A comparison of the molecular specificities of whole cell and endonuclear phosphatidylcholine synthesis

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Abstract Deuterated choline-d_9 labelling of IMR-32 cells enabled comparison of the molecular specificities of whole cell and endonuclear phosphatidylcholine synthesis after 96 h polyunsaturated fatty acid supplementation. Surprisingly, while cell phosphatidylcholine synthesis and remodelling reflected a pattern of polyunsaturated fatty acid accretion, the saturated endonuclear phosphatidylcholine pool was only transiently labelled with polyunsaturates. Periodic endonuclear accumulations of the lipid second messenger diacylglycerol, mobilised from unsaturated phosphatidylinositol or saturated phosphatidylcholine, accompany cell proliferation. Non-specific incorporation into endonuclear phosphatidylcholine and selective removal or remodelling of unsaturated molecular species may form part of a single 'off switch' recycling all endonuclear diacylglycerol accumulations.

Key words: Phosphatidylcholine synthesis; IMR-32 nuclei; Deuterium labelling; Electrospray ionisation mass spectrometry

1. Introduction

Unlike cells in vivo, where homeostatic mechanisms actively resist change in phospholipid composition [1], cultured cells progressively lose capacity to counter the external manipulation of membrane phospholipid molecular species. For phosphatidylcholine (PtdCho), remodelling mechanisms involving the sequential actions of phospholipases and specific lyso PtdCho acyl transferases augment any synthetic specificity de novo to yield characteristic membrane composition [2]. An extensive literature defines the effects of fatty acid supplementation upon these parameters [3–6]. So, for example, HL-60 cells cultured in the presence of unsaturated and polyunsaturated fatty acid (PUFA), for 4 days at 30 μM, preferentially incorporate exogenous fatty acid into selected PtdCho molecular species, thereby increasing their fractional representation [7]. However, it is unclear to what extent specialised sub-cellular phospholipid compartments such as that in the nuclear matrix [8–11] may preserve composition [12] irrespective of bulk membrane variation.

In IMR-32 neuroblastoma cells, highly purified nuclei support autonomous PtdCho synthesis de novo [12] that is responsible for around 6% of whole cell PtdCho. Unlike bulk PtdCho, synthesised on the ER, endonuclear PtdCho retains more than 60% disaturated molecular species [12]. Several functional rationales may underpin the pattern of saturated endonuclear PtdCho synthesis [12] and a catalytically active, endonuclear isoform of CTP:phosphocholine cytidylyl transferase (CCTα) is essential for the survival of proliferating cells [13], confirming the pathway's pivotal role in growth and division. Indeed, export and caspase-mediated degradation of CCTα accompanies apoptosis [14] consistent with endonuclear capacity for PtdCho synthesis mandating cell viability.

Hydrolysis of endonuclear PtdCho and phosphatidylinositol (PtdIns) provide the periodic diacylglycerol (DAG) accumulations that accompany cell proliferation [11]. Consequently, in growing cells with progressive endonuclear phospholipid accretion, endonuclear DAG composition is determined by signalling-related accumulations from highly saturated PtdCho or highly polyunsaturated PtdIns [11] together with the products of lipid phosphate phosphatase action on phosphatidic acid and any DAG synthesis de novo. With no proven endonuclear PtdIns synthesis, the downstream fate of polyunsaturated DAG formed by hydrolysis of PtdIns is uncertain but may in principle be accessible for incorporation into PtdCho. However, while remodelling of newly synthesised mono- and di-unsaturated endonuclear PtdCho increases saturation [12], the concurrent depletion of PUFA-containing species [12] may also indicate the operation of a molecular selectivity mechanism that precludes PUFA–DAG incorporation into endonuclear PtdCho. Tandem electrospray ionisation mass spectrometry (ESI-MS) following 9 deuterated methyl choline (choline-d_9) labelling [12,15] enables endonuclear PtdCho metabolism to be probed with unparalleled specificity and sensitivity. Here we compare whole cell and endonuclear PtdCho synthesis specificity in the face of prolonged, elevated PUFA exposure.

