ISSN: 2279 - 0594

Journal of Biomedical and Pharmaceutical Research

Available Online at www.jbpr.in

CODEN: - JBPRAU (Source: - American Chemical Society)

Index Copernicus Value: 63.24

PubMed (National Library of Medicine): ID: (101671502)

Volume 6, Issue 3: May-June: 2017, 01-06

Research Article

Interaction of phospholipase enzymes with autophagic processes; importance for cell signaling and therapeutics

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Received 02 April. 2017; Accepted 03 May 2017

ABSTRACT

Phospholipases are inherently involved in signalling mechanisms of various neurotransmitters and hormones. Cytosolic phospholipase ($cPLA_2$) initiates pro-inflammatory lipid mediator pathways which play a critical role in host defense and in inflammation. The crosstalk between the two pathways remains unclear. In this article, the important roles of the three phospholipases i.e PLC, PLD and PLA_2 are investigated in the autophagic processes and their link to intracellular kinetics of signalling molecules. While autophagy delivers cytoplasmic constituents to autophagosomes and is involved in innate and adaptive immunity. The $cPLA_2$ and its metabolite lipid mediators have been shown to induce autophagy in the macrophage cell line and in primary monocytes. Cysteinyl leukotrienes D4 and E4 and PGD2 also induced these effects. The autophagy is independent of changes in mTOR or autophagic flux. cPLA2 and lipid mediator-induced autophagy is ATG5 dependent. These data suggest that lipid mediators seem to play a role in the regulation of autophagy, demonstrating a connection between the two seemingly separate innate immune responses, induction of autophagy and lipid mediator generation.

Keywords: PLA_2, PLD, PLC, autophagy, lipid, cell, drug

Introduction

Phospholipase D (PLD) catalyses the breakdown of phosphatidylcholine into choline and phosphatidic acid. PLD-generated phosphatidic acid (PA) participates in the formation of the initial autophagosome. The formation of the isolation membrane is the earliest event in autophagosome generation. PLD_2 could play a key role through the regulation of the generation of PA pools, which have been shown to regulate positively formation of autophagosomes [1]. Additionally, it has been shown that PLD_2 influences the curvature of membranes through PA [2]. This fact, in particular, has the potential to promote autophagy, as negative curvature is an element that can lead to fusion of lipids, which eventually promotes the formation of the initial autophagosome [3]. It has been suggested that PLD will also lead to the reuptake of plasma membranes via endocytosis, leading to precursors of the autophagosomes. PLD_2 also has been shown to localize on the edges of the Golgi apparatus, indicating a role in cellular trafficking [4]. This could be accomplished through the membrane. There are several possible connections between autophagy and phospholipids other than PA. Lipid metabolism and the small G protein Arf6, which is involved in autophagosome formation, directly target PLD [5]. Additionally, Arf6 is a stimulator of catenin-induced transcriptional activity [6]. This could establish an additional feedback loop with PLD2. Inhibition of DAG kinase induces autophagy, mainly because the lack of this kinase is compensated by an increase of PLD so that homeostasis of PA is maintained in the cell [7]. Inducers of phagocytosis, are known to be activators of PLD2 [8] and are responsible for triggering autophagy meaning that PLD could possibly do this by direct regulation of phospholipid generation and by signaling through other means, such as PKC involvement. Guanine nucleotide exchange factor—the later acts upon small GTPases of the cellular motility machinery,

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such as Rac and Rho [9]. PLD is under expression control by mTOR [10], and mTOR is a key component of autophagy [11]. This article evaluates the role of various phospholipase enzymes in the autophagic process and prospects for therapeutic intervention.

