Cytoplasmic Lipid Bodies of Neutrophils: Formation Induced by cis-Unsaturated Fatty Acids and Mediated by Protein Kinase C

Peter F. Weller,* Sandra W. Ryeom,* Susanne T. Picard,* Steven J. Ackerman,* and Ann M. Dvorak‡

Departments of *Medicine and ‡Pathology, Harvard Medical School and the Harvard Thorndike Laboratories, Charles A. Dana Research Institute, Beth Israel Hospital, Boston, Massachusetts 02215

Abstract. Lipid bodies, nonmembrane-bound cytoplasmic inclusions, serve as repositories of esterified arachidonate and are increased in cells associated with inflammatory reactions. We have evaluated stimuli and mechanisms responsible for lipid body formation within human polymorphonuclear leukocytes (PMNs). Arachidonic acid and oleic acid stimulated dose-dependent formation of lipid bodies over 0.5–1 h. Other C20 and C18 fatty acids were less active and demonstrated rank orders as follows: cis-unsaturated fatty acids were much more active than trans-fatty acids, and activity diminished with decreasing numbers of double bonds. Lipid bodies elicited in vitro with cis-fatty acids were ultrastructurally identical to lipid bodies present in PMNs in vivo. Lipid body induction was not because of fatty acid–elicited oxidants or fatty acid–induced ATP depletion. Cis-fatty acid–induced activation of protein kinase C (PKC) was involved in lipid body formation as evidenced by the capacity of other PKC activators, 1-oleoyl-2-acetyl-glycerol and two active phorbol esters, phorbol myristate acetate, and phorbol 12,13 dibutyrate, but not an inactive phorbol, to induce lipid body formation. The PKC inhibitor, 1-O-hexadecyl-2-O-methyl-glycerol, inhibited PMN lipid body formation induced by oleic and arachidonic acids and by 1-oleoyl-2-acetyl-glycerol and phorbol myristate acetate. Other PKC inhibitors (staurosporine, H-7) also inhibited lipid body formation. Formation of lipid bodies in PMNs is a specific cellular response, stimulated by cis-fatty acids and diglycerides and apparently mediated by PKC, which results in the mobilization and deposition of lipids within discrete, ultrastructurally defined cytoplasmic domains.

LIPID bodies are nonmembrane bound, cytoplasmic inclusions present within neutrophilic (49) and eosinophilic (48) leukocytes, fibroblasts (52), endothelial cells (23), and other cell types (23). The genesis and functions of lipid bodies are largely unknown in most cells. In both neutrophils (polymorphonuclear leukocytes; PMNs)[1] (49) and eosinophils (48), lipid bodies become more prominent in number and size when these leukocytes are engaged in inflammatory responses. Increased numbers of lipid bodies in human PMNs associated with various infectious, neoplastic, and other inflammatory reactions have been demonstrated both within biopsied tissues and in PMNs from blood and exudative effusions (13, 49). In addition, PMNs from experimentally elicited peritoneal exudates in rabbits, but not PMNs collected concurrently from rabbit peripheral blood, contained increased numbers of lipid bodies (36). These in vivo findings with human and rabbit PMNs indicated that

lipid body formation was a morphologic correlate of the cells' participation in inflammation. It is PMNs in sites of inflammation, not normal blood PMNs, that are likely to be subjected to activating cellular ligands and lipids and to be engaged in stimulated cellular responses, including eicosanoid synthesis and release. Whether lipid body formation represents a toxic sequela or a specific response within PMNs has not been defined; and mechanisms responsible for lipid body formation in leukocytes or other cell types have not been identified (36).

Although the composition and functions of lipid bodies in leukocytes remain uncertain, a role for lipid bodies as major intracellular sites of deposition of fatty acids, including arachidonic acid, has been indicated by electron microscopic autoradiography (18, 19, 23, 48, 49). In human PMNs (49) and eosinophils (48), lipid bodies were the predominant sites of localization of cell-incorporated, esterified [^{3}H]-arachidonate (49). Other [^{3}H]-fatty acids also localized to PMN lipid bodies (49). Similarly, incorporation of [^{3}H]-arachidonate into lipid bodies of human alveolar macrophages and mast cells (18, 19) and murine and guinea pig peritoneal macrophages (18) has been demonstrated.

Since lipid bodies in leukocytes can serve as stores of esterified arachidonate and increase in number when these

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1. *Abbreviations used in this paper:* BHT, butylated hydroxytoluene; fMLP, N-formyl-methionyl-leucyl-phenylalanine; HMG, 1-O-hexadecyl-2-O-methyl-rac-glycerol; PDBu, phorbol 12,13 dibutyrate; PDD, 4α-phorbol 12,13 didecanoate; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; PMNs, polymorphonuclear leukocytes (neutrophils); OAG, 1-oleoyl-2-acetyl-rac-glycerol; 2,4 DNP, 2,4 dinitrophenol.

cells are involved in inflammatory responses, we have investigated stimuli and mechanisms which lead to lipid body formation in PMNs. We have found that induction of lipid body formation is mediated by activation of protein kinase C (PKC) and can be elicited preferentially by cis-unsaturated fatty acids, which can activate PKC (15, 29, 30), and by other PKC activators, 1-oleoyl-2-acetyl-rac-glycerol (OAG) and specific phorbol esters (10, 22, 32). PKC activation in PMNs elicits multiple alterations in lipid metabolism, including increases in classes of diglycerides (1, 16, 35, 47) and phospholipids (28, 41, 46) and priming for stimulated release of eicosanoids (27, 28). In the context of these PKC-mediated biochemical changes in lipids, PKC activation also promotes the mobilization and deposition of lipids into discrete cytoplasmic inclusions. In combination with observations that lipid bodies are a potential intracellular source of esterified arachidonate, these findings suggest that lipid bodies in PMNs may have a role in eicosanoid production by these cells.

Materials and Methods

Materials

Fatty acids and their analogs were obtained from Sigma Chemical Co. (St. Louis, MO) or NuChek Prep (Elysian, MN). Thiocarbohydrazide, phorbol 12-myristate 13-acetate (PMA), 4α-phorbol 12,13 didecanoate (PDD), phorbol 12,13 dibutyrate (PDBu), dipalmitin, diolein, 1-oleoyl-2-acetyl-rac-glycerol (OAG), lysopalmitoylphosphatidylcholine, phosphatidylserine, phenylmethylsulfonyl fluoride, bovine erythrocyte superoxide dismutase (3,000 U/mg), bovine liver catalase (11,000 U/mg), 2,4 dinitrophenol (2,4 DNP), butylated hydroxytoluene (BHT) (Sigma Chemical, St. Louis, MO); Ficoll-Hypaque, 6% dextran in normal saline (Macrodex; Pharmacia, Piscataway, NJ); osmium tetroxide (Electron Microscopy Sciences, Fort Washington, PA); Bloom's gelatin, isopropanol, oil red O, (Fisher, Medford, MA); staurosporine (Kamiya Biomedical Co., Thousand Oaks, CA); valinomycin, H-7 (1-[5-isoquinolinesulfonyl]-2-methyl-piperazine) (Calbiochem, San Diego, CA); 1-O-hexadecyl-2-O-methyl-rac-glycerol (HMG) (Bachem Biochemicals, Philadelphia, PA); and HBSS (Microbiological Associates, Walkersville, MD) were obtained as indicated.

