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Dynamic Lipidomics of the Nucleus

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Abstract Once nuclear envelope membranes have been removed from isolated nuclei, around 6% of mammalian cell phospholipid is retained within the nuclear matrix, which calculations suggest may occupy 10% of the volume of this subcellular compartment. It is now acknowledged that endonuclear phospholipid, largely ignored for the past 40 years, provides substrate for lipid-mediated signaling events. However, given its abundance, it likely also has other as yet incompletely defined roles. Endonuclear phosphatidylcholine is the predominant phospholipid comprising distinct and highly saturated molecular species compared with that of the whole cell. Moreover, this unusual composition is subject to tight homeostatic maintenance even under conditions of extreme dietary manipulation, presumably reflecting a functional requirement for highly saturated endonuclear phosphatidylcholine. Recent application of new lipidomic technologies exploiting tandem electrospray ionization mass spectrometry in conjunction with deuterium stable isotope labeling have permitted us to probe not just molecular species compositions but endonuclear phospholipid acquisition and turnover with unparalleled sensitivity and specificity. What emerges is a picture of a dynamic pool of endonuclear phospholipid subject to autonomous regulation with respect to bulk cellular phospholipid metabolism. Compartmental biosynthesis de novo of endonuclear phosphatidylcholine contrasts with import of phosphatidylinositol synthesized elsewhere. However, irrespective of the precise temporal–spatial–dynamic relationships underpinning phospholipid acquisition, derangement of endonuclear lipid-mediated signaling from these parental phospholipids halts cell growth and division indicating a pivotal control point. This in turn defines the manipulation of compartmentalized endonuclear phospholipid acquisition and metabolism as intriguing potential targets for the development of future antiproliferative strategies. J. Cell. Biochem. 97: 244–251, 2006.

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At the Gordon Research Conference on Molecular and Cell Biology of Lipids in 1997, Professor Claudia Kent [Kent, 2005] related details of a discussion she'd had with Professor Eugene Kennedy, a pioneer of phospholipid biochemistry. She had asked if he could explain her observation that the key regulator of phosphatidylcholine (PtdCho) biosynthesis [Kent, 2005], CTP:phosphocholine cytidylyltransferase (CCT, EC 2.7.7.15), an enzyme whose properties he had first described in 1957 [Borkenhagen and Kennedy, 1957] could be found in association with the nucleus [Wang et al., 1993]. After a reflective pause, he had replied "Because PtdCho is more important than DNA!" amid the evoked laughter of 100 or so lipid biochemists acquiring a new anecdote for less enlightened colleagues, several unanswered questions troubled me: (i) Could the nucleus actually be a site of PtdCho synthesis? (ii) Do PtdCho and other membrane phospholipids still co-locate with the nucleus once the nuclear envelope is removed? And, if so, (iii) What is their function? I determined to return to the laboratory and investigate further. Eight years later, at the inaugural Gordon Research Conference on Signal Transduction within the Nucleus, I was privileged to be invited to outline work by us and others that have begun to shed light on these fascinating questions.

NUCLEAR PHOSPHOLIPID

The recognition that the nuclear matrix (NM) contains phospholipid is not new [Rose and Frenster, 1965]. Forty years since the earliest

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reports of the phenomenon, an extensive, if fragmented, literature attests to this widespread observation [Cocco et al., 1980; Maraldi et al., 1993; Albi et al., 1994; Vann et al., 1997]. Moreover PtdCho, phosphatidylinositol (PtdIns), phosphatidic acid (PtdOH), phosphatidylethanolamine (PtdEtn), and phosphatidylglycerol (PtdGro) have all been identified within this compartment along with corresponding lysolipids, diacylglycerol (DAG), sphingomyelin (SM), and acyl-CoA species. Phospholipases and lipid-dependant kinases capable of acting upon or themselves being acted upon by lipids are equally well documented. Moreover, a phospholipid-derived signaling system exists inside nuclei [Irvine, 2003] as well as phospholipid-dependant nuclear receptors and transcription factors [Forman, 2005; Li et al., 2005] fuelling considerable recent interest in phospholipid metabolism within the nucleus.

