Increased saturated triacylglycerol levels in plasma membranes of human neutrophils stimulated by lipopolysaccharide

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Abstract Neutrophils isolated from patients with bacterial infections or stimulated in vitro with lipopolysaccharide (LPS) produce a high resolution, lipid-dominated spectrum on ^{1}H-NMR spectroscopy (May et al, 1993. *J. Infect. Dis.* **168:** 386–392). We have investigated the origin of this lipid signal using NMR and chemical analyses of both whole neutrophils and purified plasma membranes. Plasma membranes from neutrophils that had been stimulated with 50 μg/ml LPS exhibited the high resolution ^{1}H-NMR signal, and contained double the triacylglycerol (TAG) content of plasma membranes isolated from resting cells. Chemical analysis of the whole cells indicated that the TAG also increased at the cellular level (1.7-fold) after stimulation with LPS. Diradylglycerol increased 2- to 3-fold in both whole cells and plasma membranes after stimulation, but was only a minor component compared with TAG. The plasma membrane protein/phospholipid ratio increased 2.6-fold, whereas cholesterol (free and esterified) was unchanged. The membranes from LPS-stimulated neutrophils exhibited increased fluidity, as judged by increased merocyanine 540 binding, consistent with a 2-fold reduction in cholesterol/phospholipid ratio. LPS induced a shift in fatty acid content of whole cell polar lipids towards more oleic acid and less palmitic acid, whereas the neutral lipid fraction contained increased amounts of palmitic and stearic acids. The TAG fraction of plasma membrane lipids contained increased amounts of palmitic acid when prepared from cells stimulated with LPS. We conclude that the ^{1}H-NMR signal in LPS-stimulated neutrophils arises from increased amounts of plasma membrane TAG with an elevated content of palmitic acid.—**May, G. L., L. C. Wright, K. Groot Obbink, P. M. Byleveld, M. L. Garg, Z. I. Ahmad, and T. C. Sorrell.** Increased saturated triacylglycerol levels in plasma membranes of human neutrophils stimulated by lipopolysaccharide. *J. Lipid Res.* 1997. **38:** 1562–1570.

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A number of mammalian cell types capable of migration and/or activation in vivo such as stimulated lymphocytes, malignant and embryonic cells generate high resolution ^{1}H-NMR spectra arising from neutral lipid and other cellular metabolites (1). Recently, we reported that human polymorphonuclear leukocytes isolated from infected purulent exudates demonstrated an intense, high resolution lipid spectrum (2). It was determined using two-dimensional COSY methodology that this spectrum includes dominant triacylglycerol (TAG) crosspeaks. The ^{1}H-NMR signal was absent from circulating neutrophils obtained from healthy subjects but could be generated in vitro by exposure to lipopolysaccharide (LPS), the predominant proinflammatory component of bacterial cell walls (3) and was found in circulating neutrophils from patients with severe infections (3).

The source of the lipid signal is believed to be rapidly tumbling "mobile" TAG in the plasma membrane and chemical analysis of purified plasma membranes from leukemic lymphoblasts (4) has confirmed the presence of TAG. We have found the high resolution ^{1}H-NMR signal to be present in enucleated and degranulated cytoplasts isolated from LPS-stimulated neutrophils (5), and have shown that lysis of cytoplasts provides a simple, rapid method for obtaining reasonably pure plasma

Abbreviations: LPS, lipopolysaccharide; TAG, triacylglycerol; COSY, correlation spectroscopy; PBS−, Dulbecco's phosphate-buffered saline without calcium and magnesium; HEPES, (N-[2-hydroxyethyl]piperazine-N′-[2-ethanesulfonic acid]); DRG, diradylglycerol; dma, dimethylacetal; PABA, *p*-aminobenzoic acid.

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membranes (6). While it has been suggested that the ^{1}H-NMR signal arises from lipid droplets in the cytoplasm (7), we have shown that lipid droplets may be induced to form in cytoplasts, without the appearance of the NMR lipid signal (5). In this report we have used our rapid method for plasma membrane isolation to confirm that the NMR-visible lipid signal is generated from TAG located in plasma membranes from LPS-stimulated neutrophils.

