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Known unknowns of cardiolipin signaling: The best is yet to come

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ABSTRACT

Since its discovery 75 years ago, a wealth of knowledge has accumulated on the role of cardiolipin, the hallmark phospholipid of mitochondria, in bioenergetics and particularly on the structural organization of the inner mitochondrial membrane. A surge of interest in this anionic doubly-charged tetra-acylated lipid found in both prokaryotes and mitochondria has emerged based on its newly discovered signaling functions. Cardiolipin displays organ, tissue, cellular and transmembrane distribution asymmetries. A collapse of the membrane asymmetry represents a pro-mitophageal mechanism whereby externalized cardiolipin acts as an "eat-me" signal. Oxidation of cardiolipin's polyunsaturated acyl chains - catalyzed by cardiolipin complexes with cytochrome *c*. - is a pro-apoptotic signal. The messaging functions of myriads of cardiolipin species and their oxidation products are now being recognized as important intracellular and extracellular signals for innate and adaptive immune systems. This newly developing field of research exploring cardiolipin signaling is the main subject of this review. This article is part of a Special Issue entitled: Lipids of Mitochondria edited by Guenther Daum.

If you are out to describe truth, leave elegance to the tailor.
[Albert Einstein]

1. Introduction

Eukaryotic cells are believed to have evolved from a chimeric combination of an archaeon and bacterium [1–3]. Their subsequent evolution to multicellular organisms changed the biosphere and geology of our planet [4,5]. This review will focus on the ancient, conserved and intriguing lipid, cardiolipin (CL) and its signaling functions. Understanding the context of how eukaryotic cells and subsequently multicellular organisms became so systemically prominent provides clues to eukaryotic cellular diversity, adaptation and survival. Evidence based on genomic studies suggests that a bacterial ancestor of the eukaryotic cell evolved into mitochondria (and chloroplasts) [3,6]. Mitochondria allow aerobic eukaryotes to generate vastly more ATP per cell than their prokaryotic ancestors. Eukaryotes have up to 4–5 orders of magnitude more energy per gene than do bacteria and thus have a huge evolutionary edge over prokaryotes in adapting to changing environments [3]. This energetic and metabolic complexity, however, requires immensely more sophisticated communications between the cellular and mitochondrial components, thus demanding and stimulating new signaling and control mechanisms and pathways.

In both mitochondria and bacteria, in addition to ionic gradients, substrate oxidation-linked electron transport through the proton-extruding respiratory complexes I, III and IV forms a trans-membrane proton gradient [7]. Transduction of this proton gradient energy to

Abbreviations: AAPH, (2,2′-azobis-2-methyl-propanimidamide, dihydrochloride); CL, cardiolipin; cyt *c*, cytochrome *c*; DAMPs, danger-associated molecular patterns; IMM, inner mitochondria membrane; IMS, mitochondrial inter membrane space; LC, liquid chromatography; MLCL, monolyso-cardiolipin; MS, mass spectrometry; NL, neutral loss; OMM, outer mitochondrial membrane; PA, phosphatidic acid; PAMP, pathogen-associated molecular patterns; PG, phosphatidyl glycerol; PS, phosphatidylserine; PUFA, polyunsaturated fatty acids; ROS, reactive oxygen species; TLCL, tetralinoleoyl-cardiolipin.

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ATP synthesis via complex V is a core function of mitochondria in aerobic eukaryotic cells. Respiratory complexes I, II and III also reduce O_2 to $O_2^{\bullet -}$. Disproportionation of $O_2^{\bullet -}$ catalyzed by superoxide dismutase forms H_2O_2, a potent source of oxidizing equivalents [8], is utilized in many signaling reactions [9–12]. The importance of H_2O_2 dependent reactions catalyzed by many metalloproteins, particularly heme-peroxidases, is so high that the functional significance of superoxide dismutase enzymatic activity may be attributed not to the elimination of $O_2^{\bullet -}$ but to the synthesis of H_2O_2 [13]. Notably, not only electron transport complexes but also several mitochondrial dehydrogenase complexes, such as pyruvate and 2-oxoglutarate dehydrogenases, are major potential sources of $O_2^{\bullet -}$. The formation of $O_2^{\bullet -}$ is dependent in part on the electrochemical gradient and the redox state of electron transport components which is also dependent on oxygen and the redox balance of the cell [14]. Collectively mitochondrial and cellular oxidants are often referred to as reactive oxygen species (ROS) [15,16].

Eukaryotic cells capable of replication have mitochondria (with one known exception, [17]) and this includes anaerobic eukaryotes that do not generate ATP using a "bioenergetic" membrane. The functions of the mitochondria clearly extend beyond ATP production. Mitochondria have a bacterial type circular genome in multiple copies that is separate from but is coordinated with the nuclear genome [18,19] but the number of mitochondrial genes is less than 1% of their proteome. Significant gene loss also occurs in obligate intracellular pathogens and symbionts and even descriptions of the minimum gene size needed by engineered bacteria are now being defined [20,21]. Why the mitochondrial genome is even preserved at all is not clear, but the mitochondrial genome codes for 13 mRNA's that translate to membrane protein constituents of the respiratory chain as well as for the tRNA for a 12S and 16S ribozyme and for 22 tRNAs [18]. As mitochondria are present in most anaerobic eukaryotes, non-ATP generating functions of mitochondria, notably signaling and control mechanisms, may have dictated the evolutionary preservation of the mitochondrial genome. In spite of the likely relevance of the mitochondrial genome to non-bioenergetic functions of the organelle, all the 13 mitochondrial genes encode subunits of electron transport complexes. The enigma of the essentiality of mitochondria and mtDNA remains unresolved.