PMN Purification and Lipid Body Induction

Peripheral blood from normal donors was anticoagulated with acidified citrate; erythrocytes were removed by dextran sedimentation; and granulocytes (>95% PMNs, remainder eosinophils) were collected after centrifugation on Ficoll-Hypaque (49) and washed in calcium, magnesium-free HBSS. Residual erythrocytes were lysed with hypotonic saline, and PMNs were resuspended in calcium, magnesium-free Tyrode's buffer (0.1% wt/vol) dextrose, 0.1% NaHCO₃, 0.02% KCl, 0.8% NaCl, 0.005% NaH₂PO₄, pH 7.5) with 0.1% gelatin. Incubations were done with gelatin and not albumin to minimize protein binding of fatty acids and were done without exogenous calcium to avoid effects of calcium on the solubility or membrane partitioning of fatty acids (45). In representative experiments, cell viability was checked with trypan blue staining, and for each of the fatty acids was >90%. PMNs (10⁶/ml in 3 ml) were incubated at 37°C for 60 min, a time which yielded near maximal lipid body formation for both arachidonic and oleic (49) acids. To minimize toxicity to cells, incubations with PMA or with inhibitors of PKC were limited to 30 min; and with PKC inhibitors, stimulating fatty acids and OAG were used at submaximal (2.5 μM) concentrations. Possible cytotoxicity was also assessed by evaluating morphology of fixed PMNs during lipid body counting. Fatty acids, diacylglycerides, and lysophosphatidylcholine, dissolved in ethanol, were used at a final 0.1% ethanol concentration. Control cells were incubated likewise in 0.1% ethanol. Within all experiments, incubations with 1 or 2.5 μM oleic acid were included as positive control conditions for PMN lipid body formation. Phorbol esters, HMG, valinomycin, and staurosporine were dissolved in dimethylsulfoxide; control cells were incubated in 0.1% dimethylsulfoxide. H-7 was dissolved as a stock solution in distilled water. HMG, staurosporine, and H-7 were added prewarmed at 37°C to 37°C prewarmed PMNs for 15 min before

adding fatty acids or other stimuli. Superoxide dismutase and catalase solutions were heat inactivated for 15 min in boiling water.

Lipid Body Staining and Enumeration

PMNs (∼5 × 10⁴) were cytocentrifuged (Cytocentrifuge, Shandon, Pittsburgh, PA) (1,000 RPM, 5 min) onto glass slides. In early studies, slides, while still moist, were fixed by exposure to OsO₄ vapors, and then stained for 2–4 h in oil red O (6 parts 0.5% [wt/vol] oil red O in isopropanol, 4 parts water), rinsed briefly in isopropanol, and then mounted with Apathy's medium. In later studies, greater contrast of lipid bodies was obtained by staining slides sequentially with ferrocyanide-reduced OsO₄, thiocarbohydrazide and OsO₄ (53). Slides were fixed in 3.0% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4 (15 min), rinsed twice in cacodylate buffer (3 min), stained in 1.5% K₄Fe(CN)₆ in 1.0% aqueous OsO₄ (30 min), rinsed three times in water, immersed in 1.0% thiocarbohydrazide (5 min), rinsed three times in water, restained in 1.0% OsO₄ in 0.1 M cacodylate (3 min), rinsed with water, and then dried and mounted in Apathy's medium. The morphology of fixed cells was observed, and lipid bodies were enumerated with phase contrast microscopy, and a 100× objective lens in 25–50 consecutively scanned PMNs; any eosinophils were not counted. Results were calculated as mean (±SEM) lipid bodies/PMN or as mean net (±SEM) lipid bodies/PMN, after subtracting the lipid bodies/PMN formed during control incubations.

EM of PMN Lipid Bodies

Cells for EM were processed and examined as previously detailed (17, 49). PMNs were fixed in 2% paraformaldehyde, 2.5% glutaraldehyde, 0.025% CaCl₂ in 0.1 M sodium cacodylate buffer, pH 7.4 for 1 h, washed in cold 0.1 M cacodylate buffer, and rapidly centrifuged through molten agar. The cell pellets in agar were postfixed in S-collidine–buffered osmium and stained en bloc with uranyl acetate before dehydration in a graded series of alcohols and embedding in a propylene oxide-Epon sequence. Thin sections were stained with lead citrate and examined in an electron microscope (Model 400; Philips Electronic Instruments, Inc., Mahwah, NJ).

PKC Assays

PKC activity in cytosolic fractions of PMNs was assayed by quantitating phosphorylation of a synthetic PKC substrate peptide (PKC assay system; Amersham Corp., Arlington Heights, IL). After hypotonic erythrocyte lysis, PMNs, purified as described above, were washed with HBSS and resuspended at 5 × 10⁷/ml in 50 mM Tris-HCl, pH 7.5, 2 mM EGTA, 50 mM 2-mercaptoethanol, and 1 mM phenylmethylsulfonyl fluoride. PMNs were disrupted using an ice-cold homogenizer with multiple passes to achieve ∼75% cell breakage; and cytosol was recovered after centrifugation (150,000 g for 90 mins, 4°C). Assays were performed as specified by the manufacturer except that phosphatidylserine and diolein were substituted for the supplied lipid mixture. The standard assay mixture contained 25 μl cytosolic fractions, 2 μl without or with either HMG (in varying concentrations), 50 μM H-7 or 50 μM staurosporine, 2 μl 2 μg/ml diolein, and 25 μl 12 mM calcium acetate, 30 mM dithiothreitol, 900 μM PKC substrate peptide in 50 mM Tris-HCl, pH 7.5, with 0.05% sodium azide and 20 μg/ml phosphatidylserine. Reactions were started by adding 25 μl of magnesium ATP buffer (150 μM ATP, 45 mM magnesium acetate, 10 μCi/ml ³²P-γ-ATP (3,000 Ci/mmol, Amersham Corp.), in 50 mM Tris-HCl, pH 7.5), incubated for 15 min at 25°C, and then stopped with acidic reaction-quenching reagent. Phosphorylated substrate was trapped on peptide-binding paper, washed with acetic acid, and counted by liquid scintillation spectrometry. Assays were done within conditions of linearity for time and protein. In repeated experiments measured PKC activity was >94% calcium and phospholipid dependent (assessed in 12 mM EGTA without added diolein, phosphatidylserine, and calcium) and ranged ∼0.15 nmol phosphate incorporated/min/mg protein. Results are derived from means of triplicate or quadruplicate assays for each condition.

