

that if IL-3 were missing in the circulation of donors with nonreleasing basophils, their basophil response to other stimuli would also be diminished. A more direct study of the IL-3 hypothesis is currently hampered by technical issues, but the data generated thus far are not supportive.

In summary, the cause of the nonreleasing basophil "phenotype" has not yet been found. However, we now know that these basophils only poorly generate the second messengers typically occurring in releasing basophils. Both the PKC and $[Ca^{++}]$ responses are blunted, but the data also suggest that the influx of calcium is particularly blunted in the nonreleasing basophil. Although the data do not rule out a number of possible explanations for the weak signaling in these cells, we currently favor the possibility that a component of signal transduction operating early in the reaction is either inactive or missing in these donors' basophils.

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Lipid bodies: Intracellular sites for eicosanoid formation

Peter F. Weller, MD, and Ann M. Dvorak, MD *Boston, Mass.*

From the Departments of Medicine and Pathology, Beth Israel Hospital, Harvard Medical School.

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Reprint requests: Peter F. Weller, MD, Beth Israel Hospital, Dana 617, 330 Brookline Ave., Boston, MA 02215.

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Lipid bodies are non-membrane-bound, lipid-rich cytoplasmic inclusions that develop in a diversity of cell types.¹ Cytoplasmic lipid bodies, which are morphologically distinct structures, are roughly spherical, usually 0.2 to 2 μ m diameter accumulations of lipid and protein. Although lipid bodies lack a delimiting membrane, they often possess a more electron-dense peripheral shell and

Abbreviations usedOAG: 1-Oleyl-2-acetyl-*rac*-glycerol

PGH: Prostaglandin H

PMA: Phorbol 12-myristate 13-acetate

are found enmeshed in cytoskeletal elements.² Whereas lipid inclusions exist in steroid-producing cells and in preadipocytes and can serve as storage sites of esterified cholesterol, in most cells little is known about the origins, composition, or functions of lipid bodies.

In limited numbers, lipid bodies are normal cytoplasmic constituents of many cells, including neutrophils,³ eosinophils,⁴ lymphocytes,¹ mast cells,⁵ macrophages,⁵ endothelial cells,¹ and fibroblasts.¹ Lipid bodies are typically sparse in normal cells but increase in numbers and size in cells associated with inflammation. Normal blood neutrophils contain an average of less than 1 lipid body per cell, whereas eosinophils from normal donors contain an average of ~5 lipid bodies per cell.^{2,4} However, the solubility of lipid bodies in conventional alcohol-based hematologic stains (e.g., Wright's and Giemsa stains) causes lipid bodies to be dissolved and missed. Hence leukocyte lipid bodies are not usually seen on routine light microscopy with commonly used hematology stains, even when lipid body-rich leukocytes are obtained from inflammatory exudates.⁶ In contrast, if lipid is first preserved by exposure to osmium tetroxide before staining^{6,7} or if staining is effected with the lipophilic fluorescent dye Nile red,⁸ then lipid body accumulations within leukocytes can be recognized and enumerated. With such specific staining, lipid body numbers can be recognized by light or electron microscopy to be increased in leukocytes associated with various inflammatory and immunologic reactions.¹ For instance, with appropriate lipid fixation, we have demonstrated that in comparison with the normal content of ~1 and ~5 lipid bodies per cell in normal blood neutrophils and eosinophils, respectively,^{2,3} peripheral blood neutrophils from patients with infections and eosinophils from patients with eosinophilia contained many more lipid bodies per cell,^{2,3} in accord with our electron microscopic observations.^{1,3,4} Analogously, increased numbers of lipid bodies in human neutrophils associated with various infectious, neoplastic, and other inflammatory reactions *in vivo* have been demonstrated both within biopsied tissues

and in neutrophils from blood and exudative effusions.^{3,6} In addition, neutrophils from experimentally elicited peritoneal exudates in rabbits, but not neutrophils collected concurrently from rabbit peripheral blood, have been shown to contain increased numbers of cytoplasmic lipid bodies.⁹ These *in vivo* findings with neutrophils, as with eosinophils⁴ and other cells,^{1,5,10} demonstrate that lipid body formation is a natural event in many cell types and a morphologic correlate of the cells' participation in inflammation.

