MRS RESONANCES AS INDICATORS OF ALTERED PHOSPHOLIPID AND NEUTRAL LIPID METABOLISM IN LIVING CELLS

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Following the first reported detection of lipid signals in the proton NMR spectra of intact murine leukemia cells [1], multiple research efforts have been devoted to elucidate biochemical nature, structure and biological role of the corresponding components in cells and tissues. The signals were attributed to fatty acyl chains of neutral lipids endowed of a sufficiently high degree of isotropic motion to be detected in the time window of high resolution (HR) ^{1}H NMR spectroscopy. By contrast, in spite of possible residual segmental motions, the fatty acyl chains of phospholipids embedded in membrane bilayer structures typically give much broader signals, due to dipole-dipole interactions, and are therefore undetectable in HR NMR spectra.
The use of both one- and two-dimensional sequences allowed effective separation and assignment of individual signals arising from NMR-visible mobile lipids (ML). ML quantification in intact cells has usually been based upon measurement of the resonances arising from fatty acyl chains'saturated methylene segments ($-(CH_2)_n-$ at 1.3 ppm) either referred to the terminal CH_3 group at 0.9 ppm (which has however the disadvantage of overlapping with the methyl moiety of some amino acids) or to an endogenous compound such as lysine at 1.7 ppm or to an external reference. The signals from vinylic (5.35 ppm), allylic (2.04 ppm) and bis-allylic (2.80 ppm) groups allow quantification of the average degree of unsaturation of ML chains.
ML formation has been detected in a number of mammalian cell systems, including some (but not all) cancer and oncogene-transformed cells [2-6], embryo-derived cells [6-8], as well as activated lymphocytes, stimulated lymphoid cells and co-cultered lymphocytes/monocytes [9-13].
Changes in ML levels have been reported in drug-resistant compared with drug sensitive cells [14,15], in cells cultured at high density, subjected to pH stress or to proliferation arrest, [16-20], in apoptotic cells [21-28] and in cells exposed to cytotoxic antitumor drugs [29-34].
Such a large variety of physiological and pathological conditions has represented for years a true challenge to the effort of identifying common molecular mechanisms responsible for the formation of NMR-visible ML in intact cells.

Biogenesis and structure of NMR-detected mobile lipids

A model proposed by Mountford and Wright [35], based on the formation of neutral lipid domains intercalated with phospholipids in membrane bilayer configuration of cancer and stimulated cells fostered for over fifteen years a wealth of pioneering studies.
A relevant advance in the field was represented by the detection of ML signals in the ^{1}H NMR spectra of myeloma cells induced to the formation of cytoplasmic lipid droplets (LD) following cell loading with oleate [16]. Further evidence on the correlation between intracellular LD and ML signal profiles was reported in fibroblasts [6] and in apoptotic lymphoblasts [23,24,26], by combining NMR analyses with transmission electron microscopy (TEM) examinations (on osmium-stained cells and on freeze-fracture replicas) or with fluorescence microscopy of cells

stained with a lipophilic dye. Notably, TEM of freeze-fracture replicas allowed clear detection of protein particles at the periphery of the formed LD [6,23].
It is today commonly accepted that a predominant fraction of NMR-lipid signals in intact cells mainly derive from isotropically tumbling neutral lipids in cytoplasmatic LD (size of the order of micron(s)), although much smaller contributions may also be given, in some cell systems, by amorphous lipid vesicles of about 60 nm embedded in the plasma-membrane [6,23] or by raft domains [36-38].
According to classical biochemistry, major lipid components of cytoplasmic LD are triacylglycerols (TAG), cholesterylesters (CE), diacylglycerols (DAG) and free fatty acids (FFA). The presence of glycerolipids in ML pools is confirmed by the clearcut detection of the signals typical of esterified glycerol moiety in 2D ML spectra [vicinal and geminal cross-peaks].
Recent studies clarified that, contrary to traditional views, LD are not static, but dynamic bodies, whose formation, lipid and protein composition, growth, fusion, transport, breakdown and utilization are under control of hundreds of genes [39]. In particular, genomic and proteomic studies today allow functional characterization of these molecular mechanisms in relation to pathology and therapy [40]. By detecting and monitoring LD and ML formation in intact cells under different conditions of cell proliferation, stress, and induction to cell death, NMR provides a unique tool to complement lipidomic and proteomic investigations with metabolic information.