Translocation of cytoplasmic phospholipase A_2 to the plasma membrane during activation of neutrophils is associated with release of arachidonic acid from membrane phospholipids (8), thus providing a substrate for the formation of bioactive lipids, which modulate cellular responses. We reported previously that significant phospholipid remodelling occurs in whole neutrophils, and plasma membranes isolated from them, on stimulation with 50 μg/ml LPS (6). TAG can serve as a repository of arachidonic acid for transfer to phospholipids (9). We analyzed the fatty acid composition of both neutral lipids (mostly TAG and cholesteryl ester) and polar lipids (mostly phospholipids) in intact neutrophils and isolated plasma membranes to investigate the transfer of fatty acids to membrane lipid species. Our finding that both cellular and plasma membrane TAG are enriched in saturated fatty acids, such as palmitic, suggested that unsaturated fatty acids, such as arachidonic or oleic, were transferred from TAG to phospholipids and bioactive molecules. The NMR-visible TAG could then serve as a store of fatty acids for re-acylation of both lipids and palmitoylated proteins at the termination of the stimulatory process, and as a protective mechanism against the toxic effects of excess free fatty acids.

MATERIALS AND METHODS

Neutrophil preparation and stimulation

Neutrophils were isolated from the peripheral blood of healthy Blood Bank volunteers as previously described (5). Erythrocytes were removed from the platelet-rich plasma by sedimentation with 3.5% dextran and neutrophils were separated from other leukocytes on Ficoll-Paque. Residual erythrocytes were removed by hypotonic lysis and neutrophils were resuspended in calcium- and magnesium-free phosphate-buffered saline (PBS−). The purity of the preparations (>90%) was monitored by Coulter counter (Hialeah, FL) and cell viability was assessed by trypan blue exclusion. There was no difference in viability (>95%) between control and LPS-treated cells). Stimulation with LPS (50 μg/ml) from *Salmonella typhimurium* (Sigma, St. Louis, MO) was accomplished during incubation (37°C, 1 h) with gentle agitation in Dulbecco's modified Eagle's medium containing bicarbonate, 10% pooled, heat-inactivated, filtered human serum (type AB) buffered with 20 mM HEPES, pH 7.4.

Preparation of plasma membranes

These were prepared by our previously described method (6). Briefly, enucleated and degranulated cells (cytoplasts) were prepared from resting and LPS-stimulated neutrophils by the method of Roos, Voetman, and Meerhof (10) and subjected to hypotonic lysis in 10 mM HEPES, pH 7.4. The plasma membranes were collected by centrifugation at 179,082 g_{max} using a Beckman Ti 50 rotor, and were judged from marker enzyme activities to be of similar purity from both resting and LPS-stimulated cells (6).

Lipid extraction and analysis

Neutrophil lipids were extracted from whole cells or plasma membranes with chloroform and methanol containing butylated hydroxytoluene as antioxidant and non-lipid contaminants were removed with Sephadex G-25 as previously described (4); TAG, cholesterol (free and esterified), and phospholipid phosphorus were assayed as previously described (4). Diradylglycerol (DRG) was estimated in lipid extracts using the Biotrak kit supplied by Amersham (based on the diacylglycerol kinase-mediated reaction between DRG and ^{32}P-ATP). Whole cell and plasma membrane lipids were fractionated by silica gel Sep-pak chromatography (Millipore-Waters, Milford, MA) into neutral and polar lipids. These were hydrolyzed and converted to fatty acid methyl esters using the BF_3–methanol method (11) with C 25:0 fatty acid as internal standard, and quantified by capillary gas chromatography using a Hewlett-Packard 5890 Series II chromatograph fitted with a flame ionization detector and a fused silica capillary column (DB-225, 30 m, J & W Scientific, Folsom, CA). Plasma membrane total lipids were subjected to preparative thin-layer chromatography in a solvent system of petroleum ether (BP 60–80°C)–diethyl ether–acetic acid 90:15:1 (v/v/v). The TAG fraction was eluted from the plates with chloroform–methanol 2:1 (v/v) and directly transesterified to fatty acid methyl esters using anhydrous acetyl chloride (12) and C 25:0 as internal standard. Capillary gas chromatography was carried out on a fused carbon-silica column (30 m × 0.250 mm; film thickness 0.25 μm), coated with cyanopropylphenyl (DB-225, J & W Scientific, supplied by Activon Scientific, Sydney, Australia). Fatty acid methyl esters were identified by comparison of their retention times with authentic fatty acid methyl ester standards (Nu Chek Prep Inc., Elysian, MN). Relative fatty acid compo-

sitions were calculated from the areas under the peaks, after normalization to the peak area of the internal standard. There was no qualitative or quantitative difference between the fatty acid methyl esters prepared by the BF_3–methanol and acetyl chloride methods, although we found there was less background "noise" with the latter.