For much of the time since NM-associated phospholipid was first established, analytical limitations meant that characterization focused upon determining the content and proportional representations of defined headgroups. Key to enhanced understanding and interpretation of some of these observations within the nucleus has been the analytical advance in small molecule mass spectrometry over the past decade. Historically, lipid analyses required solvent extraction and laborious chromatographic separation followed by chemical and biochemical analyses of resolved components. Latterly, tandem electrospray ionization mass spectrometry (ESI-MS/MS) has rendered phospholipid analyses down to the level of individual acyl/alkyl/alkenyl species relatively facile, much more sensitive and largely unambiguous [Han and Gross, 2003; Pulfer and Murphy, 2003]. Moreover, dynamic tracking of phospholipid synthesis and turnover in vivo which formerly relied upon following the flux of radionuclide-labeled substrates through resolved phospholipid has found enhanced sensitivity and rigour in the application of stable isotope labeling of phospholipid class-specific "probes" [Hunt and Postle, 2005].

WHAT IS A "NUCLEAR" LIPID?

Systematic evaluation of nuclear lipid composition and metabolism data is fraught with problems of terminology, methodology, and of cell-specific variations. The presence of phospholipid specifically within the NM is often the subject of confused interpretation by those unfamiliar with the area. Misconceptions stem largely from widespread imprecise use of the term "nuclear." Consequently, "nuclear lipid" has variously been employed by investigators (Fig. 1) to designate either: (i) a pool confined solely to the nuclear envelope (NE) and associated membrane fragments attached to isolated nuclei, (ii) the portion exclusively found within the NM, or (iii) some combination of both, empirically determined by the extent of detergent-mediated NE removal during nucleus preparation.

Designation (i), essentially NE lipid, is frequently employed by those who disregard or discount the possibility of NM-associated lipid. However, it is increasingly clear that such a position for nuclear lipid is experimentally untenable even if it does not fit theoretical preconceptions. A distinct pool of phospholipid is irrefutably dispersed within the NM and, as evidence-based scientists, we have to accept and explain its presence. In order to avoid ambiguity between options (ii) and (iii), however, I use the adjective "endonuclear" to specify phospholipid that co-locates with the NM following demonstrated NE removal and I variously deploy "nuclear" or "perinuclear" where the extent of any NE or peripherally associated endoplasmic reticulum (ER) depletion is uncertain.

QUANTITATING ENDONUCLEAR PHOSPHOLIPID

Endonuclear PtdCho like its extranuclear counterpart comprises the major phospholipid class within that defined compartment [Hunt et al., 2001]. Quantitative estimates from independent studies of both freshly isolated rat hepatocytes [Vann et al., 1997] and cultured human IMR-32 neuroblastoma cells [Hunt et al., 2001] suggest endonuclear PtdCho comprises approximately 6% of the whole cell pool [Vann et al., 1997; Hunt et al., 2001]. However, the PtdCho that is confined to the NE, and which is removable by detergent, represents twice that found in the endonuclear compartment (12% vs. 6%) and elevates the proportion of whole cell PtdCho represented as "nuclear" in rat hepatocytes to around 18% of total [Vann et al., 1997]. This clearly offers scope for cross-contamination since NE PtdCho is enriched in polyunsaturated fatty acid (PUFA)-containing species