Phospholipase D and autophagy during starvation:

Recent data strongly suggests that mammalian PLD1 facilitates the maturation of autophagosomes (APs) during starvation-induced autophagy. Indeed, it has been shown that PLD1 is predominantly associated with endosomes in normal medium and that a pool of this enzyme translocates onto LC3-positive structures upon nutrient-deprivation. Because these compartments are also positive for endosomal markers, and it was speculated that they may originate from the heterotypic fusion of endosomes with APs and thus correspond to *bona fide* amphisomes, as seen in other instances [12]. EM analysis provided strong support to this hypothesis, as the immunoreactivity of PLD1 predominantly localized to the outer membrane of AP-related structures, suggesting that the asymmetric distribution of PLD1 may simply reflect its redistribution from the limiting membrane of endosomes to the outer membrane of amphisomes following fusion of endosomes with APs. While the mere translocation of PLD1 from endosomes to amphisomes does not prove an implication of this lipid enzyme in the maturation of APs, the functional analyses indicate that interfering with the function of PLD1 significantly alters the metabolism of LC3-II as well as the size and number of APs, thus pointing to a relevant role of this enzyme in autophagy.

Autophagy has been implicated previously in both health promoting and disease-associated states. It has been thought to be a cellular homeostatic mechanism. Autophagy has been described in a variety of processes, including neoplasia, neurodegeneration, myopathies, development, aging, and innate and adaptive immune responses [13-14]. Lipid mediators also may play a role in regulating immune and inflammatory responses [15]. Recent results have linked two events, autophagy and cPLA2-initiated lipid mediator generation, which may bridge two aspects of the innate immune response. It is suggested that lipid mediator induced inflammation may in part regulate the induction of autophagy. Lipid mediators are most likely key participants in the pathogenesis of inflammatory diseases [16-17]. Our inhibitor and lipid mediator stimulation data suggest that autophagy was induced by the lipid products from two multienzyme pathways downstream of $cPLA_2$.LTB4 and LTC4 are products of the 5-LO pathway of AA metabolism [18]. LTC4 is converted extracellularly to LTD4 and LTE4. Consequently, LTC4 ,LTD4, and LTE4 are together referred to as CysLTs.

Cell culture

Cells were cultured under 5% CO_2 at 37°C. For example the U-2 0S cells (ATCC HTB-96) are grown and assayed in McCoy's 5A medium supplemented with 10% dialyzed FBS, 25 mM HEPES, 0.1 mM NEAA, 1 mM Sodium Pyruvate, and 100 U/mL Penicillin/100 µg/mL Streptomycin. HEK293T and GripTite 293 cells (Life Technologies, R79507) are grown in DMEM supplemented with 10% dialyzed FBS, 2 mM GlutaMAX-I, 0.1 mM NEAA,1 mM Sodium Pyruvate, and 100 U/mL Penicillin/100 µg/mL Streptomycin. GripTite 293 cells stably expressing GFP-tagged LC3B are generated using the Jump-In targeted integration system (Life Technologies, A14150). For starvation conditions, growth media was removed and replaced with Earle's Balanced Salt Solution (EBSS) supplemented with 20 mM HEPES and 2 mM each of $CaCl_2$ and $MgCl_2$. SH-SY5Y (ATCC CRL-2266) cells were grown in DMEM/F12 medium supplemented with 10% dialyzed FBS and 100 U/mL Penicillin/100 µg/mL Streptomycin.

TR-FRET detection

TR-FRET detection can be conducted according to a previously described technique [19].To prepare complete 6X lysis/detection antibody solution, LanthaScreen 6X Cellular Assay Lysis Buffer (Life Technologies, A12891) is supplemented with a 1:33 dilution of protease inhibitor cocktail (Sigma, P8340) and 6 nM Tb-labeled antibody. To assay, lysis/detection antibody is applied to the wells to give a 1X final concentration and the cell lysates are incubated at room temperature for about 1–2 hr before reading the TR-FRET. Plate readers used in this study are as follows: EnVision (PerkinElmer), PHERAstar *Plus* (BMG LABTECH), Infinite F500