2. Materials and methods

2.1. Materials

Arachidonic acid (AA, 20:4 *n*-6), docosahexaenoic acid (DHA, 22:6 *n*-3) and fatty acid-free albumin were purchased from Sigma-Aldrich. Deuterated choline (choline-d_9) was purchased from C/D/N Isotopes (Pointe-Claire, QC, Canada). Tissue culture plasticware and reagents were from Life Technologies (Paisley, UK). Varian Bond Elut aminopropyl (NH2) solid phase extraction columns were supplied by Phe-

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Abbreviations: AA, arachidonic acid (20:4, *n*-6); choline-d_9, 9 deuterated methyl choline; DAG, diacylglycerol; DHA, docosahexaenoic acid (22:6, *n*-3); ESI-MS, electrospray ionisation mass spectrometry; PtdCho, phosphatidylcholine; PtdIns, phosphatidylinositol; PUFA, polyunsaturated fatty acid

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nomenex (Macclesfield, Cheshire, UK). Chemicals and biochemicals were from Merck (Lutterworth, Leicestershire, UK) or Sigma-Aldrich (Poole, Dorset, UK).

2.2. Methods: IMR-32 cell culture, fatty acid supplementation and preparation of nuclei

IMR-32 human neuroblastoma cells were cultured as described previously [12]. Fatty acid supplements were prepared at 3 mM (×100 concentration) coupled to fatty acid-free albumin at a 2:1 molar ratio in Hank's balanced salt solution without Ca^{++}/Mg^{++} adjusted to pH 7.0 and filter sterilised through 0.2 μm filters. For supplementation experiments cells were trypsinised and plated 24 h prior to commencing fatty acid exposure. Both supplemented and control culture media were refreshed at 48 h. Seeding densities were chosen to ensure that cells with the highest recorded growth at 96 h remained sub-confluent. Whole cell and membrane-free nuclear fractions were prepared exactly as described previously [12].

2.3. Deuterated choline labelling of cells

Newly synthesised PtdCho in whole cells and nuclei was determined after 3 h incubation of whole cells with excess choline-d_9 [12].

2.4. PtdCho purification and mass spectrometric analysis

Following the introduction of PtdCho 14:0/14:0 internal standard as described [12], chloroform/methanol extraction and solid-phase NH2 column purification, the PtdCho fraction was analysed by ESI-MS using a Quatro Ultima triple quadrupole mass spectrometer (Micromass, Wytheshaw, UK). Endogenous PtdCho was selectively determined by precursor scans of the phosphocholine fragment (m/z +184), while newly synthesised PtdCho was determined by equivalent precursor scans of the d_9-phosphocholine fragment (m/z +193) [12]. To account for previously reported decrease in response with increasing m/z values [16], a formula for correcting reduced response was determined experimentally as: $a = 2\times10^{13}b^{-3.9873}$, where a = reduced response factor relative to a value of 1.00 for PtdCho14:0/14:0 and $b = m/z$ value. The formula was constructed following triplicate parent scan analyses of equimolar PtdCho 14:0/14:0 plus seven other PtdCho molecular species (saturated, monounsaturated and diunsaturated) covering the full m/z range reported at four different concentrations over a 1000-fold span. MS/MS responses were calibrated daily using this mix. The differential fragmentation of PUFA-containing species [17], which can result in significant overestimation at high concentration [17], did not dramatically confound here, probably due to the very low PtdCho concentrations demanded by endonuclear phospholipid analyses. Whole cell PtdCho concentrations were routinely diluted to give similar total ion intensities to those achieved with corresponding nuclear fractions, and comparison of m/z+184 parent scans with total positive scans over the same m/z range showed no dramatic discrimination of any molecular species. It was not possible to determine these parameters for d_9-choline containing PtdCho species. These were consequently assumed to behave as their normal choline analogues.

2.5. Cell proliferation assays

Cell proliferation assays were performed in 96-well, flat-bottomed tissue culture plates using the CellTiter 96 aqueous One solution cell proliferation assay (Promega) with initial seeding densities chosen empirically to span the full dynamic range of the kit after 96 h.

