in mass by $\Delta m/z = 8.05$ were observed (see asterisks in Figure 3B). To assess the mechanism responsible for the generation of these ions with eight deuterons, rather than nine, choline-d_9 was spotted onto the MALDI plate. The mass spectra revealed that while the largest peak corresponded to choline-d_9 with $m/z = 113.16$, a smaller peak with $m/z = 112.16$ was also detected. The relative intensity of this peak increased with greater laser intensities. This trend is consistent with the photolytic loss of a deuteron and subsequent addition of a proton during the ionization/desorption process. In this work, the peaks that differed by $\Delta m/z = 8.05$ were not included in the quantification. However, care was taken to maintain the laser intensity at low levels and to avoid changes during the acquisition. With relatively low and constant laser intensity, the degree of exchange can be assumed to be equivalent for all newly synthesized deuterated PCs and SMs.

Time-Dependent Incorporation of Choline-d_9 by Individual Species of PCs and SMs in FHL124 Cells. To minimize possible cell cycle fluctuations in the synthesis and turnover of PLs in cultured cells,[30−35] FHL124 cells were synchronized after reaching 50% confluence by reducing the content of FBS in the media from 10 to 0.75%. After 24 h of starvation, the medium was removed and a new one containing 100 μM choline-d_9 was added ($t = 0$). After different periods of time, cells were collected and their PLs were analyzed by MALDI TOF-MS. The values for the relative incorporation of choline-d_9 into each individual PL were calculated as the ratio of the peak area corresponding to the

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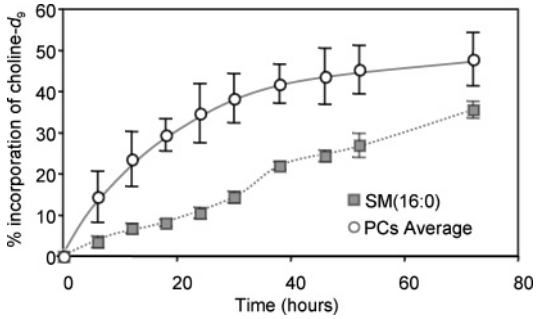

Figure 4. Time-dependent relative incorporation of choline-d_9 by PCs and SM(16:0) in FHL124 cells. To calculate the relative incorporation for each individual PL, the peak area corresponding to the deuterated PL was divided by the sum of peak areas associated with both nondeuterated and deuterated species of that particular PL. The values obtained for each of six PC species (32:0, 32:1, 34:1, 34:2, 36:1, 36:2) were then averaged (white circles). The solid squares represent the percentage of incorporation of choline-d_9 by SM(16:0). All data are included in the Supporting Information.

deuterated species and the sum of peak areas for both the deuterated and nondeuterated species for that particular PL. For example, to quantify the relative amount of newly synthesized SM(16:0), the area of the peak at $m/z = 844.53$ corresponding to the deuterated $[SM(16:0)\text{-}d_9 + Cs]^+$ was divided by the sum of areas corresponding to both nondeuterated $[SM(16:0)\text{-}h_9 + Cs]^+$ at $m/z = 835.47$ and deuterated species of SM(16:0). These values are listed for various times in Table 2 of the Supporting Information. The relative levels of choline-d_9 incorporation into each of the various PCs were not significantly different. Figure 4 shows the average of the relative uptake of choline-d_9 by the most abundant PCs (32:0, 32:1, 34:1, 34:2, 36:1, 36:2). For SMs, only data for SM(16:0)-d_9 are included. The large RSD for SM(24:1)-d_9 obscured the trend followed by the most abundant SM.

During the initial period after addition of exogenous choline ($t = 0-6$ h), the rates of incorporation of choline-d_9 into the various PCs were calculated and found to be not statistically different. The average of these rates, 2.4 ± 0.3% choline-d_9 incorporation per hour, was 4 times higher than that determined for SM(16:0), 0.6 ± 0.2% choline-d_9 incorporation per hour. These differences in the initial rates of generation of SM and PCs are consistent with the reported uptake of cholines by PCs first, followed by the subsequent hydrolysis of the phosphorylcholine headgroup and transfer to ceramides to form SMs.[20−22] At longer culture times, the rate of appearance of deuterated PCs decreased, reaching a value of 0.2 ± 0.2% after 48 h. For SM(16:0), on the other hand, the incorporation of choline-d_9 did not decrease during the experimental time but exhibited fluctuations. For example, after 24 h, the rate of appearance of SM(16:0)-d_9 increased but then decreased after 38 h. This behavior may be associated with changes in the incorporation or breakdown of this PL during different stages of the cell cycle. Studies are underway to confirm this possibility.

