LEUKOCYTE LIPID BODIES — STRUCTURE AND FUNCTION AS "EICOSASOMES"

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ABSTRACT

Lipid bodies are cytoplasmic inclusions that develop within leukocytes, including eosinophils and neutrophils, associated with inflammation. Our investigations of the formation and function of lipid bodies have revealed that they are distinct, inducible endoplasmic reticulum–derived, membrane- and ribosome-containing organelles with diverse functional roles in inflammatory responses of leukocytes. Leukocyte lipid bodies contain all enzymes required for synthesizing cyclo-oxygenase- and lipoxygenase-derived eicosanoids. Lipid body formation, rapidly inducible *in vitro* and *in vivo* by specific intracellular signaling pathways, enhances leukocyte formation of cyclo-oxygenase– and lipoxygenase-derived eicosanoids. Lipid bodies are discrete sites of eicosanoid synthesis, as documented for immunolocalized leukotriene C_4, leukotriene $B_{4,}$ and prostaglandin E_2. Lipid body–derived eicosanoids function as both paracrine and intracrine mediators of inflammation. Based on combined proteomic and ultrastructural studies, leukocyte lipid bodies are complex organelles with internal membranes and ribosomes. Structurally and functionally leukocyte lipid bodes are distinct from lipid droplets in adipocytes.

INTRODUCTION

Lipid bodies (LBs) are lipid-rich cytoplasmic "inclusions" that form in diverse cell types ranging from yeast and *Drosophila* to mammalian cells. In mammalian cells, LBs (also called lipid droplets) are characteristic of adipocytes and steroidogenic cells (1). Although little had been known about the origins, composition, or functions of LBs (2), in the last decade or so there has been a renaissance of interest in LBs and lipid droplets (3) that led to the recognition that these structures are multifunctional organelles (1,3–6). This broader recognition of LBs as organelles was bolstered by proteomic analyses of LBs from several cell types (7–12). Unexpected

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results from LB proteomic analyses included a common recognition that LBs contained many Rab guanosine triphosphate hydrolase (GTPase) proteins potentially involved in vesicular trafficking. In addition, LB-associated proteins of the PAT/PLIN family, perilipins, adipophilin, tail-interacting protein of 47 kDa (TIP47) and S3-12, initially associated only with adipocyte LBs, were uniformly found with LBs in other cells (12,13). Thus, although there was an evolving recognition of likely commonalities in the biogenesis and structure of LBs in varied mammalian cell types, the origins and range of functions of LB organelles, especially those in leukocytes and not adipocytes, remained ill understood.

Leukocyte LBs are often not recognized because LBs dissolve in common alcohol-based stains, such as Wright's and Giemsa hematological stains. In limited numbers, LBs are normally present within mammalian cells, including neutrophils (polymorphonuclear leukocytes [PMNs]), eosinophils, mast cells, macrophages, endothelial cells, and fibroblasts. LB numbers uniformly increase in cells participating in inflammatory processes (5,14–19). Our interests in LBs were occasioned both by the prominence of LB organelles within leukocytes associated with inflammation *in vivo* and by the roles that these lipid-rich organelles might play in arachidonic acid (AA) metabolism by leukocytes and other cells involved in inflammation.

RESULTS

LB Formation *In Vitro*

As we have shown, leukocyte LB formation is a rapid, highly regulated process involving specific agonist-elicited signal transduction pathways (5,20–27). Thus, LBs are inducible "early response" organelles that may participate in inflammation-associated responses of leukocytes.

Signaling pathways mediating leukocyte LB formation. Our studies delineating mechanisms of leukocyte LB induction identified several agonists and intracellular signaling pathways (20–22,25,28). For PMNs and eosinophils (Figure 1), one pathway is initiated by receptor-mediated platelet activating factor (PAF) signaling via 5-lipoxygenase (5-LO) activation to generate 5-hydroxyeicosatetraenoic acid (5-HETE), which then signals via a pertussis toxin (PTX) –inhibitable receptor-dependent mechanism. The second pathway initiated by *cis*-fatty acids is aspirin (ASA), salicylate and non-steroidal anti-inflammatory drug (NSAID) inhibitable, but cyclo-oxygenase 1 (COX1) and cyclooxygenase 2 (COX2) independent. Cell-permeant diglyceride constitutes a third