3. Results and discussion

3.1. Effect of fatty acid supplementation upon cell proliferation and membrane PtdCho synthesis

To exclude the possibility of PUFA-mediated derangement of cell-growth rates and associated membrane synthesis we first established that prolonged PUFA supplementation (30 μM for 96 h) did not dramatically alter proliferation or PtdCho synthesis (Fig. 1).

Control cell counts increased 3–4-fold while remaining sub-confluent over 96 h, in agreement with cell counts at trypsinisation (control cell growth is shown as 100% at each time point). After 24 h PUFA supplementation cell proliferation

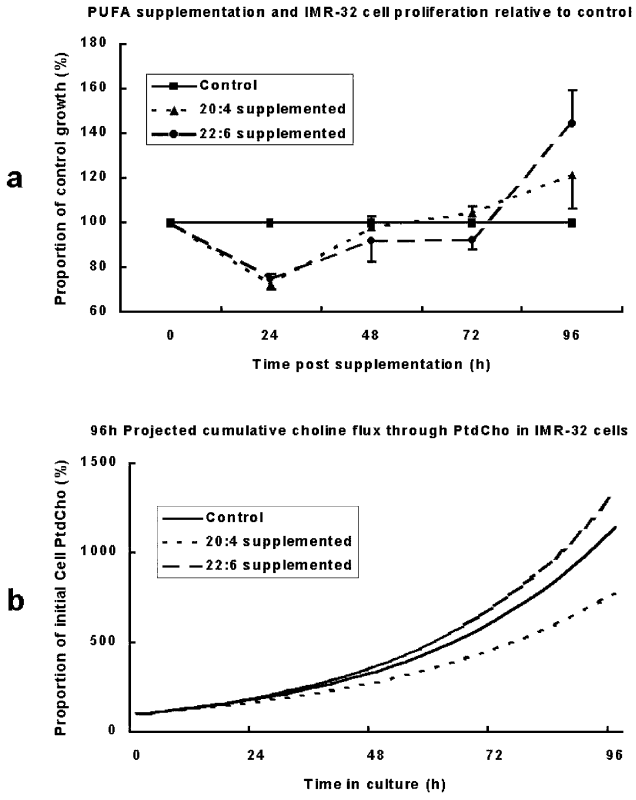

Fig. 1. Effect of PUFA supplementation on IMR-32 cell growth and PtdCho synthesis. a: Cell proliferation relative to sub-confluent control (non-PUFA-treated) cultures was determined at 24, 48, 72 and 96 h following supplementation with 30 μM PUFA and with one complete medium change at 48 h. Data presented represents means ± S.D. (n = 4–6 for each time point). b: Projected cumulative choline fluxes through PtdCho for control and PUFA-treated cells over 96 h PUFA supplementation derived by extrapolating mean 3 h PtdCho-synthesis rates at 96 h.

was diminished compared with controls, but by 72 h was comparable in each group and over the final 24 h increased in PUFA-treated cells (Fig. 1a). Membrane accretion and cell proliferation are intimately associated [18], so any PtdCho increase in cultured cells will represent the balance of new synthesis and degradation. The proportion of whole cell PtdCho synthesis over 3 h was estimated by ratioing total ion counts from precursor scans of m/z+193 with those of precursor scans of m/z+184 [12]. Control synthesis over 3 h was 7.92 ± 3.95% (mean ± S.D., n = 4) while the corresponding figure following AA- and DHA-supplementation were 6.61 ± 4.42% (mean ± S.D., n = 6) and 8.45 ± 4.20% (mean ± S.D., n = 6), respectively. Extrapolation of the mean 3-h-synthetic rates to a full 96 h of culture (Fig. 1b) showed a cumulative choline flux through PtdCho synthesis more than double that required simply for growth-related membrane biogenesis. While these results provide evidence for extensive choline headgroup turnover under all experimental conditions, they also suggest that fatty acid supplementation for 96 h had no dramatic affect either on IMR-32 cell growth or the rate of PtdCho synthesis.