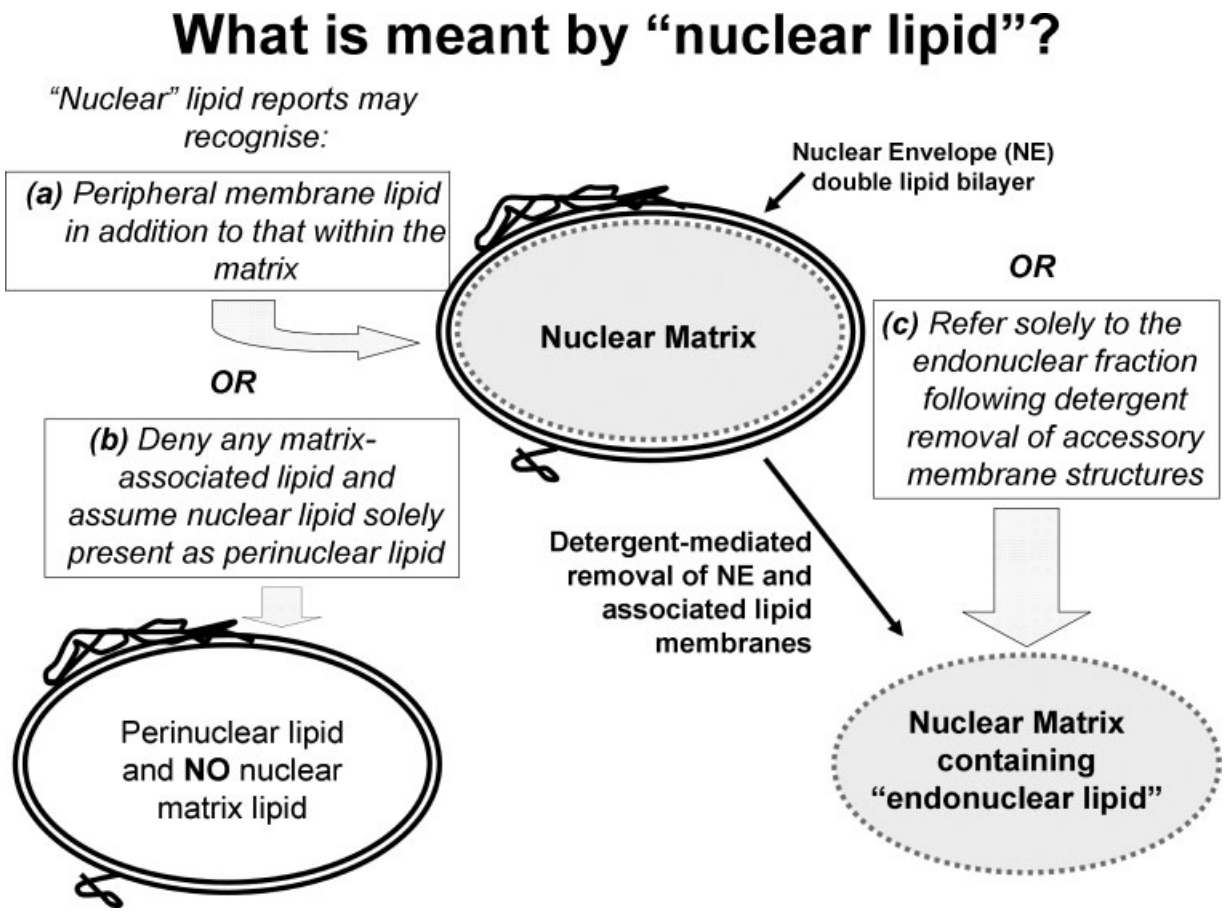

Fig. 1. The term ''nuclear lipid'' is ill-defined in much scientific literature rendering comparison of data problematic. Most confusion stems from the inclusion (**a**) or exclusion (**c**) of NE and accessory lipids. The proposal that there is no matrix associated lipid (**b**) cannot be reconciled with experimental observation. We prefer the term perinuclear to encompass NE lipid with the term ''endonuclear lipid,'' generally referring to that retained after the stripping of NE (usually by detergent), reserved for that associated with the NM.

[Surette and Chilton, 1998; Williams et al., 2000]. Moreover, it emphasizes the need for adequate NE-removal strategies in studies of endonuclear PtdCho metabolism since even small contamination the NM fraction by NE may significantly alter the analytical profile. We routinely investigate NM fractions that are detergent-stripped of NE membranes [Hunt et al., 2001].

It is also clear that in many studies the proportions of NE and endonuclear PtdCho will be subject to cell-specific, morphology-dependant, and constraint. For example, HL-60 cells, U937 cells, and other cells with relatively large nuclei and small cytoplasmic volumes may retain a greater proportion of the whole cell PtdCho in their "nuclear" fractions. Likewise, any increased cytoplasmic volumes circumscribed by relatively greater amounts of plasma membrane, as seen for example in epithelial cells, will necessarily increase the scope for greater proportions of extranuclear PtdCho.