Cumulative Effects of Addition of Myriocin to FHL124 Cells. One of the goals of this work was to determine whether MALDI TOF-MS could be applied as a quantitative tool to study PL metabolic pathways. With that purpose, we investigated the effect the addition of myriocin, a potent inhibitor of the palmitoyl transferase involved first step of the synthesis of sphingolipids,[18,19] on the composition of SMs and PCs in FHL124 cells.

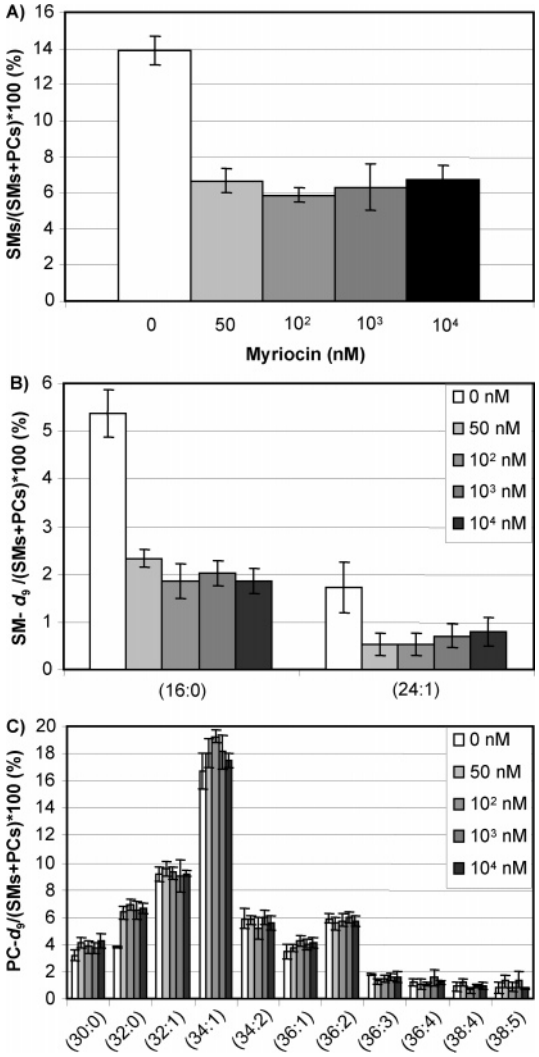

Figure 5. Effect of addition of myriocin for 72 h on the relative amounts of SMs and PCs in FHL124 cells. The average and standard deviation were evaluated from five mass spectral traces. (A) Percent of all SMs with respect to all SMs and PCs as a function of myriocin concentration (0 nM or control sample, shown by white bars). (B) Changes in the relative amounts of SM(16:0)-d_9 and SM(24:1)-d_9 as a function of myriocin concentration, and (C) Effect of myriocin addition on the acyl chain distribution of deuterated PCs.

After 72 h of exposure to the various concentrations of myriocin, a reduction in the rate of cell proliferation was observed (data not shown). Consistent with this observation, the weight of the pelleted cells decreased by ~1 mg, from 5.6 mg for the control to 4.3, 3.9, 4.1, and 3.6 mg for the cells treated with 50, 10^2, 10^3, and 10^4 nM, respectively. In addition, and relevant to this work, the analysis of positive-ion spectra showed a significant reduction in the relative amounts of SMs. As shown in Figure 5A, the amounts of all SMs relative to all SMs and PCs were more than 50% lower in the samples treated with myriocin (72 h) than in the control sample. No further decreases in the relative amount of SMs were observed with increasing concentrations of myriocin. This suggests that 50 nM is enough to cause maximal inhibition. A similar inhibitory concentration was reported when myriocin was added to mouse cytotoxic T cells.[18]

The contents of the newly synthesized deuterated SM(16:0)-d_9 and SM(24:1)-d_9, with respect to all SMs and PCs (nondeuter-

ated and deuterated species), were calculated and are shown in Figure 5B. Upon exposure to myriocin for 72 h, and for all concentrations tested, the relative amounts of both deuterated SMs were reduced significantly, by more than 50%. This is to be expected as myriocin inhibits the first step in the synthesis of SMs and should, therefore, have a similar impact on all SMs, regardless of the type of acyl chain.