3.2. Whole cell PtdCho composition and synthesis following PUFA supplementation

The molecular species compositions of control cell PtdCho

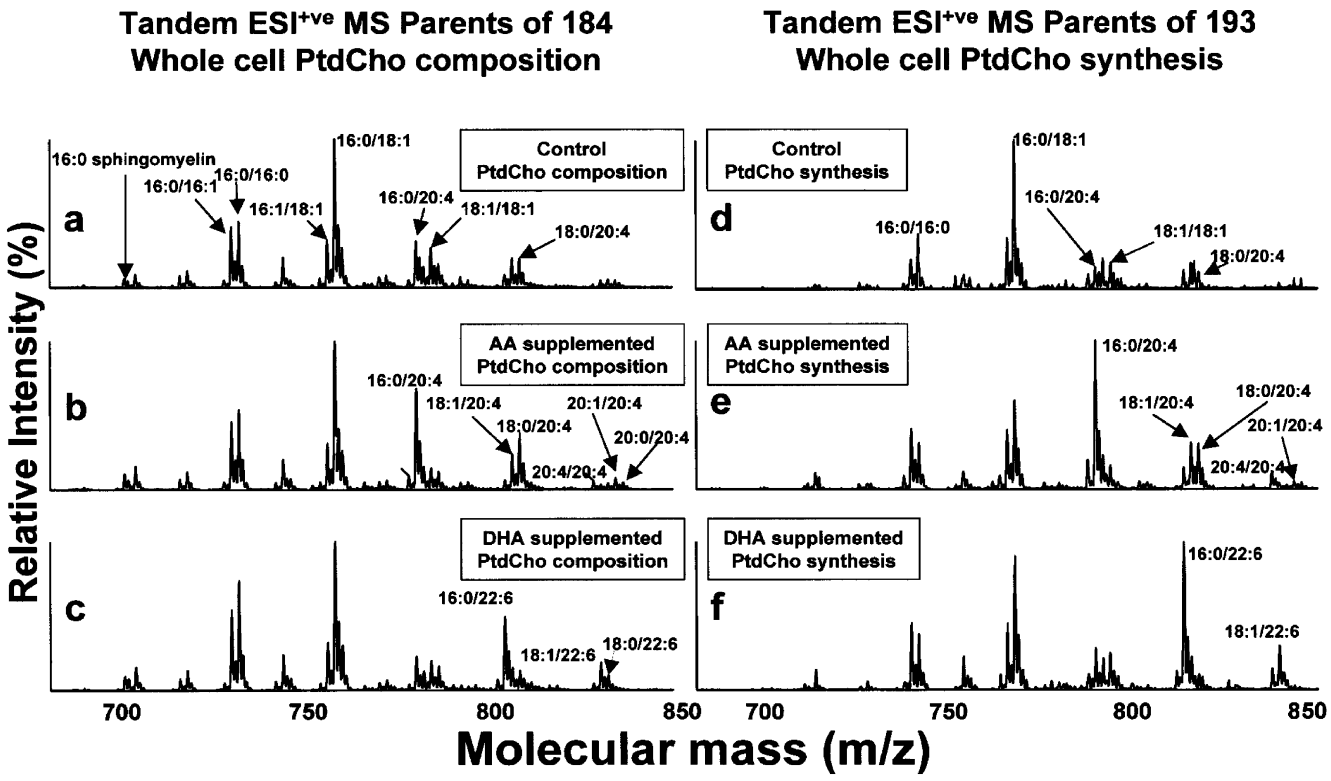

Fig. 2. ESI-MS of PtdCho from control, AA-supplemented and DHA-supplemented whole IMR-32 cells after 3 h incubation with choline-d_9. Each panel depicts diagnostic precursor scans of the phosphocholine headgroup (m/z+184, a, b and c) showing endogenous PtdCho or of the phosphocholine-d_9 headgroup (m/z+193, d, e and f) showing newly synthesised PtdCho. Panels a and d are scans from control cells, while panels b and e and panels c and f are from AA-supplemented and DHA-supplemented cells, respectively.