STATIC AND DYNAMIC PHOSPHOLIPID LIPIDOMICS

Lipidomics, the study of the composition, metabolism, and biological role of lipids in cells at the levels of molecular species, has introduced significant complexity to the analysis of cellular phospholipid metabolism. It is well established that tissue-specific, phenotypic programming of phospholipid molecular species compositions reflects functional requirements [Postle, 1998]. This is particularly apparent in the case of the major tissue phospholipid,

PtdCho as determined in "static" lipidomic profiling, the most widely used application of lipid mass spectrometry [Han and Gross, 2003; Pulfer and Murphy, 2003]. So, for example, the static liver PtdCho molecular species profile is highly unsaturated reflecting the synthesis and export of PUFA-containing lipoproteins [Hunt et al., 1996]. Lung PtdCho molecular species profiles are highly saturated in contrast due to a requirement to synthesize and export disaturated pulmonary surfactant PtdCho species [Hunt et al., 1991].

More recently, employment of deuterium labeled lipid precursors has permitted probing of dynamic aspects of lipidomics. Applications of this powerful new methodology are limited to date. However, use of stable isotope labeling to provide dynamic profiling from organelles, organs, and organisms has recently been reviewed [Hunt and Postle, 2005].

The emerging picture from detailed lipidomic studies of PtdCho including static and dynamic measurements [DeLong et al., 1999; Schneiter et al., 1999; Hunt et al., 2001, 2002; Postle et al., 2004] confirms and extends the whole cell "programmed composition related to function" concept to subcellular compartments. Dynamic analyses suggest that part of the rationale for the reported existence of spatially distinct, accessory biosynthetic pathways [Hunt and Postle, 2004] may lie in functional requirements for different patterns of some, though not all phospholipid molecular species compositions in discrete compartments. So, subcellular PtdCho synthesis is a multi-site phenomenon reflecting different molecular species profiles across the cell including within the nucleus [Hunt and Postle, 2004]. Endonuclear PtdIns, by contrast, is not synthesized in situ [Rubbini et al., 1997]. Preliminary dynamic lipidomic analyses of cell PtdIns (see below) confirms, however, acquisition of an endonuclear molecular species composition indistinguishable from that of the whole cell [Hunt and Postle, 2004; Hunt et al., 2004a] with import presumably achieved by intracellular transport from common biosynthetic loci.

THE DYNAMICS OF SATURATED ENDONUCLEAR PTDCHO ACQUISITION

As early as 1983, a report detailed apparent increases in saturated nuclear PtdCho synthesis, from [^{14}C]-labeled precursors that accompanied late mitosis [Henry and Hodge, 1983]. Although this crude dynamic lipidomic study was not conducted in NE-depleted nuclei, it suggested the potential for compartment-specific, temporal variation in PtdCho composition and synthesis. Unfortunately, it is likely that methodological limitations precluded subsequent significant advance in this area. Where analyses of PtdCho were undertaken at all, characteristic, diagnostic but relatively crude analyses of double bond content served to confirm phospholipid class or the origin of mobilized endonuclear DAG [D'Santos et al., 1999] in order to distinguish between relatively saturated endonuclear PtdCho and unsaturated endonuclear PtdIns.

Our early dynamic experiments with [^{14}C]-labeled precursors showed that isolated IMR-32 nuclei could transform a small proportion of labeled [^{14}C]-choline to PtdCho in vitro [Hunt and Burdge, 1998] and others using LA-N-1 neuroblastoma cells reported similar phenomena [Anthony et al., 2000]. More detailed analyses of the activities of CDPcholine pathway enzymes in isolated IMR-32 NM revealed that each relevant activity was partly resident in this sub-cellular compartment [Hunt et al., 2001]. CCT activity and immunoreactivity had been associated with nuclear fractions of cells for many years [Wang et al., 1993; Hunt et al., 1998; Cornell and Northwood, 2000] although its presence was rationalized as representing a reservoir of enzyme [Hunt et al., 1998; Cornell and Northwood, 2000] mobilized to the ER in response to cellular requirements for increased PtdCho synthesis. That this view is likely erroneous was subsequently suggested by compelling immunological and green fluorescent protein-tagging evidence confirming that the alpha isoform of CCT (CCTα) [Lykidis and Jackowski, 2001] is predominantly located with and confined to the nucleus of most cells [DeLong et al., 2000] with type II alveolar cells a notable apparent exception [Ridsdale et al., 2001]. This contrasts with beta isoforms (CCTβ) [Lykidis et al., 1999] showing universal extranuclear localization. Moreover, the concept of the nucleus acting merely as a reservoir of CCT is directly contradicted by the demonstration of endonuclear PtdCho synthesis.