Results

Lipid Body Induction by Fatty Acids

PMNs purified from peripheral blood of normal donors contained few lipid bodies (Fig. 1 A), an average of 0.7 lipid bodies/PMN (mean for 500 PMNs from 20 leukocyte isola-
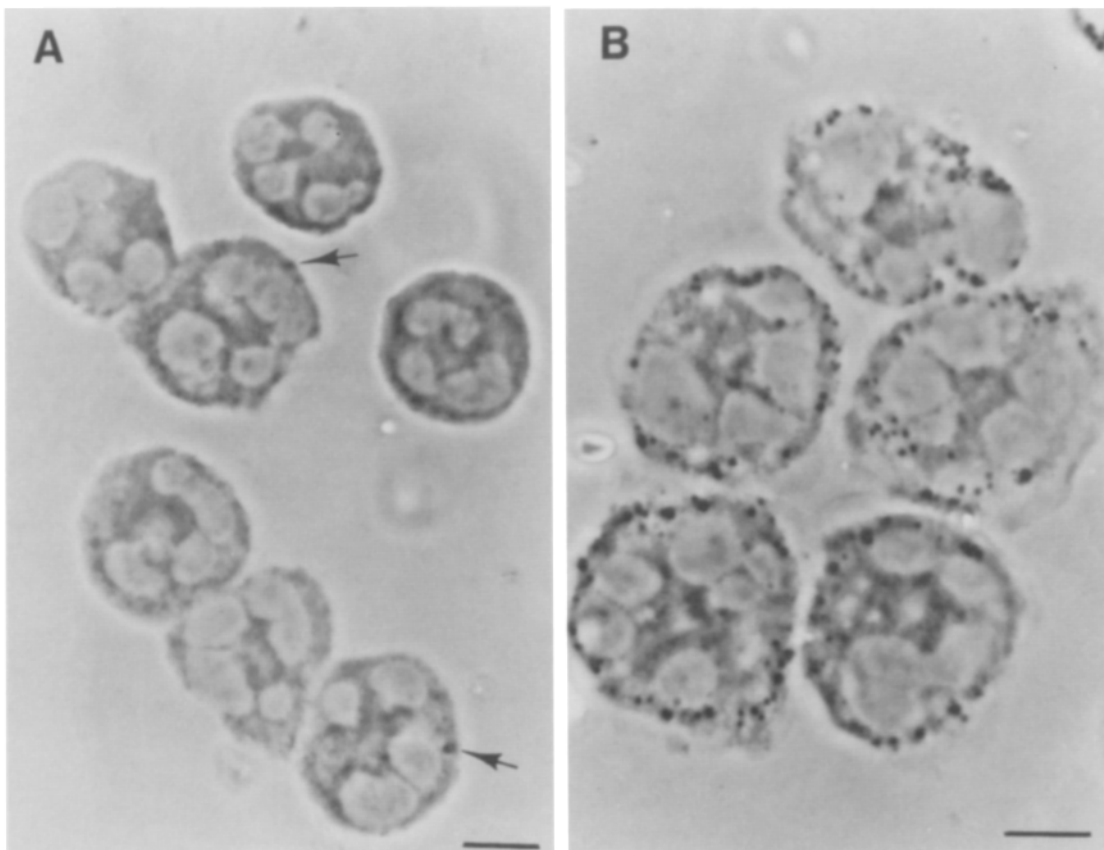
Figure 1. Unstimulated (*A*) and OAG-stimulated (*B*) PMNs stained with reduced osmium-thiocarbohydrazide-osmium to show cytoplasmic lipid bodies. PMNs were incubated for 60 min with control 0.1% ethanol (*A*) or 10 µM OAG (1-oleoyl-2-acetyl-*rac*-glycerol) (*B*). In *A*, lipid bodies are noted with arrows; in *B*, multiple darkly stained lipid bodies are present within each PMN. Bars, 10 µm.

tions). Since PMNs incubated with oleic acid had rapidly formed new lipid bodies over 30–60 min (49), we investigated lipid body formation in PMNs incubated with fatty acids of varying structures. Arachidonic and oleic acids were about equipotent in eliciting dose-dependent increases in lipid body formation, but the methyl esters of these fatty acids and arachidonyl alcohol were ineffective (Fig. 2). The morphology of lipid bodies elicited in vitro in PMNs incubated with these fatty acids was ultrastructurally identical (Fig. 3), including size range, consistency and electron density after osmium fixation, to native lipid bodies formed in vivo in blood and tissue PMNs (49). In addition, lipid bodies in PMNs exposed to oleic and arachidonic acids had an identical investiture, not with a defined unit membrane, but with an electron dense, peripheral lipid body "shell" partially or fully around their circumference (Fig. 3 *B*), as also found on PMN lipid bodies in vivo (49).

Induction of lipid body formation by other fatty acids with different degrees of unsaturation and double-bond geometries was examined. With 20 carbon fatty acids (Fig. 4 *A*), potency for lipid body induction increased progressively with the number of *cis* double bonds in the fatty acid. At 10 µM fatty acid, the rank order for lipid body induction was arachidonic acid (eicosatetraenoic acid, C20:4, *cis* 5,8,11, 14) ≅ homo-γ-linoleic acid (eicosatrienoic acid, C20:3, *cis*
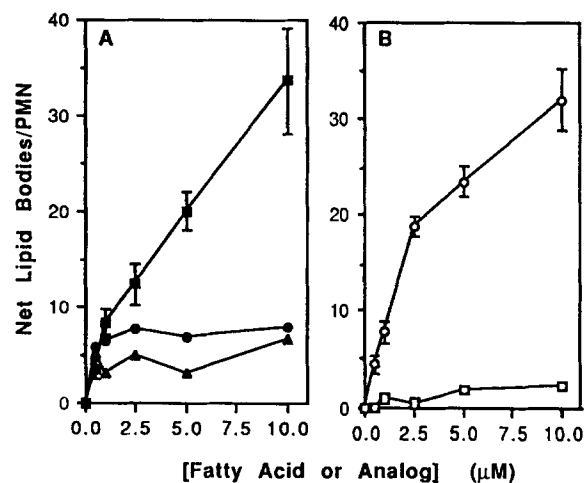