Molecular and biological mechanisms of ML formation in intact cells

The molecular mechanisms leading to ML formation can be investigated in intact cells exposed, under appropriate conditions, to selective inhibitors of pathways devoted to neutral lipid synthesis and processing.
The first contribution along this line was given by a study reporting that selective inhibition of long-chain acyl-CoA synthetase (ACS) by Triacsin C, abolished both ML and LD formation in HuT 78 cells induced to apoptosis by anti-Fas antibody [26]. These Triacsin-induced inhibitory effects did not arrest the apoptotic cascade, indicating that the biochemical mechanisms leading to ML/LB accumulation are not indispensable to the cell death program. It is however worth nothing that non-invasive detection by NMR of ML/LB formation naturally occurring in apoptotic cells may represent a very useful indicator of cell responsiveness to antitumor agents, both in vivo and in vitro. Regarding the timing of ML formation during the multi-step phenomenon of programmed cell death, it is worth nothing that two waves of ML formation have been detected in our laboratory in K562 cells induced to apoptosis by pulsed treatment with paclitaxel (3 h). The first wave was produced during the early effector phase (0-24 h) and the second in the late degradative phase (≥ 48 h), following cytochrome c release from mitochondrion and caspase 3 activation [27].
Apoptosis cannot however be seen as the only mechanism for increasing ML formation in cells. In fact experiments in our laboratory recently showed a ten-fold increase in ML formation in non apoptotic HuT 78 cells exposed to antimycin A, a selective inhibitor of complex III in the electron transport chain [41]. This overall body of evidence points to a basic role of mitochondrial dysfunction in ML/LD production in intact cells. Basic support to this hypothesis is provided by studies on breast cancer cells treated with cationic lipophilic phosphonium salts, in which parallel NMR and morphological investigations allowed detection of dose- and time-dependent LD formation, damaged mitochondria (swelling and disordered cristae) and autophagic vacuoles [33].
The pathways responsible for enzyme-mediated conversion of neutral lipids into phospholipids or for phospholipid degradation into FFA, DAG and subsequent TAG synthesis are also expected to play a basic role in the induction and modulation of ML pools and LD formation (Figure 1).

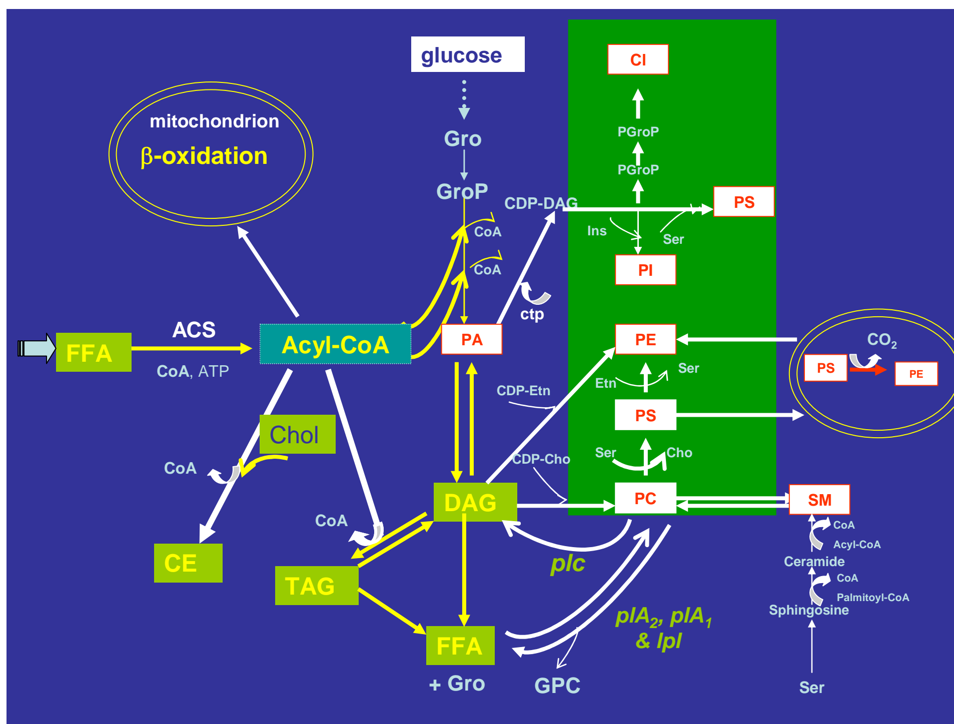

Figure 1. Schematic representation of the coordinated regulation of phospholipid biosynthesis and turnover of neutral lipids.
Metabolites: acyl-CoA, acyl-coenzyme A; CDP-Cho, cytidine diphospho-choline; CDP-DAG, cytidine diphospho-diacylglycerol; CDP-Etn, cytidine diphospho-ethanolamine; Cho, choline; Chol, cholesterol; CL, cardiolipin; CoA, coenzyme A; CTP, cytidine triphosphate; DAG, diacylglycerides; Etn, ethanolamine; FFA, free fatty acids; Gro, glycerol; Gro-3P, glycerol 3-phosphate; Ins, inositol; PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PGro, phosphatidylglycerol; P-GroP, phosphatidylglycerolphosphate; PI, phosphatidylinositol; PS, phosphatidylserine; Ser, serine; SM, sphingomyelin; TAG, triacylglycerides.
Enzymes: ACS, acyl-CoA synthetase; *plA_1*, phospholipase A1; *plA_2*, phospholipase A_2; *lpl*, lysophospholipase; *plc*, phospholipase C.

This hypothesis has been supported by in vitro experiments on fibroblasts treated with phosphatidylcholine-specific phospholipase (plc) inhibitor [6], as well as by evidence on parallel increase in ML production and phospholipase A2 overexpression in an experimental glioma induced to apoptosis by gene therapy approach [42]. Different levels of ML and glycerophosphocholine (a product of phospholipase A_2-mediated glycerophospholipid degradation) have also been reported by Delikatny et al [28] in prostate cancer cells treated in vitro with differentiating agents acting either as apoptotic (phenylbutirate) or cytostatic agents (phenylacetate).
These and similar investigations point to the role of ML signals as possible powerful candidate biomarkers of response to therapy of in vivo cancer cells. Further insights on the mechanisms underlying alterations of ML spectral profiles and therefore a better understanding of their role as indicators of pathology and therapy response require an integrated consideration of the interleaved pathways involved in neutral lipid and phospholipid synthesis and degradation.

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