To investigate whether myriocin had any impact on the synthesis of PCs, the relative levels of each deuterated PC with respect to all SMs and PCs were also calculated. As shown in Figure 5C, the addition of myriocin (at all concentrations tested) resulted in a significant increase in PC(32:0)-d_9. Indeed, its relative amount nearly doubled, from 3.78 ± 0.08% in the control group ($n = 5$) to 6.56 ± 0.23% in samples treated with myriocin ($n = 5$). This suggests that the blocking of the sphingolipid synthesis leads to a relative increase in palmitoyl chains, as they cannot be taken up to produce SM(16:0), the most abundant SM. As a consequence of this imbalance, PC(32:0), likely to have two palmitoyl chains, is synthesized in greater amounts.

As shown in Figure 5C, other species with more than two sites of unsaturation were also detected but in relatively small quantities. Because the *sn*-1 chain of PCs is usually a saturated palmitoyl (C16) or stearoyl (C18) or contains a single site of unsaturation,[36] PCs such as PC(36:3), PC(36:4), PC(38:4), and PC(38:5) are expected to have linoleoyl or arachidonoyl *sn*-2 chains. Since the hydrolysis of these PCs provides bioactive linoleic and arachidonic acids, efforts are underway to improve the sensitivity in the detection of these relevant species. In addition, to establish the exact number and position of the unsaturation sites, either postsource decay or a tandem MS/MS should be incorporated.[37,38]

Time-Dependent Incorporation of Choline-d_9 by PCs and SMs in FHL124 Cells Treated with Myriocin. After 24 h of starvation, cells that were ~50% confluent were cultured with 100 μM choline-d_9, without (control) and with 50 nM myriocin ($t = 0$). The relative amounts of each deuterated PC and SM species with respect to all PCs and SMs were calculated from spectra collected in vitro and in situ at various times between $t = 0$ and $t = 72$ h. The comparison of PC species in the control and treated samples only revealed significant changes in the relative amounts of PC(32:0)-d_9 that increased from 3.3 ± 0.1% in the control ($n = 4$) to 3.8 ± 0.2% in samples treated with myriocin for 46 h ($n = 4$) (data not shown). On the other hand, and as shown in Figure 6, a decrease in the relative amount of both SMs was observed. The SM(16:0)-d_9 decreased from 2.65 ± 0.03% in the control ($n = 4$) to 2.03 ± 0.23% in the cells treated with myriocin for 38 h ($n = 4$). This decrease was enhanced after 72 h, at which time the SM-(16:0)-d_9 was 3.4 ± 0.1% in the control ($n = 4$) and diminished to 2.1 ± 0.1% in the treated sample ($n = 4$). The reduction in the relative levels of SM(24:1)-d_9 was less pronounced or more difficult to detect as a result of the higher RSDs associated with components in relative minute amounts. The lack of significant changes in the levels of SMs during the first 1.5 days of exposure to myriocin was also observed in mouse cytotoxic T cells. Indeed, cell apoptosis caused by the reduction of sphingolipid levels was only observed after 32 h, once the cells had undergone one or two divisions.[18]

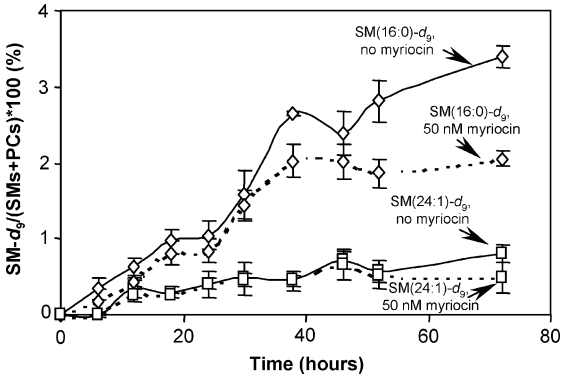

Figure 6. Time-dependent incorporation of choline-d_9 by SMs in FHL124 cells fed with 50 mM myriocin. Data are presented as the average ± standard deviation ($n = 4$) of the relative content of SM-(16:0)-d_9 and SM(24:1)-d_9 with respect to all SMs and PCs (nondeuterated and deuterated). The solid and broken lines show the time dependence for the control samples (0 nM myriocin) and treated samples, respectively.