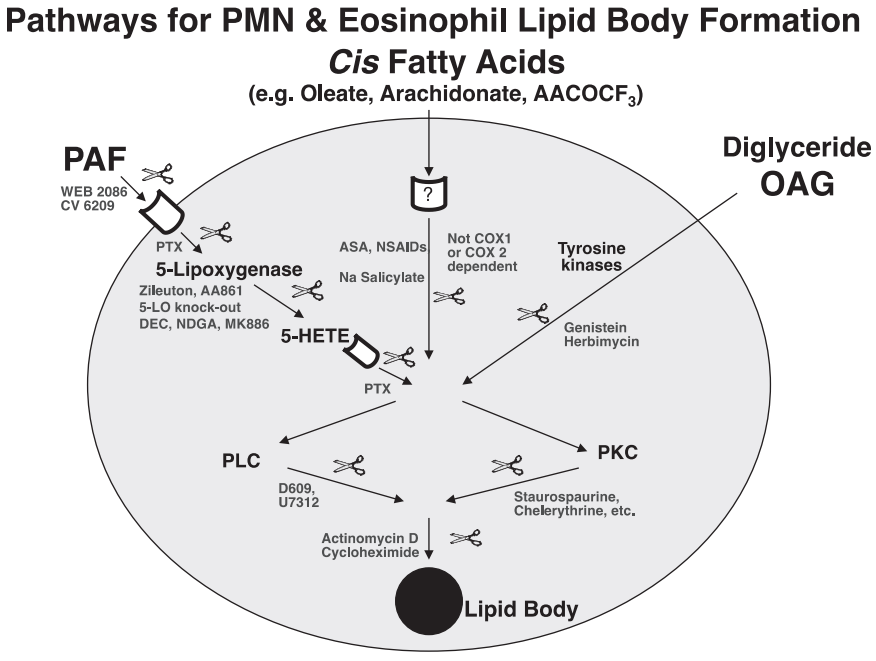

FIG. 1. Pathways of lipid body formation in neutrophils (polymorphonuclear leukocytes) and eosinophils. Stimuli include platelet activating factor, *cis* fatty acids and diglycerides (OAG). Abbreviations: PAF, platelet activating factor; WEB, ; CV, ; PTX, pertussis toxin; 5-LO, 5-lipoxygenase; DEC, diethylcarbamazine; NDGA, ; MK866, ; ASA, aspirin; NSAIDs, non-steroidal anti-inflammatory drugs; COX, cyclo-oxygenase; OAG, oleoyl-acetyl glycerol; PLC, phospholipase C; PKC, protein kinase C; 5-HETE, 5-hydroxyeicosatetraenoic acid; D609, ; U7312, .

initiating signal. For all three pathways, common downstream signaling requires in part protein kinase C and phospholipase C activation and new mRNA and protein synthesis. In addition for eosinophils, eotaxin/RANTES acting via CCR3 activate downstream phosphatidylinositol 3 kinase (PI3K) and ERK 1/2 and p38 MAP kinases to elicit LB formation. Eosinophils from normal subjects after exposure to granulocyte-macrophage colony stimulating factor behave like eosinophils from hypereosinophilic syndrome donors in which PAF-induced LB formation is tyrosine kinase-dependent (20). Interleukin 5 (IL-5) and immunoglobulin G stimulate eosinophil LB formation and leukotriene C_4 (LTC_4) production via endogenous PAF formation (29). IL-16 by stimulating eotaxin/RANTES release stimulates LB formation and LTC_4 production (30). Thus, leukocyte LB formation is a highly regulated, rapid cellular response mediated by several distinct activating and signaling mechanisms.

LB Formation *In Vivo*

While normal blood PMNs and eosinophils contain ~1 and ~5 LBs/cell, respectively, blood PMNs from patients with infections and eosinophils from eosinophilic patients contain many more LBs/cell (14,15,31). Recruited PMNs and eosinophils in airways, tissues, blood, and exudative effusions of humans and experimental animals with infectious and inflammatory reactions typically contain abundant LBs (15–17,21,32,33). LB formation in leukocytes *in vivo* is a common correlate of their participation in inflammation.

LBs as "Eicosasomes" — Sites of Arachidonate Localization and Eicosanoid Formation

We hypothesized that LBs have roles in the regulated formation of AA-derived eicosanoids in leukocytes. COX pathways, mediated by COX1 or COX2, form prostaglandins (PGs) and thromboxanes. The 5-LO pathway in PMNs, eosinophils, monocytes, mast cells, and macrophages, forms 5-HETE and leukotrienes (LT). The formation of eicosanoids that are not stored pre-formed within cells is tightly regulated; and enhanced formation of eicosanoids, necessary for their intracrine, autocrine, and paracrine activities, occurs at cell membranes in response to activating stimuli. Recognized intracellular sites of eicosanoid formation have included the membranes of the nuclear envelope and cytosolic LBs (34–36).