We were subsequently able to investigate endonuclear PtdCho synthesis, compare it with whole cell PtdCho synthesis, and explore in general terms the molecular mechanisms that

underpin its saturation enrichment. The acyl substitution profile of PtdCho synthesized de novo via the CDPcholine pathway [Kent, 2005] depends upon the composition of accessible DAG incorporated [McMaster and Bell, 1997], while downstream remodeling of newly synthesized molecular species accounts for fine "tailoring" [Choy et al., 1997]. A limited capacity for molecular selectivity while manifest enzymatically in vitro [Henneberry et al., 2002], is clearly augmented in vivo. This is seen, as noted above, in NE PtdCho composition where enrichment in arachidonyl (20:4, n-6) species of multiple phospholipids occur [Surette and Chilton, 1998; Williams et al., 2000] alongside a cell requirement for the generation of eicosanoids and related lipid signals from this membrane. Here, a specific ER/golgi-associated phospholipase A_2 (PLA_2) independent of any associated with eicosanoid generation is likely to be intimately involved in enzymatic phospholipid remodeling, which permits arachidonate enrichment [Balsinde, 2002]. Spatially proximal, NM-associated PtdCho synthesis, by contrast, is independent of ER and directed towards retention of highly saturated molecular species [Hunt et al., 2001]. So, the molecular species profile of endonuclear PtdCho synthesis via the CDPcholine pathway is initially more unsaturated than the equilibrium composition [Hunt et al., 2001, 2002] and, moreover, is determined in part by the composition of DAG supplied at the cholinephosphotransferase step [Hunt et al., 2002]. Remodeling or removal mechanisms act to actively exclude arachidonyl and other PUFA chains from the equilibrium mixture [Hunt et al., 2001, 2002]. From these studies, it was clear that endonuclear PtdCho synthesis lacks any significant specificity for DAG species incorporation but that it rather incorporates accessible DAG species of any composition and then uses combinations of acyl remodeling or selective removal of PUFA containing species to achieve saturation enrichment. Endonuclear PtdCho synthesis and active exclusion of PUFA-containing species is a common feature of all proliferating cells examined [Hunt et al., 2001, 2002, 2004a; Hunt and Postle, 2004]. The rationale for the synthesis, remodeling, and retention of highly saturated endonuclear PtdCho, while excluding more unsaturated species remains uncertain although its very ubiquity does imply an important functional requirement.