Protein estimations

Protein content of cells and membranes was measured using the Bio-Rad Coomassie blue reagent with bovine serum albumin as standard.

Fluidity measurements

The binding of merocyanine 540 (Sigma) was used to assess membrane fluidity by the method of Bratton (13). After treatment, the cells were washed and resuspended in PBS. Triplicate 50-μl aliquots (10^6 cells) were mixed with 50 μl of HBSS containing BSA to give a final BSA concentration of 1%. Ten μl of merocyanine 540 (diluted 1 in 10 with HBSS containing 1% BSA to 100 μg/ml) was added and the cells were incubated for 3 min at room temperature. Nine hundred μl of HBSS (without BSA) was added and the samples were analyzed immediately by flow cytometry on an EPICS Profile II Analyzer (Coulter). Fluorescence was monitored through a 570 ± 35 nm band pass filter and forward angle light scatter and side scatter were measured from each sample. Data were collected as log FL2 measurements.

^{1}H magnetic resonance spectroscopy (^{1}H-NMR)

Neutrophils were washed twice in PBS− made in ^2H_2O. The final cell count was 5×10^7 cells per 400 μl. Plasma membranes prepared from 4×10^8 cells were resuspended in 400 μl of PBS− made in ^2H_2O. ^{1}H-NMR spectra were recorded as previously described (3–5) using a Bruker AM 360 MHz spectrometer equipped with an Aspect 3000 computer, with 1.6 mM *p*-aminobenzoic acid (PABA) as internal standard. All crosspeak volumes were measured at the same intensity and standardized against the volume of the PABA crosspeak.

Statistical analysis

Calculations of significance were performed using the computer program InStat (GraphPAD Software Pty. Ltd, San Diego, CA).

RESULTS

Human peripheral blood neutrophils, when stimulated with LPS (50 μg/ml), and cytoplasts prepared from them, produce an increase in the intensity of the ^{1}H-NMR lipid signal (5). Plasma membranes were prepared from both resting and LPS-stimulated cells to ascertain the location of the lipid signal. The increase in lipid signal in the stimulated cells is clearly visible in the one-dimensional spectrum of membranes prepared from them, compared with the spectrum of membranes prepared from resting cells (**Figs. 1a and b**). The lipid crosspeaks A, B, E, F, and G′ are prominent in the COSY spectrum of the stimulated membranes, with G and C just visible (Fig. 1b). Other crosspeaks in the spectra have previously been assigned to metabolites, such as lactate, taurine, or other amino acids (3). Crosspeak D, which arises from protons of the methylene group between two double bonds, was not visible. In the control membranes only crosspeaks A and G′ are faintly visible (Fig. 1a). Quantitation of TAG, based on volumes of the F crosspeak in the COSY spectra (5) revealed values of 6.1 ± 1.5 and 0.93 ± 0.78 (n = 3, $P < 0.01$) for membranes from stimulated and resting cells, respectively. These volumes are ratios obtained by assessing the intensity of the F crosspeak by integration, using standard Bruker integration procedures, compared with the intensity similarly obtained for the internal standard, PABA.

Chemical analyses of both whole cells and plasma membranes were undertaken to investigate the biochemical changes that contribute to the increase in NMR lipid signal on stimulation with LPS. In whole cells, there were significant increases in both TAG and diradylglycerol (DRG; the assay method does not distinguish between 1,2 diacyl and 1-alkyl, 2-acyl glycerols) and a decrease in whole cell phospholipid content when expressed on the basis of cell number (**Table 1**). There was no change in phospholipid when expressed as nmol/mg lipid, probably because of the slight decrease in cellular total lipid (not statistically significant). In addition, on stimulation there were no changes in the whole cell free and esterified cholesterol, or protein content. The amount of esterified cholesterol was extremely variable in these cells and below the level of detection after stimulation (Table 1). The results of esterified cholesterol analyses were confirmed by high performance liquid chromatography (L. Danckwerts, unpublished data). The cholesterol/phospholipid ratios in Table 1 agree well with those for whole polymorphonuclear leukocytes reported in the literature, e.g., 0.54, 0.54, 0.55 (refs. 14, 15, 16, respectively).