MECHANISMS OF LIPID BODY FORMATION

Although observations indicated that lipid bodies were more prominent in cells associated with inflammatory reactions, it was unknown whether lipid body formation represented an adverse reaction or a physiologic response within these cells. With use of human peripheral blood neutrophils, we have evaluated the stimuli and mechanisms that lead to lipid body formation. *In vitro*, lipid body formation can be elicited within 15 to 30 minutes by exposures of cells to *cis*-polyunsaturated fatty acids, including arachidonic acid.^{2,3} That this lipid body formation is not a manifestation of cellular injury or simply attributable to excess substrate arachidonyl fatty acid has been indicated in neutrophils by showing that lipid body formation is stereochemically dependent on the structures of exogenous fatty acids.² Exposures of neutrophils to arachidonic and oleic acids, in a dose-dependent (0 to 10 μ mol/L) manner, elicited lipid body formation within 30 minutes,² and these fatty acid-elicited lipid bodies were morphologically identical at the ultrastructural level with native lipid bodies.³ In contrast, neither arachidonyl alcohol nor methyl esters of arachidonate or oleate elicited lipid body formation. With a series of C20 and C18 fatty acids, the potency of these fatty acids generally correlated with their structures. Saturated fatty acids, which are taken up by cells and can be incorporated into lipid bodies,³ failed to stimulate lipid body formation.² With unsaturated fatty acids, those with *trans* double bonds exhibited little lipid body-inducing activity, in contrast to fatty acids with *cis* geometry double bonds.² Potency of *cis*-unsaturated fatty acids as lipid body inducers increased generally with increasing numbers of *cis* double bonds.² These findings demonstrated that lipid body induction elicited by fatty acids was structurally and stereochemically restricted.

Possible mechanisms for lipid body formation

stimulated by *cis*-unsaturated fatty acids were evaluated. Although *cis*-fatty acids can elicit the generation of superoxide anion by neutrophils, studies with superoxide dismutase and catalase demonstrated that neutrophil respiratory burst-derived oxidants actually inhibited and did not stimulate lipid body formation. Similarly, neither lipid peroxides nor depletion of cell adenosine triphosphate could be implicated in the formation of lipid bodies, which was time, temperature, and energy dependent.²

Because *cis*-fatty acids can stimulate protein kinase C activation, other stimuli of protein kinase C were evaluated as lipid body inducers in neutrophils.² 1-Oleoyl-2-acetyl-*rac*-glycerol (OAG), a cell-permeant diglyceride activator of protein kinase C, was a potent stimulus of lipid body formation. Similarly, lipid bodies were induced to form in neutrophils by phorbol 12-myristate 13-acetate (PMA) and phorbol 12,13-dibutyrate, but not by a control phorbol esters (4 α -phorbol 12,13-didecanoate), which was not an activator of protein kinase C. Inhibitors of protein kinase C, including 1-O-hexadecyl-2-O-methyl-*rac*-glycerol H-7, and staurosporine, inhibited lipid body formation elicited by all stimuli, including *cis*-fatty acids, diglycerides, and phorbol esters.² These findings indicated that protein kinase C activation is involved in lipid body formation and that the role of protein kinase C was not simply to contribute to the intracellular release of fatty acids, which themselves cause lipid body formation. Lipid body formation was rapid (within 30 to 60 min), not attributable to deleterious metabolic effects of agonists, not necessarily dependent on exogenous lipid, and inhibitable with protein kinase C inhibitors.² Thus lipid body formation represents a coordinated cellular response, mediated by protein kinase C activation, that eventuates in the mobilization and deposition of lipids and proteins in discrete, ultrastructurally defined intracellular domains.²

LIPID BODIES AS SITES OF ESTERIFIED ARACHIDONIC ACID

Lipid bodies can serve as intracellular sites of deposition of arachidonyl lipids. By using electron microscopic autoradiography to localize ³H-arachidonate incorporated by cells, lipid bodies have been demonstrated to constitute major sites of localization of cell-incorporated arachidonate.¹ In both eosinophilic⁴ and neutrophilic³ leukocytes, after incubations of cells with nanomolar concentrations of ³H-arachidonate (which were insufficient concentrations to stimulate new lipid body

formation), ultrastructural autoradiographic studies documented that lipid bodies were the predominant structures containing ³H-arachidonate, with minimal labeling of cell membranes. Other ³H-fatty acids, palmitic and oleic acids, were also incorporated into lipid bodies in these leukocytes.^{3, 4} Similarly, incorporation of ³H-arachidonate into lipid bodies of human alveolar macrophages and mast cells^{5, 10} and murine and guinea pig peritoneal macrophages⁵ has been demonstrated. That the ³H-arachidonate present within leukocyte lipid bodies was not free fatty acid was indicated by parallel analyses of total cellular ³H-arachidonyl lipids.^{3, 4} As expected, little ³H-arachidonate remained as free fatty acid; rather, it was esterified within classes of glycerolipids, with most ³H-arachidonate present in classes of phospholipids. By inference, lipid bodies would have contained esterified arachidonate.