Figure 2. Lipid bodies in PMNs incubated for 60 min with C20 fatty acids (*A*) and C18 fatty acids (*B*) and their analogs. C20 fatty acids (*A*) include arachidonic acid (■), arachidonyl alcohol (▲), and arachidonyl methylester (●); C18 fatty acids (*B*) include oleic acid (○) and oleoyl methylester (□). Mean net lipid bodies/PMN in 25–50 cells are corrected for lipid bodies (usually 1–3/PMN) in PMNs incubated in 0.1% ethanol alone. For arachidonic and oleic acids and arachidonyl methylester, results are means (±SEM) of six, eight and two experiments, respectively.
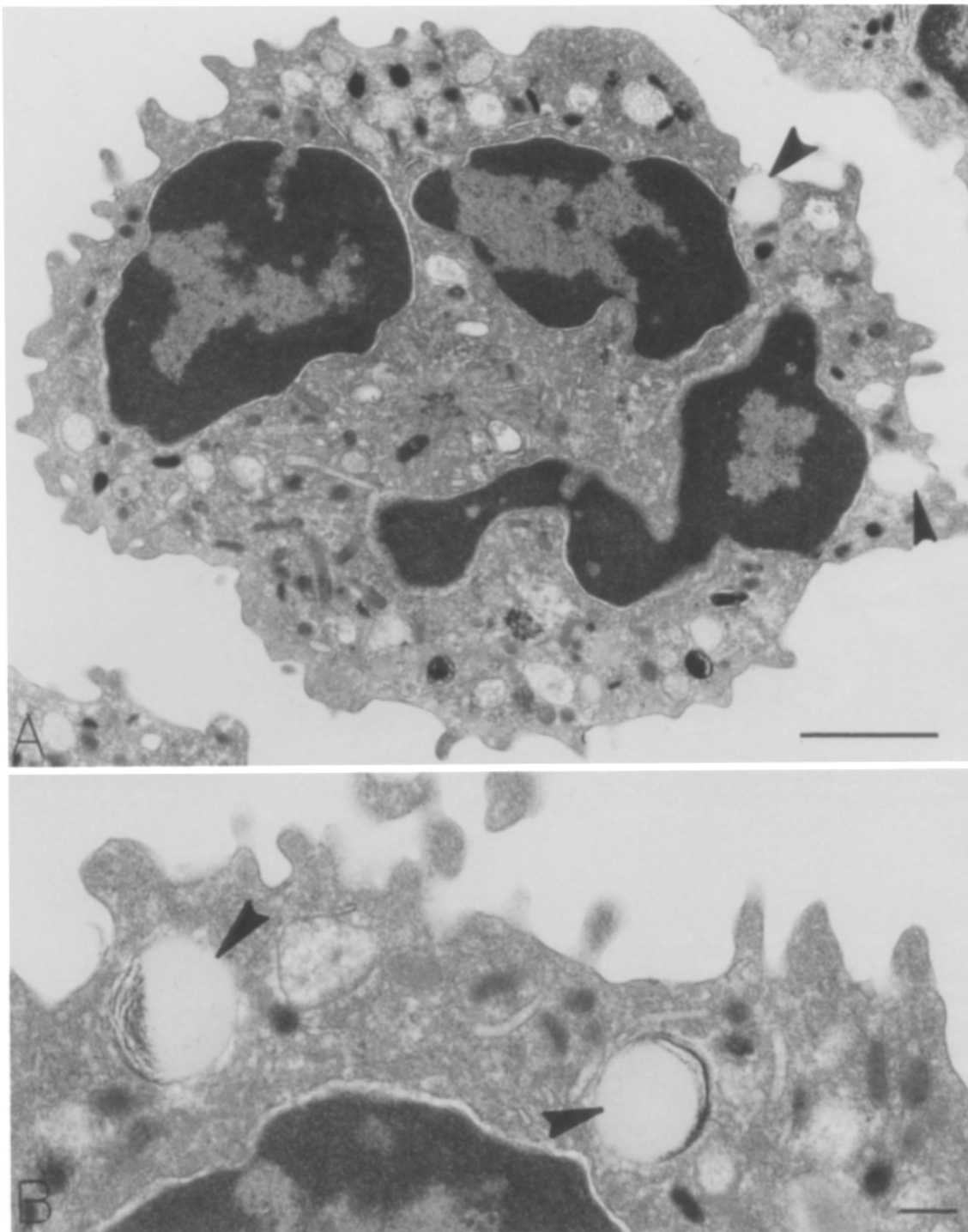
Figure 3. Electron micrographs of oleic acid–induced lipid bodies in PMNs. PMNs incubated for 60 min with 5 μM oleic acid (*A*) or 1 μM oleic acid (*B*) show cytoplasmic lipid bodies (*arrowheads*) which in *B* are partially invested with more electron dense lamellae, or a "shell." Bars, (*A*) 1 μm; (*B*) 0.2 μm.

8,11,14) > eicosadienoic acid (C20:2, *cis* 11,14) > eicosenoic acid (C20:1, *cis* 11) > eicosadienoic acid (C20:2, *trans* 11, 14) > eicosenoic acid (C20:1, *trans* 11) > eicosanoic acid (C20:0). The rank order with increasing degrees of unsaturation was also evident with 18 carbon fatty acids (Fig. 4 *B*), as was the importance of the geometry of double bonds. With