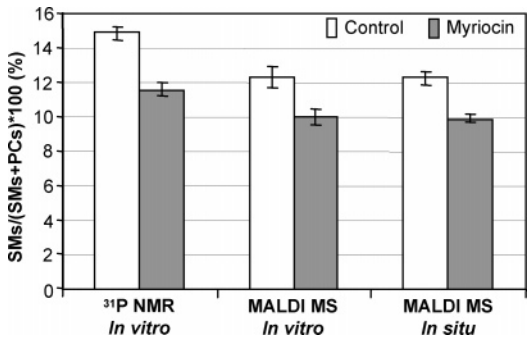

Figure 7. Comparison of quantitative results obtained by ^{31}P NMR spectroscopy and MALDI TOF-MS in vitro and in situ. The relative amounts of SMs (with respect to all SMs and PCs) in FHL124 cells cultured for 52 h diminished upon treatment with 50 nM myriocin.

To evaluate the accuracy of the quantification based on MALDI TOF-MS results, control and myriocin-treated samples at $t = 52$ h were analyzed by ^{31}P NMR spectroscopy. Except for PCs and SMs, there were no significant differences in the relative amounts of the other PL classes. The changes induced by myriocin in the relative amounts of SMs with respect to all SMs and PCs are shown in Figure 7 and are based on ^{31}P NMR spectral data and MALDI TOF-MS results, both in vitro and in situ. ^{31}P NMR results indicated a decrease in the relative content of SMs from 14.8 ± 0.4% in the control sample ($n = 5$) to 11.6 ± 0.4% in the sample treated with myriocin ($n = 5$). From in vitro MALDI TOF-MS data ($n = 5$), the content of SMs was reduced from 12.3 ± 0.4 to 10.0 ± 0.5% and a similar decrease, from 12.3 ± 0.4 to 9.9 ± 0.2%, was obtained from data acquired in situ ($n = 5$). The comparison of values obtained by ^{31}P NMR and MALDI TOF-MS reveals that the relative amounts for SMs were slightly lower when calculated from MALDI TOF-MS data. This is due to the lower ionization efficiency of SMs with respect to PCs when PNA is used as the matrix.[25] However, as shown in Figure 7, comparable trends were observed with the use of both techniques.

From a biological perspective, the reduction in cell growth induced by myriocin seems to be contradictory to our proposed roles for PCs and SMs in the control of mammalian lens growth.[5,6]

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Indeed, because we observed faster growth in lenses with higher levels of PCs and lower levels of SMs, the inhibition of SM synthesis was expected to increase the relative levels of PCs and thus enhance cell growth. However, different sphingolipid metabolites have been shown to either increase or decrease cell proliferation.[19,22,39−44] Therefore, a systematic study designed to inhibit different steps in the de novo synthesis and catabolism of sphingolipids should be pursued to elucidate the possible effect(s) of each metabolite on the rate of mitosis of lens epithelial cells.

CONCLUSIONS

The feasibility of using MALDI TOF-MS and deuterated precursors to monitor choline-PL metabolism in cultured cells has been demonstrated. Furthermore, the comparable accuracy and precision of in vitro and in situ measurements make this approach a quantitative and sensitive tool for the direct analysis of choline-containing PLs in whole cells. While this study was carried out with a single deuterated precursor and only one inhibitor, one can envision the addition of multiple deuterated precursors and other inhibitors that could provide details into anabolic and catabolic pathways involving various PL classes. However, differences in ionization efficiency for the various PL classes make the use of external standards imperative for absolute quantification.

One of the greatest advantages of current mass spectroscopic approaches over the more conventional use of isotopic labeling followed by chromatographic separation(s) is the ability to capture changes in acyl chain composition directly, without further separation/analysis steps.[12,45,46] Whereas in this work we have only reported the overall length and level of unsaturation, the use of postsource decay or tandem MS/MS can provide rigorous structural information[37,38] from which acyl chain specificity at each metabolic step could be revealed. Furthermore, these approaches can also resolve the possible presence of isomeric ions, such as ether-linked and alkenyl-linked PCs.[12,47]

One of the foreseen challenges in the analysis of the mass spectral data obtained with multiple precursors is the quantification. Care should be exercised to remove isotopic contributions and account for the possible presence of partially deuterated species. Addressing these challenges is a worthwhile effort given the relevance of the information to enhancing our understanding of lipid metabolism.

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SUPPORTING INFORMATION AVAILABLE

Phospholipid species detected in positive and negative modes by MALDI TOF MS. Phospholipid composition determined by ^{31}P NMR spectroscopy. Comparison of percentage for each PC and SM evaluated from mass spectral data acquire in vitro and in situ, and values for the incorporation of choline-d_9 by each individual species of PCs and SMs after different culture times. This material is available free of charge via the Internet at http://pubs.acs.org.

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