Both TAG and DRG increased by approximately 2- and 3-fold, respectively, in pure plasma membranes isolated from LPS-stimulated cells, when expressed as nmol/mg lipid (**Table 2**). The TAG content of neutrophil plasma membranes is lower than values obtained previously (4) from a range of cultured cell lines (77–

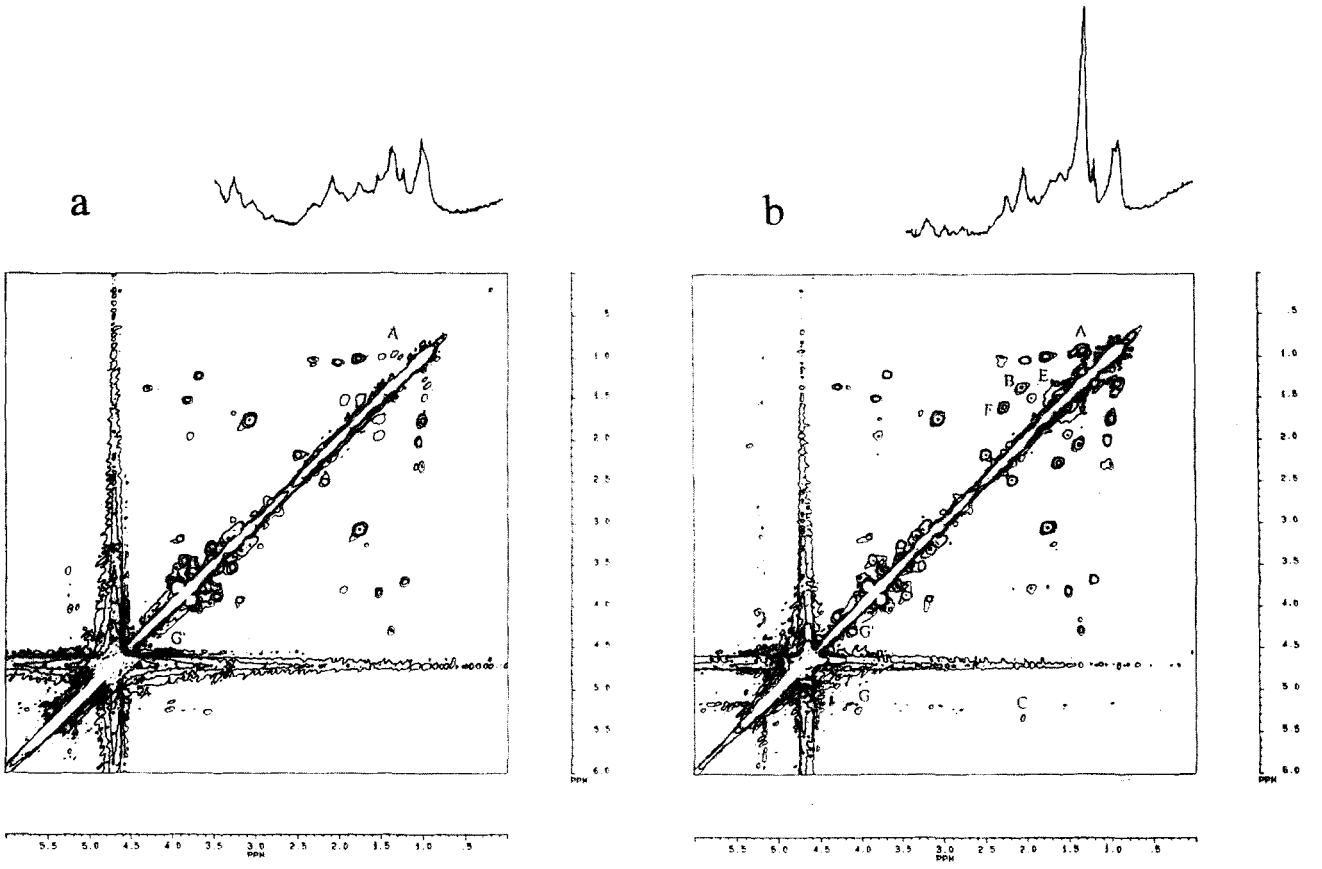

Fig. 1. Symmetrized 2D COSY spectrum of purified plasma membranes isolated from (a) control cells and (b) from neutrophils stimulated with LPS (50 μg/ml). The plasma membranes were suspended in PBS/D_2O and the spectra acquired on a Bruker AM-360 spectrometer at 37°C with samples spinning (NS = 192, NE = 200, spectral width = 4000 Hz). Data were processed with a sine-bell window function in the t_1 domain and a Gaussian (LB = −30, GB = 0.12) in the t_2 domain. Lipid acyl chain and glycerol backbone connectivities are indicated (A–G′), and have been assigned as previously described (4). The relevant part of the one-dimensional ^{1}H NMR spectrum is displayed above the COSY spectrum. The spectral width was 4000 Hz, the acquisition time was 1.024 s, and a line broadening of 3 Hz was applied.