and corresponding newly synthesised pools after 3 h incubation with choline-d_9 (Fig. 2a, d) were essentially identical to those seen previously [12]. Newly synthesised PtdCho composition resembled the endogenous pool, but further tailoring was obvious from the equilibrium composition. For example, PtdCho 16:0/18:1 was more abundant in newly synthesised cell PtdCho (Fig. 2d) than in endogenous PtdCho (Fig. 2a), suggesting the operation of post-synthesis acyl remodelling. However, despite minor variation, a lack of change in total PUFA-containing PtdCho between newly synthesised and equilibrium compositions suggests only slight involvement of these species in the remodelling.

Following prolonged PUFA supplementation, cells tolerated a wide variation in the molecular species compositions of their endogenous PtdCho, with increased concentrations of unsaturated species after both AA (Fig. 2b) and DHA (Fig. 2c) supplementation. In both cases, the *sn*-1 palmitoyl (16:0), *sn*-2 PUFA was the species most elevated. After AA-supplementation PtdCho 16:0/20:4 content increased from the control value of 7.2 ± 0.7% (mean ± S.D., $n = 4$) to 12.5 ± 1.6% (mean ± S.D., $n = 6$) while DHA-supplementation increased the content of PtdCho 16:0/22:6 from 3.2 ± 1.2% (mean ± S.D., $n = 4$) to 8.9 ± 2.2% (mean ± S.D., $n = 6$). Despite some small relative changes within the disaturated species their total proportion did not vary between any of the groups.

The pattern of newly synthesised cell PtdCho data following supplementation was even more enriched in unsaturated species that that observed in the equilibrium composition. For both AA and DHA supplementation, the *sn*-1 16:0, *sn*-2 PUFA supplanted PC16:0/18:1 as the dominant species synthesised (Fig. 2e, f) with *sn*-1 oleoyl (18:1), *sn*-2 PUFA and *sn*-1, *sn*-2 di-PUFA enrichments also noted. For example, while newly synthesised PC16:0/20:4 in control cells represented 8.7 ± 2.3% (mean ± S.D., $n = 4$) this increased to 18.0 ± 3.5% (mean ± S.D., $n = 6$) upon supplementation with 30 μM AA for 96 h. Likewise PtdCho 16:0/22:6 comprised 4.2 ± 1.0% (mean ± S.D., $n = 4$) of newly synthesised PtdCho in control cells, but rose to 15.2 ± 4.9% (mean ± S.D., $n = 6$) following DHA supplementation. The fractional synthetic rates of both these species were considerably greater than their equilibrium concentrations after supplementation of 12.5 ± 1.6% (mean ± S.D., $n = 6$) and 8.9 ± 2.2% (mean ± S.D., $n = 6$), respectively. Clearly, the post-3-h labelling change following PUFA supplementation suggests that remodelling here involves degradation of the whole PUFA-containing PtdCho rather than simply remodelling the *sn*-2 position [7]. In addition, the wide range of molecular species incorporated into newly synthesised PtdCho demonstrates a lack of molecular specificity in whole cell PtdCho synthesis de novo. The biologically accessible DAG pool determines newly synthesised PtdCho composition and prolonged PUFA exposure has enriched the representation of PUFA-containing DAG. Downstream remodelling/degradation then produces characteristic whole tissue or subcellular membrane divergence in PtdCho composition.