LBs are sites of substrate arachidonate and eicosanoid-forming enzyme localization. We have shown that eicosanoid precursor AA localizes within LBs in many leukocyte types (14,15,25,37,38) and that AA was esterified in phospholipids of eosinophil LBs (38). Esterified AA localization within PMN LBs has been confirmed using Raman microscopy (39). A key enzyme needed to release AA, cytosolic phospholipase A_2, activating kinases (MAP and PI3 kinases), and all relevant eicosanoid-forming enzymes (COXs, 5- and 15-LO, 5-LO–activating protein, and LTC_4 synthase) have been localized to LBs (20–22,24,31,32,40–49). These findings support the capacity of LBs to serve as distinct sites for eicosanoid formation.

Lipid bodies correlate with enhanced ("primed") leukocyte eicosanoid formation and release. In studies of PMNs, there had been a paradox. In response to the Ca^{++} ionophore, A23187, PMNs generated large quantities of leukotriene B_4 (LTB_4) and 5-HETE; whereas more natural, receptor-mediated stimulation, for example, N-formylmethionyl-leucyl-phenylalanine (fMLP), elicited little eicosanoid

formation (50,51), unless PMNs are first "primed." Priming consists of pre-incubating PMNs with oleoyl-acetyl-glycerol (OAG), PAF, AA, phorbol myristate acetate or lipopolysaccharide (50–53). Priming agents also augment eicosanoid formation by A23187-stimulated PMNs (21,24,54,55). Our studies established that increased leukocyte LB formation consistently correlated with primed responses for enhanced eicosanoid formation. With each class of LB inducer (e.g., eotaxin/RANTES, PAF, OAG, *cis*-fatty acids, lipopolysaccharide), numbers of induced LBs correlated with enhanced magnitudes of released COX- and 5-LO–derived eicosanoids (21,22,24,31,47,56,57). Conversely, inhibition of LB induction correlated with suppression of enhanced eicosanoid release (21,22,24,27,56). For instance, ASA and NS-398 inhibition of oleate-induced LB formation suppressed generation of 5-LO (not itself ASA or NS-398 inhibitable)-derived leukotrienes (24,27). Thus, induction of LB formation uniformly correlated with enhanced primed generation of eicosanoids, and inhibition of LB formation correlated with diminished capacity to form both COX- and LO-derived eicosanoids.

To further ascertain a role for LBs in enhanced eicosanoid formation independent of nuclei, we established that: 1) anucleate eosinophil cytoplasts form LBs in response to PAF stimulation, 2) LB numbers in cytoplasts correlated with levels of primed LTC_4 and prostaglandin E_2 (PGE_2) released by cytoplasts following A23187 challenge, and 3) LBs in anucleate eosinophil cytoplasts were sites of immunolocalized 5-LO, COX, and LTC_4 synthase proteins (58). Thus, in the absence of nuclei and nuclear envelopes, LBs were sites of enhanced eicosanoid formation.

Direct localization of eicosanoid synthesis at LBs. Previously, intracellular sites of eicosanoid formation had never been directly demonstrated in any cell, but were only inferred based in immunolocalization of eicosanoid-synthesizing enzymes. Because immunolocalization of eicosanoid-forming proteins might not reflect their state of enzymatic activity, we developed a novel method to immunolocalize newly synthesized LTC_4 at its sites of formation within eosinophils. We localized newly synthesized LTC_4 at LBs in eosinophils stimulated *in vitro* with eotaxin-1, RANTES, or anti-CD9 monoclonal antibody and at LBs in eosinophils elicited *in vivo* by allergic challenges (22,30,32,33,59). In contrast, in eosinophils activated by A23187 alone, newly formed LTC_4 was localized at nuclear membranes (22,59), concordant with the perinuclear immunolocalization of 5-LO protein in A23187-activated eosinophils (60). Our colleagues and collaborators utilized our same eicosanoid immunolocalization methods to likewise localize newly synthesized PGE_2 and LTB_4 directly at LBs in macrophages (56,57,61). Likewise, PGD_2 formation has been localized to LBs in eosinophils (62).

These findings provided the first direct "smoking gun" evidence that LBs are sites of synthesis of both 5-LO- and COX-derived eicosanoids.

Functions of Leukocyte LB-Derived Arachidonate and Eicosanoids

One function of induced LBs is to serve as distinct sites for enhanced synthesis of COX- and/or LO-derived eicosanoids that are paracrine mediators of inflammation (63). As noted above, our studies correlated elicited formation of LBs with enhanced extracellular release of COX- and 5-LO–pathway-formed eicosanoids by PMNs, eosinophils, and macrophages. Utilization of depots of LB-derived AA for generating paracrine-acting mediators, rather than AA derived from perinuclear membranes, would obviate stoichiometric structural perturbation of nuclear membranes.