163 nmol/mg lipid) and peripheral blood lymphocytes stimulated with pokeweed mitogen (491 nmol/mg lipid). The phospholipid content of plasma membranes from stimulated cells was increased 2-fold and total cholesterol was unchanged, resulting in a decrease in the total cholesterol/phospholipid ratio from 0.9 to 0.5 (Table 2 and ref. 6). Esterified cholesterol was unchanged in membranes after stimulation, but the protein content of the membranes was significantly increased, leading to an increased protein/lipid ratio (2.6-fold, Table 2).

To test whether the decreased cholesterol/phospholipid ratio (Table 2) correlated with a more fluid plasma membrane, we measured the binding of the impermeant fluorescent dye, merocyanine 540. This dye binds most strongly to cells with a loosely packed (more fluid) outer monolayer. The log mean fluorescence intensity (in arbitrary units) for resting neutrophils was 5.74 ± 1.12, whereas that for the LPS-stimulated population was 8.10 ± 2.72, an increase of 41.2%. These results are the means and SD of three experiments performed in triplicate (significant difference, $P = 0.0245$, 2-tailed, paired t test).

Having established that stimulation of cells with LPS yielded plasma membrane preparations with an increased NMR-detectable lipid signal and that this correlated with an increase in plasma membrane TAG (DRG is only a minor component and would contribute little to the spectrum), we sought to determine which changes in cellular lipid composition contributed to formation of the additional membrane TAG. The whole cell polar lipids (predominantly phospholipid) and neutral lipids (predominantly TAG and esterified cholesterol) were analyzed separately. While there was no change in arachidonic acid content of the polar lipids from LPS-stimulated cells, there was a significant decrease in the proportion of palmitic acid (16:0) and increase in the proportion of oleic acid (18:1) (**Table**

TABLE 1. Lipid and protein content of whole neutrophils before (control) and after stimulation with LPS

Component	Control	Stimulated
Total lipid (mg/10^8 cells)	1.78 ± 0.24	1.49 ± 0.37
Protein (mg/10^8 cells)	4.09 ± 0.60	3.55 ± 0.30
Protein/lipid	2.35 ± 0.55	2.48 ± 0.54
Phospholipid		
nmol/mg lipid	549 ± 110	518 ± 110
nmol/10^8 cells	958 ± 89	749 ± 90[a]
Triacylglycerol		
nmol/mg lipid	40 ± 8	67 ± 30[b]
nmol/10^8 cells	71 ± 12	94 ± 30[b]
Free cholesterol		
nmol/mg lipid	266 ± 80	279 ± 51
nmol/10^8 cells	467 ± 142	408 ± 96
Esterified cholesterol		
nmol/mg lipid	18 ± 115[d]	BLD
nmol/10^8 cells	13 ± 201[d]	BLD
Diradylglycerol (nmol/10^8 cells)	0.59 ± 0.35	1.00 ± 0.44[c]

Neutrophils were stimulated with LPS (50 μg/ml) for 1 h at 37 °C. Analyses are presented as means ± SD for 6 experiments. Molar calculations are based on a mean molecular weight of 750 Da for phospholipid, and on triolein, cholesteryl oleate, and diarachidonin for TAG, esterified cholesterol, and diradylglycerol, respectively. BLD, below the level of detection.

[a]Significant with unpaired *t*-test, $P < 0.01$.
[b]Significant with unpaired *t*-test, $P < 0.05$.
[c]Significant with paired *t*-test, $P < 0.01$.
[d]The errors here are large because of the extreme variability of the samples and because esterified cholesterol is obtained by subtraction of free from total cholesterol. The remaining (unassayed) lipid weight in this table consists mainly of glycolipids (16).