10 μM fatty acid, potency for lipid body induction was oleic acid (C18:1, *cis* 9) > γ-linoleic acid (C18:3, *cis* 6,9,12) > linoleic acid C18:2, *cis* 9,12) > linoleidic acid (C18:2, *trans* 9,12) > elaidic acid (C18:1, *trans* 9) > stearic acid (C18:0). Effects of unsaturated fatty acids were additive. Whereas PMNs incubated alone with 1 μM oleic acid or 1 or 2.5 μM
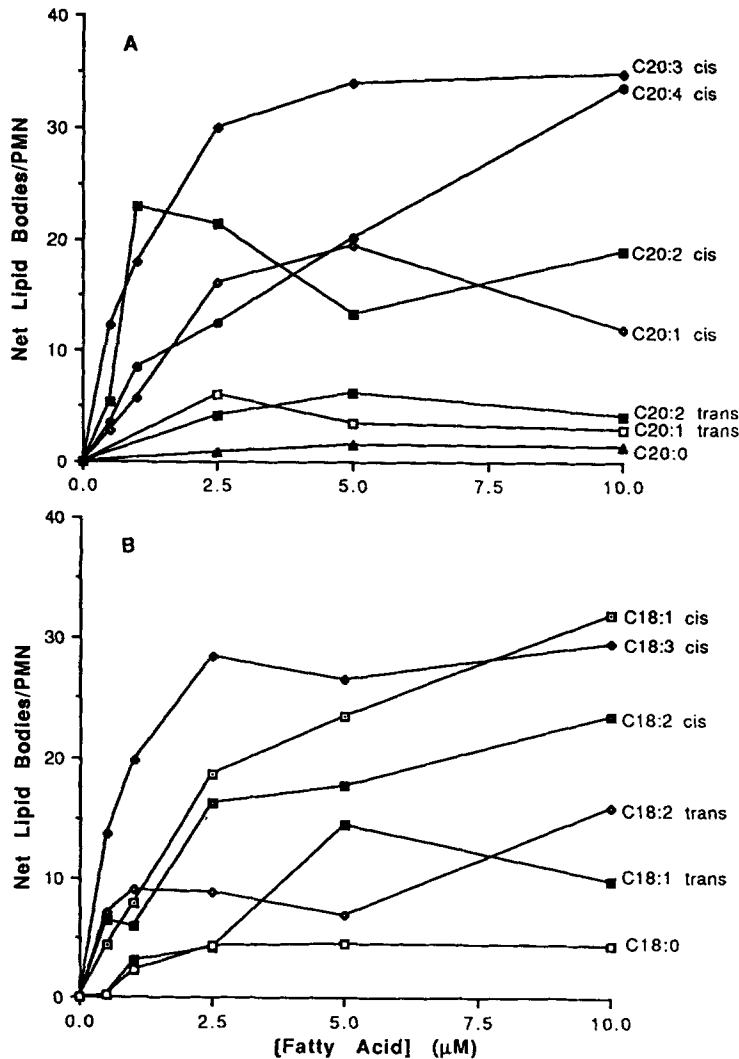
Figure 4. Induction of PMN lipid body formation by C20 fatty acids (*A*) and C18 fatty acids (*B*). C20 fatty acids included arachidonic acid (eicosatetraenoic acid, C20:4, *cis* 5,8,11,14), homo-γ-linoleic acid (eicosatrienoic acid, C20:3, *cis* 8,11,14), eicosadienoic acid (C20:2, *cis* 11, 14), eicosenoic acid (C20:1, *cis* 11), eicosadienoic acid (C20:2, *trans* 11,14), eicosenoic acid (C20:1, *trans* 11), and eicosanoic acid (C20:0). C18 fatty acids included oleic acid (C18:1, *cis* 9), γ-linoleic acid (C18:3, *cis* 6, 9,12), linoleic acid (C18:2, *cis* 9,12), linoleidic acid (C18:2, *trans* 9,12), elaidic acid (C18:1, *trans* 9), and stearic acid (C18:0). Mean lipid bodies/PMN in 25–50 cells were corrected for lipid bodies (between 1–4/PMN) in PMNs incubated with control 0.1% ethanol. Results are means from six experiments with arachidonic acid, from two each with 11,14 *trans*-eicosadienoic, 11 *trans*-eicosenoic, and eicosanoic acids, from eight with oleic acid and three with linoleic acid.

arachidonic acid contained 10.4 ± 3.2 ($\pm$SEM), 9.9 ± 3.5 and 17.3 ± 4.3 lipid bodies/PMN, respectively, combinations of 1 μM oleic acid with 1 or 2.5 μM arachidonic acid yielded 16.6 ± 4.8 and 27.7 ± 7.0 lipid bodies/PMN. In contrast to *cis*-unsaturated fatty acids, the fully saturated fatty acids, palmitic (C16:0) and myristic (C14:0), two diglycerides, dipalmitin and diolein, and lysophosphatidylcholine, at concentrations up to 10 μM, failed to induce lipid body formation (data not shown).

Mechanism of Lipid Body Induction

Since lipid body formation was not elicited simply by exposures to exogenous lipids but was structurally dependent on the composition and stereochemistry of stimulating fatty acids, potential mechanisms of lipid body formation were sought from the recognized effects of fatty acids, especially *cis*-unsaturated fatty acids, on PMNs.

Potential Toxic Effects of cis-Fatty Acids. Focal cytoplasmic accumulations of lipids might have developed as adverse consequences of fatty acid-stimulated metabolic events in PMNs. Specific fatty acids, notably *cis*-unsaturated fatty

acids, stimulate the respiratory burst of PMNs (4, 5), and the potencies of various polyunsaturated fatty acids as stimuli for PMN superoxide anion release (4, 5) were approximately the same for lipid body induction. To evaluate whether fatty acid–elicited superoxide anion or H_2O_2 contributed to lipid body formation, superoxide dismutase and catalase were added during incubations of PMNs with oleic acid. These enzymes, in amounts sufficient to consume superoxide anion or H_2O_2 released by stimulated PMNs, did not inhibit oleic acid–induced lipid body formation but rather augmented lipid body formation (Fig. 5 *A*). In the presence of active, but not heat inactivated, superoxide dismutase plus catalase, oleic acid–stimulated lipid body numbers were greater than without these enzymes. In addition, the antioxidant, BHT, did not inhibit, but also significantly enhanced, oleic acid–induced lipid body formation (Fig. 5 *B*).

Oxidant stress of cells evoked by exposures to H_2O_2, superoxide anion or PMA-stimulated PMNs can deplete cellular ATP (44), and *cis*-fatty acids can uncouple mitochondrial oxidative phosphorylation (3, 38). *cis*-Unsaturated, but not saturated, fatty acids have depleted cellular ATP and conse-
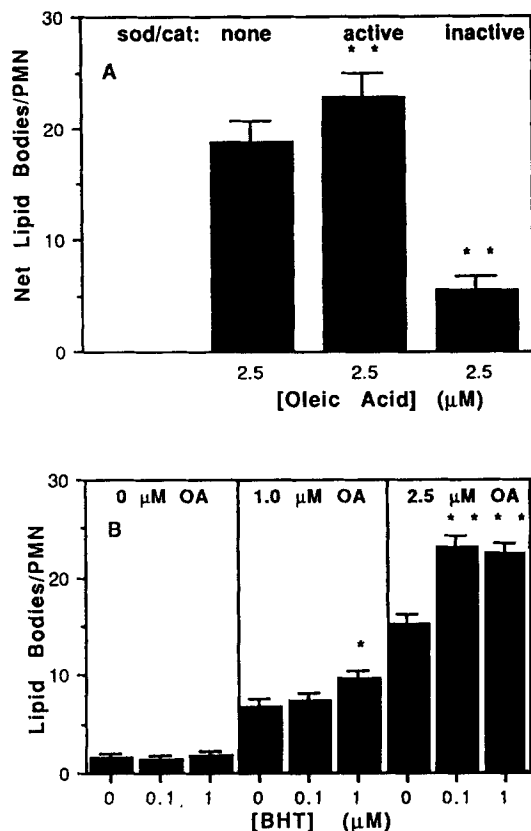
Figure 5. The effects of superoxide dismutase (sod) and catalase (cat) (A) and the antioxidant BHT (B) on oleic acid–induced lipid body formation in PMNs. In A, PMNs were incubated for 30 min with and without 2.5 µM oleic acid alone or with active or heat-inactivated superoxide dismutase (5,000 U/ml) and catalase (25,000 U/ml). Data are from one of three representative experiments with similar results. In B, PMNs were preincubated for 30 min at 37°C with the indicated concentrations of BHT and then washed and incubated for 30 min with oleic acid (OA). Comparable results were found when BHT was not washed from the cells before oleic acid addition. Mean (±SEM) lipid bodies/PMN were enumerated in 25–50 PMNs. Statistical comparisons were by two-tailed student's t tests for comparisons between the same concentrations of oleic acid with and without BHT or sod/cat. (*) $p < 0.05$; (**) $p < 0.001$.