A second functional role for leukocyte LBs is to provide AA and eicosanoids that act as intracrine signal-transducing mediators. The first evidence for this intracellular mediator role for LTC_4 arose from our studies. Our LTC_4 immunolocalization technique with its heightened sensitivity detected low level LTC_4 formation at LBs in eotaxin-1-stimulated eosinophils when no extracellular LTC_4 release was detectable (22,30,64). Eotaxin-1 stimulates the release of preformed IL-4 from eosinophil granules (30,65). We established that eotaxin-1–elicited release of IL-4 is: 1) dependent on intracellular activation of 5-LO to form LTC_4 at LBs, and 2) intracellular (not extracellular) LTC_4 functions as an obligate signal-transducing mediator (64). Moreover, we have documented that eosinophil granule membranes express functional cysteinyl leukotriene (CysLT) receptors that mediate secretion from within eosinophil granules (66). Thus, for the first time, we demonstrated an intracrine, signal-transducing role for a CysLT and established that the regulated formation of LTC_4 at LBs is critically involved in controlling a response of eosinophils, including their capacity to secrete the cytokine IL-4.

Another intracrine signaling role for LBs was revealed by Raman microscopic studies that showed that AA-rich LBs in PMNs rapidly move to and associate with phagosomes, suggesting that LB-derived AA functions to locally activate nicotinamide adenine dinucleared phosphate, reduced (NADPH) oxidase at phagosomes (39). Thus, LBs are functional as sites of regulated formation of eicosanoids for their paracrine mediator functions and are intimately involved in intracellular signal transduction fully pertinent to leukocyte functioning in inflammation.

Consonant with a central role for leukocyte LBs as principal sites of eicosanoid formation, increasingly findings in other cell types, including colon cancer cells (67), *Pseudomonas* ExoU toxin-stimulated airway

epithelial cells (68), and *Mycobacteria* Bacillus Calmette Guerin (BCG) vaccine challenged macrophages *in vivo* (61), are documenting that LBs in varied cell types have central and primary functions in the synthesis of eicosanoids (69–71). These findings fully substantiate the functional importance of LBs in leukocytes, in common with other cells, in eicosanoid synthesis pertinent to host inflammatory and immune responses, and exemplify how our focused studies of LBs in leukocytes likely have broader implications for other cells.

DISCUSSION

LBs in leukocytes and other cells have engendered considerable recent interest (4,67,72,73). For leukocyte LBs involved in eicosanoid formation, we have significantly advanced our understanding of these inducible organelles. We established that LTC_4, LTB_4, PGD_2, and PGE_2 are formed at LBs. We have provided evidence that LBs have functional roles pertinent to allergic inflammation as a source of eicosanoids with both extracellular-paracrine and intracellular-intracrine roles as mediators of leukocyte responses pertinent to allergic inflammation. Our proteomic/electron microscopy studies revealed endoplasmic reticulum (ER)–like membranes and ribosomes in LBs. Recognition of ER membranes within leukocyte LBs resolves a conundrum about how membrane-associated eicosanoid-forming enzymes could function at LBs. In accord with their content of ribosomal and mRNAs, we demonstrated that LBs are sites of *de novo* 5-LO protein synthesis. In addition to their roles in generating eicosanoid lipid mediators, active intra- and extracellularly, leukocyte LBs likely have roles in cytokine-mediated responses. Moreover, the leukocyte LB proteome in concert with proteomes of LBs from other cells supports the capacities of leukocyte LBs to be multifunctional organelles. Whereas lipid droplets in adipocytes and other cells are recognized to contain neutral lipids surrounded by a monolayer of phospholipid membranes (74), our studies of leukocyte LBs differentiate them from most lipid droplets. With our insights that leukocyte LBs are complex ER-derived organelles, leukocyte LBs differ in form and function from lipid droplets found in adipocytes.

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DISCUSSION

Moore, New York City: Thank you Peter, it's wonderful to see some real hematology presented at the meeting. Are there any congenital abnormalities of lipid bodies to explain any problems handling infections or inflammation that have been described?

Weller, Boston: Probably not. Not that we know of in terms of the lipid bodies that form within leukocytes, there aren't. There are though other congenital inherited abnormalities that probably affect lipid droplet formation. These are due to alterations in some of the associated proteins that form within lipid droplets.