(Tecan), Infinite 200 PRO (Tecan). Guidelines for the use and interpretation of assays for monitoring autophagy in higher eukaryotes.. Instrument-specific Tb TR-FRET setup is performed as detailed at 'www.invitrogen.com/instrumentsetup'. The data shown is normally collected on an EnVision with TRF laser light source, with emission collected at 500 nm (Tb donor signal) and 520 nm (GFP FRET acceptor signal) in time-resolved fluorescence mode (100 µs delay, 200 µs integration time). For each well, a TR-FRET Emission Ratio is determined by dividing the acceptor emission (520 nm) by the donor emission (500 nm) value. Assay Window is calculated by dividing the treated data by the untreated control. Curve fitting and EC_{50} value calculations are performed with GraphPad Prism software using a nonlinear regression equation for variable slope sigmoidal dose-response.

Phospholipase and autophagy and drug target:

PLD modulates autophagy in different manners, depending on specific cell type and subcellular localization: On one hand, PLD/PA interact with mTOR, PI3K-Ras/ERK signaling pathways, both regulate negatively autophagy. On the other hand, PLD1 has been shown to foster autophagy by co-localized with LC3 during starvation. Such a dual role has been previously described for Vps34, a lipid enzyme that is required for autophagy, yet also stimulates mTOR [20]. Rapamycin-based therapeutics effectively decreased cyst growth and preserved renal function in a variety of animal models for PKD. However, conflicting results were obtained in clinical trials. Two clinical trials did not show a beneficial effect in autosomal dominant polycystic kidney disease (ADPKD) patients, both in early and more progressive disease stages. One of the possible factors confounding the interpretation of these results could be the dose of mTOR inhibitors used. The levels of rapamycin tolerated in humans are lower than in mice. Based on some recent observations, elevated activation of PLD may mitigate the effect of rapamycin on human PKD cells. Indeed, combining mTOR and PLD inhibitors enhanced the rapamycin-sensitivity of PKD cells. Therefore, combination therapies that include rapamycin and strategies that suppress PLD activity could be used to target mTOR signaling in PKD [21-22]. Autophagy is a catabolic process in which cell components are degraded to maintain cellular homeostasis by nutrient limitations. Defects of autophagy are involved in numerous diseases, including cancer. It has been demonstrated a new role of phospholipase D (PLD) as a regulator of autophagy. PLD inhibition enhances autophagic flux via ATG1 (ULK1), ATG5 and ATG7, which are essential autophagy gene products critical for autophagosome formation [23]. PLD inhibition significantly sensitized *in vitro* and *in vivo* cancer regression via genetic and pharmacological inhibition of autophagy, providing rationale for a new therapeutic approach to enhancing the anticancer efficacy of PLD inhibition. Collectively, this shows a novel role for PLD in the molecular machinery regulating autophagy. The lys49PLA_2 a myotoxic PLA_2 from the 'Bothrops moojeni' has potential for treatment of parasitic diseases like Schistoma mansoni and Leishmania species and also against cancers cells of the human breast adenocarcinoma (SK-BR-3) and human T leukemia cells.

Phospholipases and bacterial cell autophagy:

Listeria monocytogenes is a Gram-positive bacterial pathogen that induces its own uptake in non-phagocytic cells. *Listeria* produces two phospholipase toxins, a phosphatidylinositol-specific phospholipase C (PI-PLC, encoded by *plcB*) and a broad-range phospholipase C (PC-PLC, encoded by *plcA*), which contribute to bacterial virulence. It has long been recognized that secretion of PI- and PC-PLC enables the disruption of the double membrane vacuole during cell-to-cell spread, and those phospholipases have also been shown to augment LLO-dependent escape from the entry endosome. Since both PI- and PC-PLC cleave phosphatidylinositol (PI), the precursor to PI3P, was investigated to determine whether these molecules affected cellular levels of PI3P. It was observed that infection with wild type but not phospholipase-deficient bacteria decreased levels of PI3P, providing a potential mechanism for the phospholipase-dependent manipulation of PI3P pathways. In agreement, short (10 minute) wortmannin treatment of cells infected with phospholipase-deficient bacteria restored granule formation. Furthermore, compared to cells infected with wild type bacteria, autophagy flux was increased in cells infected with phospholipase-deficient *Listeria* [24]. Collectively, these studies indicated that secretion of PI- and PC-PLC into the cytosol by *Listeria*, interferes with the efficient

generation of PI3P resulting in stalling of pre-autophagosome structures, decreased autophagy flux and reduced bacterial targeting to autophagosomes [25].