quently inhibited 5-lipoxygenase metabolism of arachidonic acid in alveolar macrophages (33). To determine if cis-fatty acid–stimulated induction of PMN lipid bodies was mediated by depletion of ATP, several metabolic inhibitors were used to diminish ATP formation. Three agents which uncouple mitochondrial oxidative phosphorylation, 2,4 DNP, oligomycin and valinomycin, in concentrations that diminish ATP in PMNs and other cells (2, 12, 24, 44; Roos, D., M. Reiss, A. J. M. Balm, A. M. Palache, P. H. Cambier, and J. S. van der Stijl-Neijenhuis. 1979. Adv. Exp. Med. Biol. 121:29–36.), did not themselves elicit lipid body formation (data not shown). Further, the numbers of lipid bodies elicited with low (1 µM) concentrations of oleic or palmitic acids were not increased to levels attained with higher (2.5–10 µM) cis-fatty acid concentrations (Table I). EM demonstrated that these oxidative phosphorylation inhibitors, while causing mild injury to PMNs, altered neither the ultrastructural morphology nor the numbers of lipid bodies (not shown). An inhibitor of glycolysis, 2-deoxyglucose, did

Table I. Effects of Inhibitors of ATP Formation on Fatty Acid-induced PMN Lipid Body Formation

		Net lipid bodies/PMN	
		Oleic acid	Palmitic acid
2,4 DNP	0 M	5.6 ± 1.0	−0.4 ± 0.4
	10^{-5} M	5.1 ± 1.3	1.2 ± 0.7
	10^{-4} M	6.7 ± 0.8	−0.5 ± 0.4
	10^{-3} M	8.6 ± 0.9	1.0 ± 0.6
Oligomycin	0 µg/ml	4.4 ± 0.6	−
	25 µg/ml	6.3 ± 0.9	−
Valinomycin	0 M	10.4 ± 1.2	−1.6 ± 0.3
	10^{-8} M	9.4 ± 1.1	0.2 ± 0.8
	10^{-7} M	5.8 ± 1.0	−2.9 ± 0.4
	10^{-6} M	17.1 ± 2.0	−2.0 ± 0.2
2 Deoxyglucose	0 M	19.2 ± 2.1	−1.4 ± 0.2
	10^{-4} M	19.9 ± 2.0	−1.4 ± 0.4
	10^{-3} M	3.7 ± 1.0	−4.0 ± 0.4
	10^{-2} M	3.9 ± 0.8	−1.9 ± 0.1

PMNs in 5.6 mM dextrose buffer were incubated with 1.0 and 5.0 µM oleic or palmitic acids and inhibitors as described in Materials and Methods. For 2 deoxyglucose, which inhibited oleic acid–induced lipid body formation, results with 5 µM fatty acids are presented. For other inhibitors, which did not promote lipid body formation, presented results are with submaximal 1 µM fatty acid concentrations. Results, mean (±SEM) lipid bodies/PMN from 25–50 PMNs minus lipid body numbers in control cells incubated with each inhibitor and 0.1% ethanol (between 2–5 lipid bodies/PMN in different experiments), are derived from separate experiments with each inhibitor.

not promote oleic (or palmitic) acid–mediated lipid body formation, but instead significantly ($p < 0.0001$, t test) inhibited oleic acid–induced lipid body formation (Table I).

cis-Fatty Acids and PKC Activation. Because fatty acids, especially cis-fatty acids, can activate PKC, other stimuli of PKC were evaluated. In contrast to the ineffectiveness of dipalmitin and diolein, the cell permeable, PKC-activating diglyceride, OAG, was a potent stimulator of lipid body formation (Figs. 1 B and 6 A). The PKC-activating phorbol ester, PMA, induced PMN lipid body formation within 30 min with maximal lipid body formation at 20 nM (Fig. 6 B). Another phorbol ester, PDBu, at 2 and 20 nM, also elicited lipid body formation, with about 1/6th the molar potency of PMA; whereas PDD, a phorbol not activating PKC, elicited no induction of lipid bodies in concentrations up to 200 nM (data not shown). In the presence of superoxide dismutase plus catalase, greater numbers of lipid bodies were formed in response to PMA (Fig. 6 B) and PDBu, but not PDD (data not shown), as well as to OAG, arachidonic acid (data not shown), and oleic acid (Fig. 5 A).

PKC Inhibition and Fatty Acid-induced Lipid Body Formation. To further evaluate the role of PKC activation, the effects of several PKC inhibitors on fatty acid–induced lipid body formation were assessed. HMG, an effective inhibitor of PKC-mediated processes in human PMNs (25), potently inhibited lipid body formation induced by 2.5 µM concentrations of oleic acid, arachidonic acid, and OAG, 20 nM PMA (Fig. 7) and 20 nM PDBu (data not shown). HMG, at only 3 µM, produced about 50% inhibition of lipid body formation induced by each of these stimuli. HMG, in the concentrations used, inhibited cytosolic PKC activity of disrupted PMNs (Fig. 7 C). Other PKC inhibitors were also tested. With 1 µM staurosporine, lipid body formation induced by 2.5 µM arachidonic acid, oleic acid, and OAG was inhibited (mean ± SEM, $n = 3$) 39 ± 21%, 48 ± 10%, and 23 ±
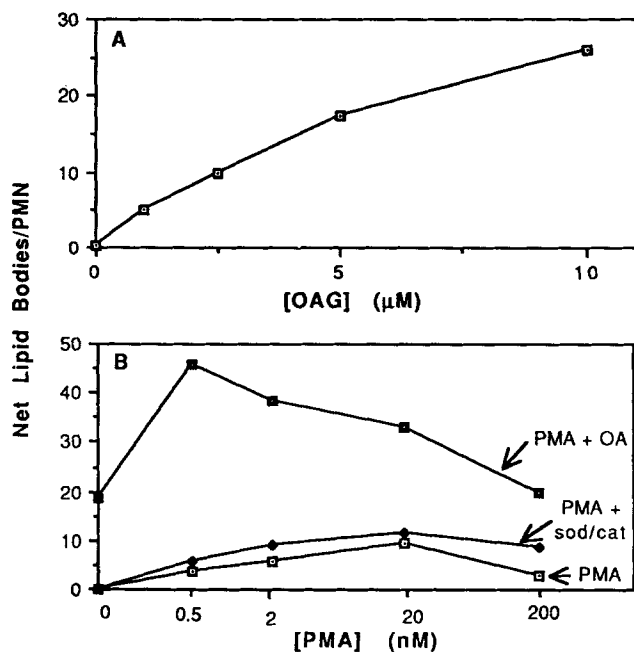
Figure 6. Induction of lipid body formation by OAG (A) or by PMA (B) alone or with superoxide dismutase/catalase (sod/cat) or oleic acid (OA). PMNs were incubated for 60 min with OAG or for 30 min with PMA. Where indicated, superoxide dismutase (5,000 U/ml) and catalase (25,000 U/ml) or 2.5 μM oleic acid were included with PMA. Mean net lipid bodies/PMN in 25–50 PMNs were corrected for lipid bodies (~1 and 10/PMN, without and with superoxide dismutase/catalase, respectively) in control PMNs incubated without stimuli. Data represent the means from triplicate experiments for PMA with superoxide dismutase/catalase and from duplicate experiments for OAG, PMA, and PMA with oleic acid.
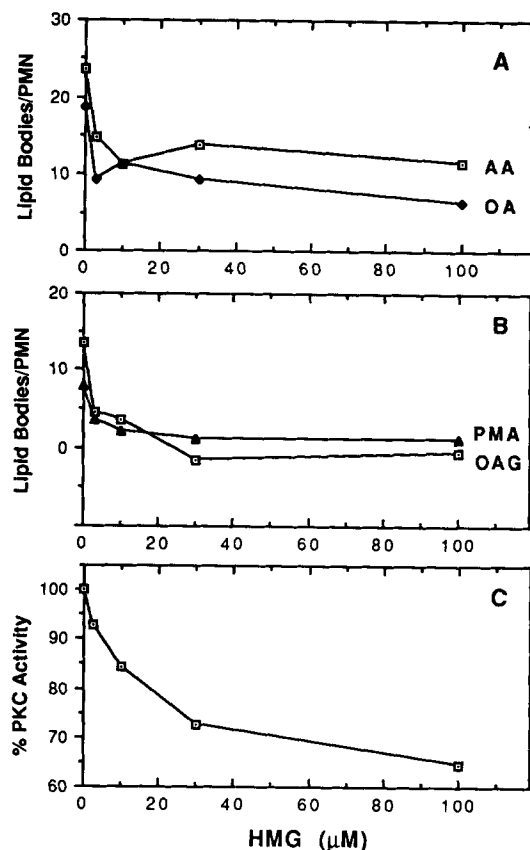