While maintaining their ATP generating functions mitochondria must undergo biogenesis, mitophagy and turnover of their complex membrane systems to optimize their many functions. The proteins comprising the respiratory chain, mitochondrial transport systems and protein synthesis along with a cohort of mitochondrial specific lipids are ancient and highly conserved mitochondrial constituents. A hallmark mitochondrial lipid present in all "bioenergetic" prokaryotic and mitochondrial membranes is CL. Cardiolipin is a unique double-charged tetra-acyl chained phospholipid that is conserved in bacteria and mitochondria. Functions ascribed to CL include maintaining a non-spheroidal membrane structure, binding specifically to membrane proteins, respiratory and super-complex stabilization and signaling [22–31]. The signaling features of CL are the focus of this review. The mitochondrial CL differs from its prokaryotic precursor by having diverse polyunsaturated acyl side chains. In addition to structurally altering the membrane bilayer, polyunsaturated acyls are also oxidation targets giving rise to myriads of signaling lipid mediators [32] The initially synthesized nascent mitochondrial CLs may include a variety of acyls (32 fatty acid residues in Lipid Maps (www.lipidmaps.org) generating a highly diversified molecular speciation of CLs. In several tissues, however, the nascent CLs with different acyls (hetero-acylated CLs) undergo subsequent synthetic transformations - a process also called remodeling - to form CLs with identical acyls, homo-acylated CLs [33–36]. This stage of the biosynthesis requires remarkable specificity of the engaged enzymatic catalysts as it has to overcome the entropy-driven diversification of randomly integrated acyls in favor of stochastically almost impossible selection of only few homo-acylated CL species. The predominant presence of tetra-linoleoyl CLs in the heart, skeletal muscles and liver of mammals [35,37,38] illustrates the extraordinary transacylase selectivity of the process. While it is evident that high levels of selectivity is presumably associated with some function of homo-acylated polyunsaturated fatty acid (PUFA) CLs, their actual unique role remains unidentified. It is noteworthy, however, that this exceptional and "energetically expensive" selectivity may be a mammalian evolutionary achievement as phylogenetically more ancient eukaryotic organisms, like cold blooded fish, do not display the selective accumulation of homo-acylated CLs in heart and liver. In Fig. 1A-B the hetero-acylation of CLs detected in the brain, heart and skeletal muscle of Northern Red Snapper *(Lutjanus campechanus)* is contrasted with bovine heart which is mostly homo-acylated. Fig. 1 compares the diversification and the preponderance of different molecular species of CL in the fish and bovine heart. The fish has a CL hetero-acylation that more closely resembles the diverse range of CL seen in the mammalian brain – which has the highest tissue diversification in mammalian tissues [39–41]. The enormous diversity of Cls in the heart of a fish (Northern Red Snapper) contrasts with extremely high dominance of homo-acylated tetralinoleoyl-CL in the bovine heart.

While CLs and oxidized products derived from them are now recognized as a rich and diverse source of cell signals, the significance of the diversification vs uniformity of CL is still enigmatic and represents an exciting field of future research [35,42–44]. In this context, CLs and their oxidation products should be recognized as a novel cellular signaling language that is part of and integral to, many cellular functions [22,24,26,45]. The complexity required for a harmonized spatiotemporal organization of the myriads of reactions in a multi-cellular organism as well as interactions with extra- and intracellular bacterial pathogens, involving CL, is apparent. This includes signaling during cell death, mitophagy, innate immunity and potentially many hereto unknown signaling functions. With increasing signaling complexity, the potential for defects derived from CL biosynthesis resulting in pathology is already apparent in Barth Syndrome [33,35,46–48] and it is likely other diseases linked to CL structure, location and stability may subsequently be defined.

This review is not at all intended to restate or rework the many fine reviews about CL, which nicely summarize and review the explosion of new literature and knowledge about CLs [26,35,42–44,49–51]. Instead, our focus will be on issues of CL as a signal that are not well understood and require wisdom and experimental precision to unravel. We will provide a brief overview of CL structure and synthesis pertaining to its role in cellular signaling. A more detailed discussion of CL binding to proteins, especially cytochrome *c* (cyt *c*), its asymmetric distribution in the mitochondria, the scope of CL diversity and the role of CL in mitophagy, apoptosis and innate and adaptive immunity will provide opportunities for the reader to understand that CLs function as sophisticated cell signals. While our understanding of the complex differential tissue distribution of unsaturated and oxidized CL and its role in cell signaling is just now becoming apparent, we trust that the readers of this review will grasp the depth and complexity that CLs play in biology.

2. Cardiolipin, its molecular structure, homo- and hetero-acylated species, asymmetry – links with the biosynthesis

2.1. Cardiolipin structure and ionization state

Cardiolipin, (1,3-bis(sn-3′-phosphatidyl)-sn-glycerol) demonstrates a molecular asymmetry, an acyl chain asymmetry and a membrane distribution asymmetry which are integral features of its functions in mitochondria. Cardiolipin is a dimeric phosphatidic acid with a "backbone" glycerol containing a prochiral central carbon, two phosphates and 4 acyl chains (Fig. 1C) [52]. The two phosphates are ionized at physiological pH resulting in CL being a doubly-negatively charged amphiphilic lipid. While the pKa of the phosphates of CL had originally been described as strong acids and were doubly negatively charged [53], a pH titration study comparing a native and 2′-deoxy-glycerol backbone suggested

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