3.3. *Endonuclear PtdCho composition and synthesis following PUFA supplementation*

The highly saturated endonuclear PtdCho pool [12] from the same cell cultures showed some remarkable composition

and synthesis differences. Specifically, endogenous endonuclear PtdCho compositions (Fig. 3a–c) showed that the highly saturated species were tightly retained, even after 96 h PUFA supplementation. However, in both cases the *sn*-1 16:0, *sn*-2 PUFA rose from below quantifiable level to 3.2 ± 0.4% (mean ± S.D., $n = 6$) for PtdCho 16:0/20:4 in AA-treated cells and 2.2 ± 0.5% (mean ± S.D., $n = 6$) for PtdCho 16:0/22:6 in DHA-treated cells. Given the synthesis patterns below, small PUFA enrichments at equilibration are most easily explained as representative of the balance of new synthesis and active remodelling or degradation or possibly export from the nucleus. The turnover rates of endonuclear, PUFA-containing PtdCho might resolve this issue but are not accessible from these data. Alternatively highly purified nuclei may be able to tolerate small proportions of PUFA-containing PtdCho species but not those higher figures seen in the whole cell. The patterns of endonuclear PtdCho synthesis de novo seen below (Fig. 3d–f) would support either possibility.

Newly synthesised endonuclear PtdCho in control cells followed the pattern previously seen [12]. There was no apparent synthesis of PUFA-containing species, and the degree of saturation enrichment increased from newly synthesised (Fig. 3d) to the equilibration composition of PtdCho (Fig. 3a). In agreement with our previous report [12], this remodelling of newly synthesised, largely saturated PtdCho into even more saturated species represented the major endonuclear transformation in control cells [12]. By contrast, PUFA supplementation of cells stimulated the synthesis within the nucleus of significant amounts of PtdCho containing PUFA (Fig. 3e, f). For the predominant species this represented 12.9 ± 3.0% (mean ± S.D., $n = 6$) for PtdCho 16:0/20:4 in AA-supplemented cells and 9.8 ± 3.8% (mean ± S.D., $n = 6$) for PtdCho 16:0/22:6 in DHA-supplemented cells. However, the large synthetic enrichments in PUFA-containing species were not carried forward to the corresponding endonuclear equilibrium compositions (Fig. 3b, c). Examination of the endonuclear ESI-MS traces suggests that removing PUFA-containing PtdCho solely by remodelling the *sn*-2 position would require significant re-acylation with monounsaturated species to retain constant endonuclear PtdCho saturation. The predominance of endonuclear 16:0 CoA [19] and preference for 16:0 acylation in this compartment [20] provides limited scope for such remodelling, and its operation on this scale would still require an as yet uncharacterised rapid removal route for liberated PUFA. Indeed, the data suggest instead that complete degradation/removal of PUFA-containing species from the nucleus contributes to maintaining the pattern of saturation enrichment. In either case, the lack of specificity of endonuclear PtdCho synthesis at the level of DAG incorporation, unlike that of whole cell, is effectively countered by the selective removal of PUFA-containing species between synthesis and the establishment of the equilibrium composition.

3.4. Comparison of molecular specificities and implications

While both pathways showed a lack of selectivity for DAG incorporation into newly synthesised PtdCho (Figs. 2 and 3), post-synthetic tailoring of the lipid was more tightly regulated in the endonuclear compartment (Fig. 3), as evidenced by retention of highly saturated PtdCho. The maintenance of highly saturated endonuclear PtdCho is likely related to es-