TABLE 2. Lipid and protein content of pure plasma membranes isolated from neutrophils before (control) and after stimulation with LPS

Component	Control	Stimulated
Lipid (mg/10^9 cells	4.29 ± 0.64	3.30 ± 0.16
Protein (μ/10^9 cells	435 ± 71	863 ± 106[a]
Protein/lipid	0.1 ± 0.02	0.26 ± 0.04[a]
Phospholipid		
nmol/mg lipid	206 ± 12	400 ± 73[b]
nmol/10^9 cells	881 ± 119	1316 ± 223[c]
Triacylglycerol		
nmol/mg lipid	22 ± 4	43 ± 9[b]
nmol/10^9 cells	97 ± 34	143 ± 25[b]
Free cholesterol		
nmol/mg lipid	129 ± 12	128 ± 31
nmol/10^9 cells	557 ± 129	422 ± 98
Esterified cholesterol		
nmol/mg lipid	64 ± 14	65 ± 9
nmol/10^9 cells	272 ± 43	361 ± 56
Diradylglycerol (nmol/mg lipid)	2 ± 0.7	7 ± 3.9[d]

Neutrophils were stimulated with LPS (50 μg/ml) for 1 h at 37°C, then plasma membranes were isolated as in Materials and Methods. Analyses are presented as means ± SD of six experiments. The remaining lipid weight in this table is mainly glycolipid (unassayed) (16). Molar calculations are based on a mean molecular weight of 750 Da for phospholipid, and on triolein, cholesteryl oleate, and diarachidonin for TAG, esterified cholesterol, and diradylglycerol, respectively.

[a]Significant with unpaired *t*-test, $P < 0.01$.
[b]Significant with unpaired *t*-test, $P < 0.02$.
[c]Significant using the unpaired *t*-test, $P < 0.05$.
[d]Significant using the paired *t*-test, $P < 0.05$.

TABLE 3. Fatty acid composition of polar lipids isolated from whole neutrophils before (control) and after stimulation with LPS

Fatty Acid	Control	Stimulated
14:0	1.6 ± 0.2	1.7 ± 0.4
16:0	22.5 ± 2.6	18.5 ± 0.6[a]
16:0 dma	1.4 ± 0.2	1.7 ± 0.2
18:0	20.3 ± 2.5	17.1 ± 0.8
18:0 dma	2.2 ± 0.4	2.7 ± 0.3
18:1 (n–9)	27.9 ± 2.0	32.1 ± 0.7[b]
18:2 (n–6)	8.1 ± 1.1	9.5 ± 0.8
20:3 (n–6)	1.1 ± 0.3	1.3 ± 0.4
20:4 (n–6)	10.7 ± 1.5	11.1 ± 1.5

Neutrophils were stimulated with LPS (50 μg/ml) for 1 h at 37°C. Values are expressed as percentages of the total fatty acid content and represent the means and SEM of nine experiments. Dimethyl acetals, formed from plasmalogen-containing lipids, are represented by dma. Polar lipids include fatty acids from both phospholipids and glycolipids. Minor components (≤ 1%), such as 16:1 are not included in this table.

[a]Significant by the Wilcoxon signed rank test, $P < 0.05$.
[b]Significant by the Mann-Whitney two sample test, $P < 0.02$.

3). Conversely, the neutral lipid fraction from stimulated whole cells was enriched in saturated fatty acids (16:0 and 18:0) at the expense of the unsaturated fatty acids (18:1, 18:2, 20:4 and 16:1) (**Table 4**). The plasmalogen-containing neutral lipids disappeared after cell stimulation.

There were no differences in the fatty acid composition of polar lipids from pure plasma membranes isolated from resting and LPS-stimulated cells (**Table 5**). Because of problems with sensitivity, and because there was no change in esterified cholesterol content of the membranes following stimulation, we used the total lipid from membranes prepared from $>2 \times 10^8$ cells to prepare pure TAG. Fatty acid analysis of the pure TAG revealed a significant increase in 16:0 (palmitic) acid, expressed as a percentage of the total fatty acid

TABLE 4. Fatty acid composition of neutral lipids extracted from whole neutrophils before (control) and after stimulation by LPS

Fatty Acid	Control	Stimulated
14:0	6.3 ± 1.0	8.8 ± 1.7
16:0	24.6 ± 4.2	38.6 ± 5.8[a]
16:0 dma	10.1 ± 2.5	BLD[b]
16:1 (n–7)	5.3 ± 2.7	3.4 ± 2.2
18:0	13.8 ± 1.9	22.0 ± 2.2[a]
18:1 (n–9)	29.6 ± 3.8	19.9 ± 4.1
18:2 (n–6)	7.4 ± 1.2	3.8 ± 3.8
20:4 (n–6)	4.5 ± 1.9	BLD

Neutrophils were stimulated with LPS (50 μg/ml) for 1 h at 37°C. Results are expressed as percentages of the total fatty acid content and represent the means and SEM of four experiments. Dimethyl acetals formed from plasmalogen-containing lipids are represented by dma. BLD, below the level of detection. Minor components (≤ 1%) are not included in this table.