THE DYNAMICS OF UNSATURATED ENDONUCLEAR PTDINS ACQUISITION

Notwithstanding the observations noted above that endonuclear PtdCho is more saturated than the proximal PtdIns pool [D'Santos et al., 1999], PtdIns composition is largely unchanged between endonuclear and extranuclear compartments [Hunt and Postle, 2004; Hunt et al., 2004a]. It had been proposed that endonuclear PtdIns synthesized in extranuclear compartments was transported to the nucleus by a PtdIns transfer protein (PITP) [Rubbini et al., 1997] and that the alpha isoform (PITPα) was the most likely candidate having both extranuclear and endonuclear distributions [Routt and Bankaitis, 2004]. Two strands of evidence argued against this possibility. Use of static lipidomic methodologies enabled us to establish a molecular specificity for recombinant PITPα, which showed that the protein preferentially binds shorter chain saturated PtdIns and PtdCho [Hunt et al., 2004b], whereas the molecular species profile of acquired endonuclear PtdIns has a predominantly stearoylarachidonoyl (18:0/20:4) molecular species composition [Hunt and Postle, 2004; Hunt et al., 2004a], a less favored species in binding terms [Hunt et al., 2004b]. Moreover, data from Professor Vytas Bankaitis' laboratory at Chapel Hill, NC showed that ablation of PITPα was only post-natally lethal in mice [Alb et al., 2003] and did not compromise the ability of derived MEF cells from knock-outs to proliferate. When we compared the static whole cell and endonuclear PtdIns composition profiles of PITP$\alpha^{+/+}$ and PITP$\alpha^{-/-}$ MEF cells, the compositions were unchanged [Hunt et al., 2004a]. Moreover, when we tracked the passage of deuterated myo-inositol biosynthetically chased into cell PtdIns, the rate of acquisition of endonuclear PtdIns did not appear appreciably different between wild-type and knock-out [Hunt et al., 2004a]. Together these data indicate that the endonuclear compartment does not rely on PITPα for its PtdIns import.

ENDONUCLEAR LIPID DYNAMICS AND SIGNALING

While much attention has been focused in recent years upon PtdIns-mediated signaling within the nucleus [Irvine, 2003], the potential contribution of endonuclear PtdCho synthesis to lipid second messenger (LSM) signaling

function is apparent [Hunt et al., 2001, 2002] Mobilization of endonuclear phospholipid-derived LSM during S phase of the cell cycle appears to be a ubiquitous characteristic of proliferating cells [D'Santos et al., 1998]. Periodic S phase accumulations of DAG from either PtdCho or PtdIns [D'Santos et al., 1999] are essential for continued cell growth/division, and ablation of these signals, for example, by targeted DAG kinase overexpression-mediated removal of endonuclear DAG will halt proliferation [Topham et al., 1998]. Unchecked accumulation of endonuclear DAG may also be a pro-apoptotic signal as suggested by the observation that migration of CCTα from the nucleus, which would likely halt endonuclear PtdCho synthesis, accompanies apoptosis [Lagace et al., 2002]. Accordingly, control of endonuclear DAG can be seen to be a critical component of the regulation of cell survival and proliferation. Irrespective of whether more saturated DAG is an inactivation product of bioactive phosphatidate [Hodgkin et al., 1998], LSM actions must be terminated by degradation, removal, or recycling. We have shown that endonuclear PtdCho synthesis possesses the capacity to incorporate a wide range of DAG molecular species into newly made phospholipid [Hunt et al., 2002] thereby removing a potentially potent LSM or metabolite. However, we have suggested that subsequent removal/remodeling of PUFA-containing PtdCho species could serve to "switch off" PtdIns-derived signals [Hunt et al., 2002]. The extent to which cell cycle-dependent