Direct evidence that lipid bodies contain pools of arachidonyl phospholipids was obtained when methods were developed to successfully isolate lipid bodies free of cellular membranes.¹¹ Lipid bodies were isolated from lipid body-rich human eosinophils that had been incubated overnight with ³H-arachidonic acid to achieve isotopic equilibrium.¹¹ Analyses of these isolated lipid bodies demonstrated that lipid body lipids containing ³H-arachidonate were principally phospholipids, with ³H-arachidonate present in phosphatidylcholine, phosphatidylinositol, and phosphatidylethanolamine/serine in the same proportions as found in eosinophil membranes.¹¹ In recovered lipid bodies, little ³H-arachidonate was present as free fatty acid; some ³H-arachidonate was found in triglycerides and a larger amount was present in diglycerides, possibly as a result of the actions of lipolytic enzymes occurring during lipid body isolation. Although it was possible to recover eosinophil lipid bodies free of contaminating cellular membranes,¹¹ it was not possible to quantitate the recovery of lipid bodies, because no protein marker for this structure has been identified, as conventionally used to monitor the distribution and recovery of other cellular organelles during subcellular isolations. Nevertheless, lipid bodies clearly constitute a structurally distinct pool of potential eicosanoid substrate containing arachidonyl phospholipids.

SITES OF EICOSANOID-FORMING ENZYME LOCALIZATION

In all cells, substrate arachidonate, which is not present to any large degree as free fatty acid,¹² is

esterified within glycerolipids and is mobilized principally from classes of phospholipids by the actions of phospholipases to initiate the cascades that eventuate in eicosanoid formation. Because eicosanoid precursor containing arachidonyl phospholipids are widely assumed to reside within cell membranes, it is cellular membranes in leukocytes as in other cells that are believed to constitute the sites of eicosanoid formation. Prostaglandin H (PGH) synthase (cyclooxygenase) is believed to reside within membranes,¹³⁻¹⁵ perhaps as interpreted by some¹⁶ but not all¹⁷ to have a conventional hydrophobic transmembrane spanning region. In other cell types, PGH synthase has been localized to the endoplasmic reticulum, the nuclear membrane, and infrequently to the plasma membrane.^{14, 15}

In addition to cell membranes, an alternative intracellular domain potentially involved in eicosanoid formation is the lipid body. If lipid bodies are to play a role in eicosanoid formation, then esterified arachidonate must be enzymatically liberated and either acted on locally at lipid bodies or transported from lipid bodies to other sites for eicosanoid synthesis. That lipid bodies are not solely lipid in composition but rather have enzymatically active proteins associated with them has been indicated both by our light and electron microscopic cytochemical observations and our more recent analyses of isolated lipid bodies. In various cell types, we have localized alkaline phosphatase, peroxidase, nonspecific esterase, and serine esterase activities to lipid bodies.^{1, 11, 18-21} These observations are tantalizing because some lipolytic enzymes are serine esterases, and both PGH synthase and lipoxygenases have inherent or associated peroxidatic activity.

More directly pertinent to the processes involved in eicosanoid formation, by studying the localization of PGH synthase in various cells, including murine fibroblasts and human monocytes and eosinophils, we have now established that PGH synthase is localized at lipid bodies.²²⁻²⁴ Lipid bodies within these cells, after cell permeabilization and processing designed to preserve lipid bodies, are found to specifically react with monoclonal and polyclonal anticyclooxygenase antibodies. Moreover, ultrastructural immunogold localization has detected PGH synthase on lipid bodies in various cells including human mast cells, neutrophils, and eosinophils, as well as in cells of other species.²²⁻²⁴ In addition to these immunohistochemical, indirect immunofluorescent, and ultrastructural localizations within intact, permea-

bilized cells, lipid bodies, which are isolated by subcellular fractionation free of endoplasmic reticulum and other membrane markers, express PGH synthase enzymatic activity, inhibitable with anticyclooxygenase reagents. These novel findings, therefore, provide direct evidence that lipid bodies can serve as specific domains at which eicosanoid formation can occur. Thus lipid bodies appear to serve as distinct intracellular domains at which both pools of substrate that contain arachidonyl phospholipids and a key eicosanoid-forming enzyme are localized.