Snake venom cPLA2 and autophagy:

PLA2s from snake venoms have been classified as groups I and II on the basis of their primary structure and disulfide bridge pattern [26-27]. In addition to their primary catalytic role, snake venom PLA2s show other important toxic/pharmacological effects including myonecrotic, neurotoxic and cardiotoxicity. Crotoxin (CrTX) is a cytotoxic PLA_2 isolated from the venom of the South American rattlesnake, Crotalus durissus terrificus [27-28]. It is a non-covalent complex formed by 2 non identical subunits, one acidic (subunit A) and one basic (subunit B).Subunit B is a PLA_2 formed by a single chain of 122 amino acid residues cross-linked by 7 disulfide bonds.Its cytotoxicity was independent of cell growth since both quiescent and proliferating cells had similar sensitivities. CrTX displays cytotoxic activity against a variety of murine [29] and human tumor cell lines in vitro [30-31] and appears to be highly active toward cell lines expressing a high density of epidermal growth factor receptors (EGFR) ; however, the precise mechanism of the cytotoxicity remains to be established. Thus, levels of p62 can be used as an index of autophagy activity. It has been reported that CrTX enhanced autophagy in MCF-7 cells by activating cathepsin B, D, and L, releasing cytochrome *c*, and relocating AIF into nuclei. Similar results were observed in a study; whereby CrTX elevated the expression of both LC3-II and Beclin 1 protein and decreased p62 protein levels, suggesting that autophagy may be involved in the inhibitory effects of CrTX on SK-MES-1 cells [32].

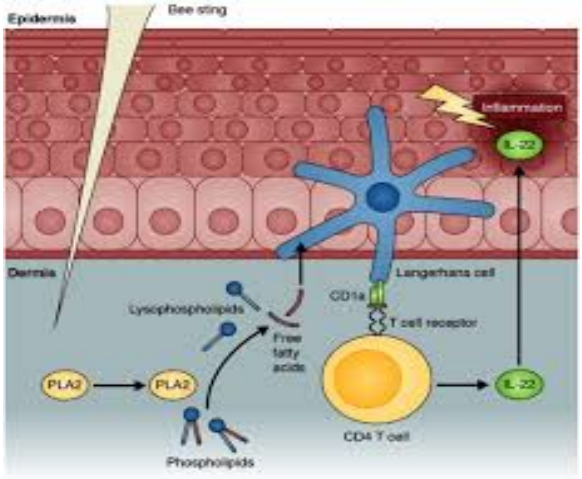

Fig.1: Effect of bee venom PLA_2 on autophagy (Courtesy :M-C F Boutrin H.A. Foster V.W. Pentreath. Jul 2008, Experimental Parasitology)

Conclusion:

It appears the phospholipases do interact in the autophagic processes and the biochemical process connecting membrane damage to amino acids starvation during infection remains to be clarified. Probably,phospholipases inhibit the autophagic flux by reducing PI3P generation. This suggests that pre-autophagosomal structure progression likely requires uncharacterized additional PI3P-dependent events. Finally, the molecular mechanisms through which PLCs down-regulate PI3P levels remains to be characterized. Lipid mediators also may play an important role in regulating immune and inflammatory responses. These results have linked two events, autophagy and $cPLA_2$-initiated lipid mediator generation, which may bridge two aspects of the innate immune response. It is suggested that lipid mediator induced inflammation may in part regulate autophagy induction.

Acknowledgements:

The authors wish to thank the Lipid research group at IICT, Hyderabad for useful scientific discussion and demonstrations.

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