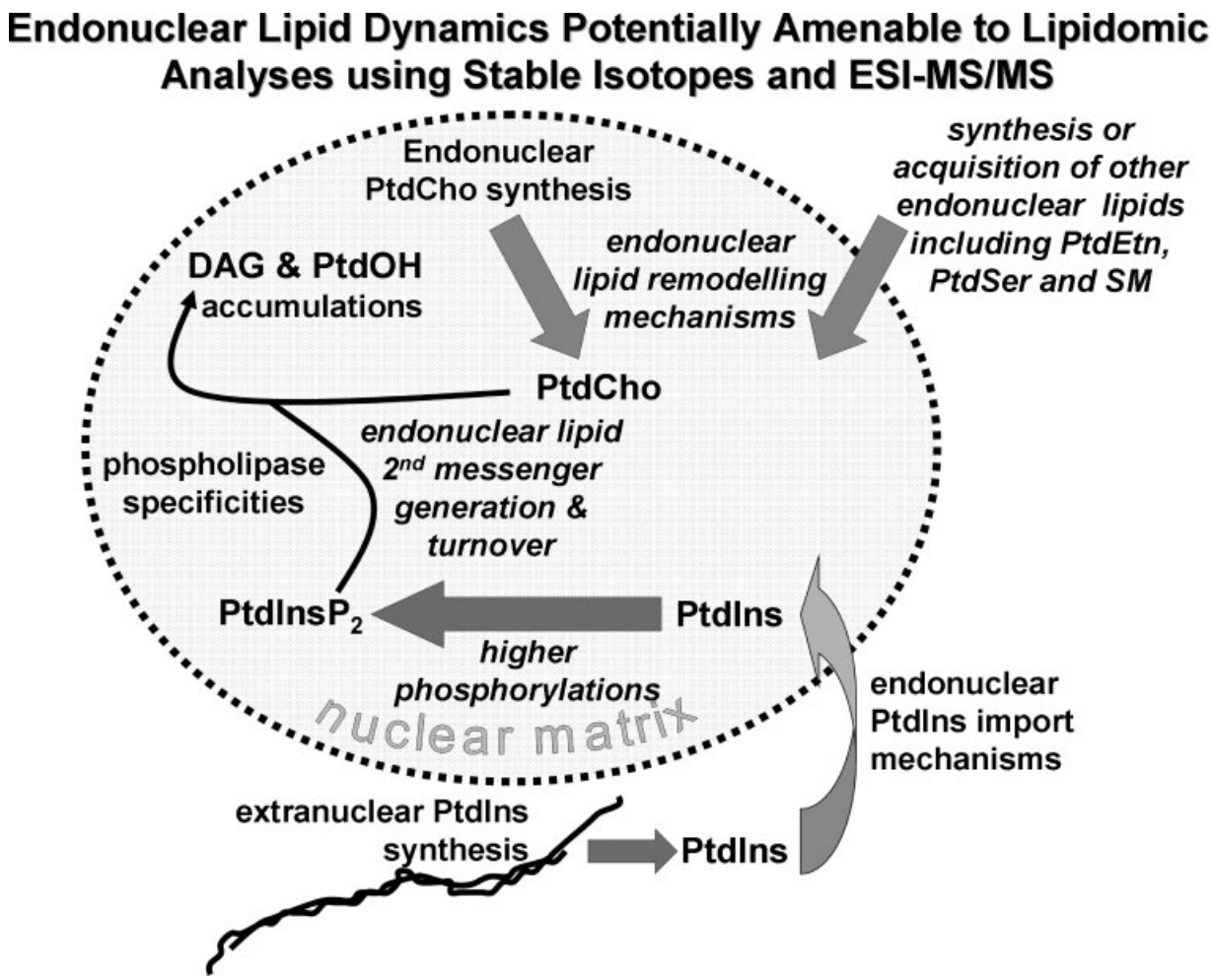

Fig. 2. In addition to tracking the flux of supplemented deuterated choline through PtdCho and SM and myo-inositol through endonuclear PtdIns and their downstream products, numerous other dynamic possibilities exist for future exploration. The flux of supplemented deuterated fatty acids through endonuclear phospholipid classes is relatively facile. In addition, appropriate head-group stable isotopes exist for PtdEtn and PtdSer. ESI-MS/MS strategies also exist or are in development for evaluating the dynamics of endonuclear DAG and PtdOH species.

changes in endonuclear PtdCho synthesis may be expressed in a temporally distinct fashion to whole cell PtdCho synthesis [Jackowski, 1994] remains a dynamic question awaiting an answer.

PROSPECTIVE

With due deference to Professors Kennedy and Kent, we have yet to show that endonuclear PtdCho is indeed "more important than DNA" but, levity apart, we have seen that the roles of endonuclear phospholipid are intimately involved in supporting cell survival and proliferation. Numerous questions concerning endonuclear lipid dynamics remain unanswered but many are amenable to the new lipidomic techniques as shown (Fig. 2). How, for example, does the NM acquire those phospholipids such as PtdIns not synthesized in situ? How is the endonuclear CDPcholine pathway activity/flux regulated? In addition, since endonuclear PtdCho biosynthesis supports proliferation, does compartment-targeted inhibition of this pathway offer a potential anti-proliferative strategy? Likewise, given a need to cyclically replace PtdIns consumed in LSM generation [Irvine, 2003], does blocking nuclear import of PtdIns offer another proliferation blocking strategy? The technological advances of recent years should enable us to address many of these questions in the near future.

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