RELEVANCE OF LIPID BODIES TO EICOSANOID PRODUCTION BY CELLS INVOLVED IN INFLAMMATION

Although stimulation of normal leukocytes, often with a nonphysiologic agonist (e.g., the calcium ionophore A23187) has been very useful in defining the pathways of eicosanoid biosynthesis *in vivo*, it is undoubtedly leukocytes and other cells associated with inflammation (i.e., those likely also to contain many lipid bodies) that are producing the greatest quantities of eicosanoids. For instance, increased lipid body numbers in kidney cells and heightened kidney prostaglandin production have been correlated.²⁵ In leukocytes and other cells associated with inflammation, the mobilization of arachidonate for eicosanoid formation may differ from that studied in more readily obtainable normal blood leukocytes.

In studies of neutrophils there has been an apparent paradox. In response to the ionophore A23187, neutrophils generate large quantities of 5-hydroxyeicosatetraenoic acid, leukotriene B₄ and its metabolites, whereas more natural, receptor-mediated stimulation, as with the chemoattractant peptide N-formyl-methionyl-leucyl-phenylalanine, routinely elicits little or no eicosanoid formation^{26, 27} unless neutrophils are first "primed." "Priming" consists of preincubating neutrophils with $\mu\text{mol/L}$ amounts of OAG²⁷ or arachidonate²⁶ or with the phorbol ester PMA.²⁸ Of note from our studies, these same priming agents, in the concentrations and time periods used, would have induced abundant lipid body formation. Analogously, preincubating cells with 10 to 50 $\mu\text{mol/L}$ arachidonate before stimulation (as is commonly done to provide ample presumed substrate) would also effectively stimulate lipid body formation. The priming agents PMA, OAG, and arachidonate also augment eicosanoid formation by A23187-stimulated neutrophils.²⁹ Thus the capacity of neutrophils to release eicosanoids in

response to physiologic stimuli may be correlated to their content of lipid bodies, and "priming" for eicosanoid release may be based on events that occur at lipid body domains.

Eicosanoids are derived from arachidonyl phospholipids assumed to reside within cellular membranes; however, which membranes, and indeed whether it is membranes that serve as storage depots of eicosanoid-precursor arachidonate, is unknown. In a recent study, prostaglandin E_2 -producing cells lost the capacity to release prostaglandin E_2 after deprivation of exogenous arachidonate for 24 to 48 hours.³⁰ Because total cellular arachidonate and relevant enzymes remained normal during this time, depletion of arachidonate from a specific but unidentified cellular pool was hypothesized.³⁰ Under the conditions used, lipid bodies would have been depleted.³¹ Another consideration concerning membrane structure is also pertinent. Using lipid bodies and not membranes as the source of eicosanoid-precursor arachidonate would obviate excessive perturbation of membranes when quantities of arachidonate are released and removed from phospholipids. In lymphocytes, the converse has been established: membrane perturbation in response to exogenous fatty acids is prevented by the formation of lipid bodies.³² Hence it is possible that relatively small quantities of arachidonate may be liberated from cell membranes when that arachidonate is destined for formation of autocrine eicosanoids or for second messenger activities, as elicited by signal-transducing mechanisms within membranes, whereas the larger amounts of arachidonate needed for eicosanoids formed as paracrine mediators would be derived from lipid body stores.

SUMMARY

Lipid bodies, therefore, represent specialized intracellular domains that form rapidly in response to agents that activate protein kinase C. These structures contain eicosanoid-precursor arachidonate esterified in specific phospholipids. Arachidonate-releasing phospholipases probably act at lipid bodies, and an eicosanoid-forming enzyme, PGH synthase (cyclooxygenase), definitely localizes to lipid bodies. In addition, the heightened presence of lipid bodies in cells likely to be producing eicosanoids as part of inflammatory reactions indicates that lipid bodies are dynamic, specialized intracellular domains with roles pertinent to the metabolic transformation of arachidonate into paracrine mediators of inflam-

mation. With their prominence in cells in association with inflammation, lipid bodies constitute specialized sites at which eicosanoid formation could occur for the heightened generation of eosinophil eicosanoid mediators of inflammation. This compartmentalization of eicosanoid formation at lipid bodies would provide a nonmembrane pool of arachidonate whose metabolic utilization could occur without perturbation of membranes if membranes were the sole stores of substrate fatty acid used for quantities of eicosanoids synthesized as paracrine mediators of inflammation. Moreover, lipid bodies would serve as sites at which the coordinated and regulated enzymatic events involved in arachidonate mobilization and oxidative metabolism could occur.

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