Schreiner, Los Altos: Thank you for a very interesting presentation. I have two questions, is there any evidence or role for these lipid bodies as extruded elements?

Weller, Boston: Excellent question and the evidence for that would largely rest with EM studies in order to detect them. In general, from what I am aware of, there really is no recognized role for these being extruded. There may be intracellular associations within cells. For instance within mast cells, lipid bodies have been shown to associate with degranulating phagosomes; and there is some data in neutrophils that actually shows that lipid bodies are quite motile within cells and move to potentially deliver activating arachidonic acid to help facilitate the respiratory burst.

Schreiner, Los Altos: Thank you, and my second question is that I am struck by the number of initiating factors that are either lipids or glycolipids particularly with respect to bacterial glycolipids. Is there any evidence that some of the initiating elements particularly bacterial glycolipids are actually part of the lipid body or incorporated as they come into the cell and play a continuing role so to speak in activity within the lipid body?

Weller, Boston: There is no indication that bacterial-derived lipids are actually incorporated into the lipid body. Curiously, part of the signaling to initiate leukocyte lipid body formation that occurs by the *cis*-fatty acids is not a reflection of the incorporation the exogenous *cis*-fatty acid into the lipid bodies. Hence, if one uses a *cis*-fatty acid that has a blocked carboxyl group, it still is acting as a signaling agent even though it cannot become esterified within neutrophil or eosinophil lipid bodies. Bacterial-derived products, including lipopolysaccharide, can signal to initiate lipid body formation.

Boxer, Ann Arbor: Excellent talk Peter. What is the natural history of the lipid bodies in malignant eosinophilia and do they play in the role in the pathogenesis of malignancy? And then you know when on peripheral blood smears we see often activated eosinophils that apparently degranulate, do lipid bodies re-accumulate in these activated eosinophils?

Weller, Boston: Good questions Larry, I think lipid bodies probably do re-accumulate within eosinophils that have degranulated so that it is a dynamic process. In terms of relationships with neoplasms, lipid bodies are not unique to leukocytes; and there is actually a literature with epithelial cells and adenocarcinomas in the GI tract that would identify lipid bodies as sites of eicosanoid production within some of the neoplasms. These lipid bodies might be one of the sites where taking aspirin or NSAID can have a therapeutic benefit by inhibiting the formation or the function of these lipid bodies.

Boxer, Ann Arbor: What about the malignant eosinophil itself? Is there any unusual morphology under EM that you could recognize?

Weller, Boston: No, with malignant eosinophils there is no alternate morphology that would distinguish a malignant eosinophil enough from an activated eosinophil that might have more lipid bodies.

Dale, Seattle: Very interesting talk….thank you Peter… neutrophil is a very short-lived cell, are these cells in the death pathway or do the lipid bodies actually promote the cell survival?

Weller, Boston: We have no data that would link the formation of these with a death pathway. We think that it's very much a functional capacity of an intact viable cell and not necessarily a cell that is heading toward death.

Schiffman, Providence: Beautiful talk Peter. Any down-the-road implications for either enhancement or blocking of the formation of lipid bodies in order to interfere with either inflammatory or infectious pathways?

Weller, Boston: Thank you. I think that is a good question. Curiously, when we began to look at the capacities of *cis* fatty acids stimulate lipid body formation, we found that aspirin blocked lipid body formation as did sodium salicylate. *Cis* fatty acid–elicited lipid body formation was also blocked in cells deficient in cyclo-oxygenase 1 and cyclo-oxygenase 2. This blockade of lipid body formation impaired the formation of both prostaglandins and leukotrienes, so there is the potential for a therapeutic intervention in the formation of lipid bodies that would then limit the quantities of eicosanoids being produced.

Michael Gershon, New York City: The question I had, since neutrophils are pretty much loaded guns, that is they finish the protein synthesis by the time they are out there in tissue….

Weller, Boston: I would question that, I think that is the old dogma and at the same time I would likewise question the limited capacities of eosinophils for further protein synthesis.

Michael Gershon, New York City: Alright, the question I was asking whether the lipid bodies get made with precursors already loaded into the cell and as a progenitor of the lipid body before it's a neutrophil that while it is still in the forming stage?

Weller, Boston: Lipid body formation in leukocytes requires in part *de novo* protein synthesis. Lipid bodies arise from the endoplasmic reticulum which folds back on itself. Local synthesis of lipid develops and overlies the folded endoplasmic reticulum and accounts for the staining with lipophilic stains or with osmium. We do think that mature neutrophils and eosinophils retain the capacity for ongoing protein synthesis and for de novo lipid body formation. Thank you.