Figure 7. Inhibition by HMG of PMN lipid body formation induced by arachidonic acid, oleic acid, OAG, and PMA. PMNs were incubated with HMG at 37°C for 15 min and then with 2.5 μM arachidonic acid (AA) or oleic acid (OA) (A), or 2.5 μM OAG or 20 nM PMA (B) for 30 min. In each experiment mean net lipid bodies/PMN in 50–75 cells were corrected for lipid bodies (between 2–5/PMN) in PMNs incubated without stimuli. Results are means from four experiments, except values at 3 and 30 μM HMG are means from duplicate experiments. In C, PMN cytosolic PKC activity was assayed in the presence of concentrations of HMG; each value is the mean from triplicate experiments.

1%, respectively; and with 40 μM H-7 and the same three stimuli, mean inhibition (n = 2) was 52 ± 18%, 91 ± 7%, and 92 ± 8%, respectively. H-7 at 25 and 50 μM and staurosporine at 50 μM inhibited PKC activity 24, 77, and 83%, respectively, when assayed on disrupted PMNs. Sphingosine (5–25 μM) could not be evaluated since it was toxic to PMNs, in accord with previous findings (26), under conditions used for lipid body induction (e.g., 30–45 min with exogenous fatty acids but no BSA). The inhibition of cis-fatty acid- and diglyceride-stimulated lipid body induction by staurosporine and H-7 supported the findings with HMG indicating that lipid body formation was mediated by PKC activation.

Discussion

Although lipid bodies occur in a wide variety of cell types, mechanisms of formation and functions of these lipid-rich cytoplasmic inclusions have been uncertain. Normal blood PMNs have few lipid bodies, ~0.7 per cell; but tissue, exudate, and blood PMNs, when associated with inflammatory reactions, contain increased lipid body numbers (13, 23, 49). Lipid bodies serve as nonmembrane stores of esterified arachidonate, as evidenced by autoradiography of PMNs (49), eosinophils (48), and other cells (18, 19, 23), and by lipid analyses of lipid bodies isolated from eosinophils (50). Lipid bodies, ultrastructurally identical to lipid bodies in

PMNs in vivo, were elicited in PMNs incubated with arachidonic and oleic acids (Fig. 3) (49). In evaluating mechanisms of lipid body formation in PMNs, our studies have indicated that activation of PKC, by these fatty acids or other stimuli, is involved in lipid body formation.

While incubation of PMNs with exogenous fatty acids provides a source of lipids for incorporation into newly forming lipid bodies, the structurally restricted capacities of different fatty acids and lipids to induce lipid bodies indicated that mechanisms other than simple availability of lipid precursors were involved in lipid body formation. Palmitic acid, like other saturated fatty acids (eicosanoic, stearic, and myristic), failed to elicit lipid body formation; but [³H]-palmitic acid, like [³H]-arachidonic and [³H]-oleic acids, is freely taken up by PMNs (34, 49) and incorporated into PMN lipid bodies (49). Thus, while some lipids evaluated, including the diglycerides, dipalmitin and diolein, and the fatty acid methyl esters, are minimally permeant to intact cells, inability to be incorporated into cells or lipid bodies would not account for the failure of saturated fatty acids to elicit lipid body induction, nor would differential incorporation explain

the compositionally and stereochemically dependent lipid body–inducing activities of various unsaturated fatty acids (Fig. 4).

The preferential lipid body–inducing activities of cis-unsaturated fatty acids were not attributable to sequelae associated with other metabolic effects of these fatty acids. In cells with active oxidative phosphorylation, including macrophages (33) and lymphocytes (3), cis-unsaturated fatty acids can uncouple oxidative phosphorylation and cause ATP depletion, which might impair fatty acid esterification (11). In PMNs three inhibitors of oxidative phosphorylation did not elicit lipid body formation and did not augment fatty acid–induced lipid body formation to levels attainable with greater fatty acid concentrations. Moreover, PMNs, in contrast to other leukocytes, derive only 0.01–0.1% of their ATP from oxidative phosphorylation (24), so inhibition of oxidative phosphorylation would not account for cis-fatty acid elicitation of PMN lipid bodies. Inhibition of glycolytic ATP generation also did not promote, but rather significantly inhibited, fatty acid–induced lipid body formation (which was also inhibited at 4°C, data not shown). The lack of inhibition of oleic acid–induced lipid body formation by BHT helped exclude roles for free radical species, products of lipid peroxidation derived from unsaturated fatty acids (20), or oxidant-induced ATP depletion (44) in the genesis of lipid bodies. Analogously, since superoxide dismutase plus catalase did not inhibit stimulated lipid body formation, but rather augmented it (as considered more fully below), respiratory burst products released extracellularly from cis-fatty acid–stimulated PMNs (4, 5) were not involved in promoting lipid body formation. Thus, lipid body formation was temperature and energy dependent but not due to cis-fatty acid–induced ATP depletion or cis-fatty acid–elicited oxidants.