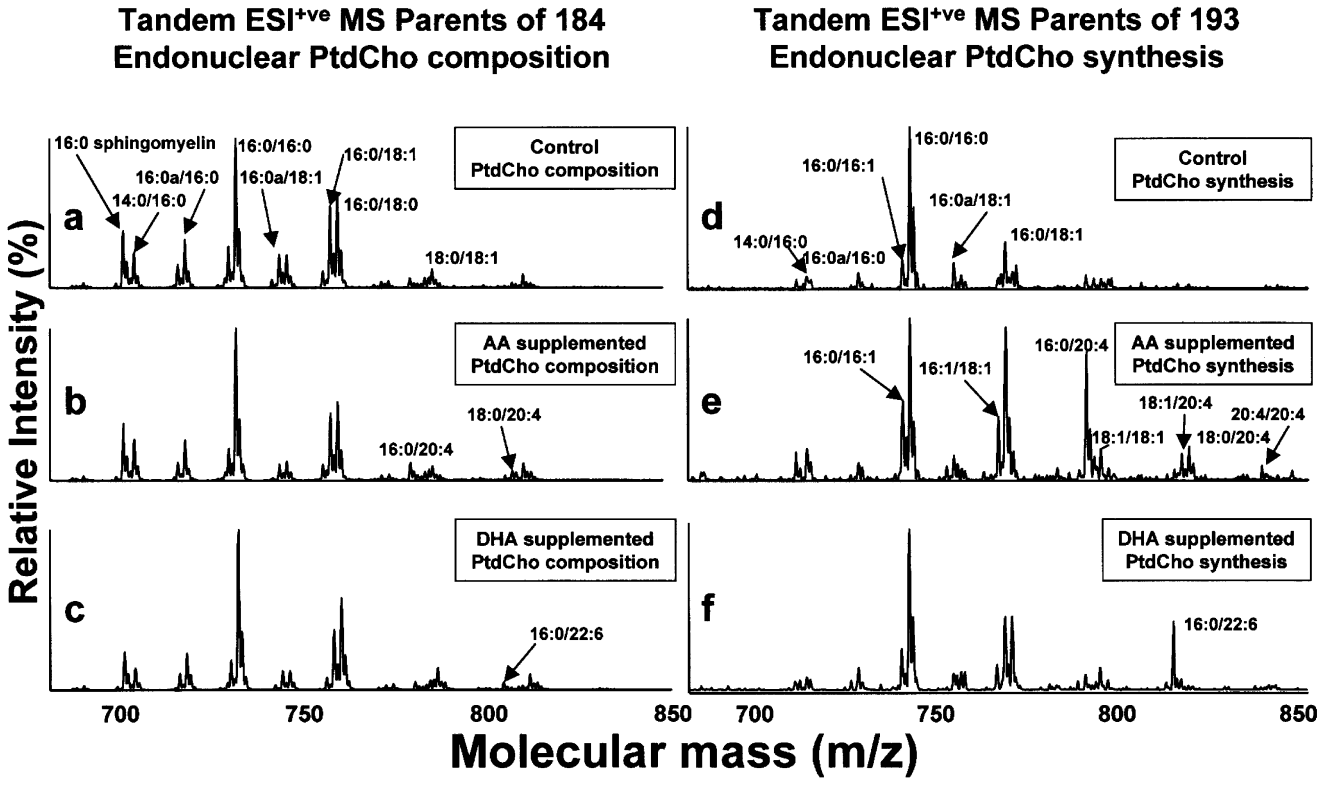

Fig. 3. ESI-MS of PtdCho from highly purified control, AA-supplemented and DHA-supplemented IMR-32 nuclei after 3 h incubation with choline-d_9. Each panel depicts diagnostic precursor scans of the phosphocholine headgroup (*m*/*z*+184, a, b and c) showing endogenous PtdCho or of the phosphocholine-d_9 headgroup (*m*/*z*+193, d, e and f) showing newly synthesised PtdCho. Panels a and d are scans from control cells, while panels b and e and panels c and f are from AA-supplemented and DHA-supplemented cells, respectively.

sential functional requirements as noted previously [12]. However, lack of stringency in terms of DAG incorporation into endonuclear PtdCho provides the synthetic pathway with a potential role in the universal termination of DAG-mediated endonuclear signalling. Periodic accumulations of predominantly saturated or unsaturated endonuclear DAG arise from PtdCho or PtdIns, respectively [11]. However, while a partial function of endonuclear PtdCho synthesis may be to remove and recycle the PtdCho-derived lipid second messenger [12], no comparable system exists for regenerating endonuclear PtdIns. The nucleus converts PtdIns to the higher phosphorylated forms, PtdInsP and PtdInsP2, necessary for IP3 and DAG liberation, but the pathway for PtdIns synthesis de novo is confined to the ER [21], from which it is transferred to the nucleus by phosphatidylinositol transfer protein [21]. In proliferating cells, endonuclear PtdCho synthesis may provide the necessary capacity for unsaturated endonuclear DAG removal and or inactivation.

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