Instead, the lipid body–inducing activities of cis-fatty acids could be accounted for by their capacities to stimulate PKC (15, 30, 39, 40, 42), including human PMN PKC (29, 43). Since PKC species, including those in human PMNs (29), can be activated by cis-unsaturated fatty acids and not by trans- or saturated fatty acids, such activation would explain the preferential activities of cis-unsaturated fatty acids as inducers of lipid body formation. In support of this mechanism, lipid body formation was stimulated by other PKC activators, OAG, a cell permeable diglyceride well recognized to activate PKC (22, 32), PMA, and PDBu, both phorbol ester activators of PKC (10), but not PDD, a phorbol without activity for PKC (10). Thus, nonfatty acid activators of PKC, as well as cis-fatty acids, were effective in stimulating PMN lipid body formation.

The capacity of PKC inhibitors to block lipid body formation elicited both by cis-fatty acids and by OAG and PMA further indicated that PKC activation was involved in lipid body formation. The PKC inhibitor, HMG, has been shown to inhibit PKC without inhibiting cAMP-dependent or Ca⁺/calmodulin-dependent kinases, to be noncytotoxic for PMNs at concentrations that inhibited N-formyl-met-leu-phe (fMLP) chemoattractant- and phorbol ester–stimulated respiratory burst, and to inhibit specific phosphorylation in PMN cytoplasts stimulated with PDBu (25). In our assays, HMG was nontoxic to PMNs, elicited no lipid body formation by itself (data not shown), and inhibited PMN PKC. HMG inhibited lipid body formation induced by arachidonic and oleic acids

and by diglyceride and phorbol activators of PKC. HMG would be freely permeable within PMNs and able to act on intracellular, possibly cytoplasmic, PKC involved in formation of cytoplasmic lipid bodies. Two other PKC inhibitors, H-7, and staurosporine, also inhibited lipid body formation. That all three PKC inhibitors blocked cis-fatty acid–mediated lipid body formation excluded the possibility that PKC's role was solely as an intermediate acting to increase intracellular-free fatty acids, which then were the primary promoters of lipid body formation. The formation of lipid bodies in PMNs, therefore, represents a response mediated by PKC as · evidenced by the abilities of several activators of PKC (cis-fatty acids, diglyceride [OAG], and phorbol esters) to induce lipid body formation and by the actions of PKC inhibitors to block lipid body formation induced by cis-fatty acids and other PKC activators. While the PKC isozymes involved in cis-fatty acid- and diglyceride-stimulated lipid body formation have not been defined in PMNs, in other cells subspecies of PKC can be differentially responsive to cis-fatty acid stimulation and, in the presence of these fatty acids, may have differing requirements for increased Ca^{2+} concentrations for activation (15, 30, 39, 40, 42).

In addition to stimulating PKC, exogenous cis-fatty acids may contribute to lipid body formation in other ways. As demonstrated with ultrastructural autoradiography and lipid analyses, exogenous [³H]-arachidonate and [³H]-oleate (as well as [³H]-palmitate) are incorporated into leukocyte lipid bodies as neutral and phosphoglycerolipids (48, 49, 50). Lipids forming these inclusions are not derived solely from exogenous fatty acids or diglycerides, however, since phorbol esters can induce lipid body formation. Phorbol ester stimulation of PMNs has increased fatty acid incorporation into phospholipids (46), promoted accumulation of phospholipid classes (41), and stimulated increases in 1,2 diacyl- and 1-O-alkyl-2-acyl-glycerols (1, 16, 35, 47). Some of these endogenously formed lipids may be mobilized for inclusion within developing cytoplasmic lipid bodies.

The augmentation of lipid body formation by superoxide dismutase and catalase was notable, since all inducers of lipid body formation studied (arachidonic, oleic, and other cis-fatty acids, OAG, and PMA) can elicit a respiratory burst in PMNs (4, 5, 22). The diminished fatty acid-, but not phorbol ester-, stimulated lipid body induction by heat-inactivated superoxide dismutase and catalase was probably because of protein binding of fatty acids by the quantities of enzymes used. The enhancing effects both of active, extracellular superoxide dismutase plus catalase (despite protein binding of fatty acid stimuli) and of BHT suggest that respiratory burst-derived oxidative products inhibit lipid body formation. Extracellular oxidants might diminish stimulating fatty acid concentrations by oxidative alterations of the cis-fatty acids (21), although it is unclear if oxidants would similarly degrade OAG and PMA. Alternatively, oxidative products might act intracellularly to inhibit lipid body formation, as suggested by the findings with BHT-treated PMNs that were washed to remove free BHT. Whatever the mechanism, superoxide anion, H_2O_2, or derivative compounds (e.g., HOCl, chloramines) appear to inhibit lipid body formation in PMNs.

Conditions for inducing lipid body formation (i.e., 30–60-min time course and specific diglyceride and cis-fatty acid stimuli) are similar to conditions used in studies of PMNs exposed to "priming" stimuli (6, 7, 8). Priming stimuli have

included cell permeable 1,2-diacyl-glycerols, PKC activators (9, 22, 32), and 1-alkyl-2-acyl-diglycerides, PKC inhibitors (7, 9, 14) (analogous to 1-O-alkyl-2-O-methyl-glycerol, HMG [14, 25], used in this study). From our results, concentrations of cell-permeant diacylglycerols found effective for priming (~20 μM) (6, 7, 8) would also have induced lipid body formation. Whether 1-alkyl-2-acyl-diglycerides elicit lipid bodies is not known; but HMG, up to 100 μM, did not stimulate lipid body formation. While both diacyl- and alkylacyl-glycerols can prime PMNs, significant differences were found between the two diglyceride classes in terms of their effects on fMLP-induced respiratory burst activity (7) and arachidonate metabolism (8). Diacylglyceride priming was specifically effective in enabling PMNs, upon stimulation with fMLP, to form [^{3}H]-arachidonate–derived eicosanoids, whereas alkylacylglycerol priming inhibited such eicosanoid formation (8). Thus, the same priming diglycerides that enabled fMLP-induced eicosanoid formation in PMNs would also have elicited lipid bodies in PMNs. In conjunction with our demonstration that lipid bodies can be a predominant site of [^{3}H]-arachidonate deposition within PMNs (48) and an intracellular localization of prostaglandin H synthase (cyclooxygenase) (Weller, P. F., and S. W. Ryeom, manuscript submitted for publication), lipid bodies may constitute a nonmembrane pool of esterified arachidonate from which incorporated arachidonate can be mobilized for eicosanoid formation.

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