[a]Significant by the two-tailed, paired *t*-test, $P < 0.02$.
[b]Significant by the two-tailed *t*-test, $P < 0.05$.

TABLE 5. Fatty acid composition of polar lipids extracted from plasma membranes of neutrophils before (control) and after stimulation by LPS

Fatty Acid	Control	Stimulated
14:0	1.1 ± 0.4	1.6 ± 0.3
16:0	18.7 ± 0.4	18.6 ± 0.5
16:0 dma	1.6 ± 0.4	1.9 ± 0.3
16:1 (n–7)	1.2 ± 0.4	1.7 ± 0.4
18:0	16.8 ± 0.8	15.6 ± 0.9
18:0 dma	3.1 ± 0.6	3.2 ± 0.5
18:1 (n–9)	33.3 ± 2.3	32.5 ± 2.3
18:2 (n–6)	9.4 ± 0.5	9.5 ± 1.0
20:0	1.5 ± 0.4	2.0 ± 0.7
20:3 (n–3)	1.4 ± 0.6	1.2 ± 0.6
20:3 (n–6)	1.3 ± 0.4	1.0 ± 0.5
20:4 (n–6)	9.1 ± 1.2	9.3 ± 1.0
22:0	1.6 ± 0.5	1.9 ± 0.8

Neutrophils were stimulated with LPS (50 μg/ml) for 1 h at 37°C, then membranes were prepared and lipids were extracted. Dimethyl acetals formed from plasmalogen-containing lipids are represented by dma. Values are presented as percentages of the total fatty acid content of the membranes and represent the means and SEM of five experiments. Polar lipids include fatty acids from both phospholipids and glycolipids.

content (**Table 6**). The fatty acids 18:1 (oleic) and 18:2 (linoleic) also showed increased levels that did not, however, reach statistical significance when expressed as a percentage of the total fatty acids, but were significant ($P = 0.01$) when the increase in total TAG fatty acid was included in the calculations (see below). The increase in palmitic acid occurred at the expense of several unsaturated fatty acids, namely 18:3, 20:1, 20:4, and 22:1. The 2.7-fold increase in relative total fatty acid content in TAG after stimulation with LPS (Table 6) was consistent with the increased TAG mass obtained by chemical analysis (Table 2). No dimethylacetals were found in the fatty acid methyl esters derived from the plasma membrane TAG, indicating the absence of TAG containing vinyl ether-linked fatty acids.

TABLE 6. Fatty acid composition of TAG extracted from plasma membranes isolated from neutrophils before (control) and after stimulation by LPS

Fatty Acid	Control	Stimulated
14:0	2.6 ± 1.1	1.6 ± 0.5
16:0	13.8 ± 2.9	23.8 ± 3.6[a]
16:1 (n–7)	2.6 ± 0.9	2.0 ± 0.3
18:0	10.6 ± 2.0	11.6 ± 1.9
18:1 (n–9)	27.8 ± 4.9	35.2 ± 1.2
18:2 (n–6)	11.4 ± 2.5	16.0 ± 0.3
18:3	1.8 ± 0.9	0.8 ± 0.3
20:1	3.6 ± 2.9	1.2 ± 0.6
20:2	2.2 ± 0.6	2.0 ± 0.3
20:3	3.2 ± 0.9	3.0 ± 1.1
20:4 (n–6)	9.2 ± 3.8	3.0 ± 1.3
22:1	5.2 ± 1.8	1.9 ± 0.6
22:6	0.8 ± 0.4	0.9 ± 0.3
Total fatty acid content	100 ± 15.4	279.5 ± 58.5[a]

Neutrophils were stimulated with LPS (50 μg/ml) for 1 h at 37°C, then membranes were prepared as in Materials and Methods. Individual fatty acid values are presented as percentages of the total fatty acid content of the membranes; the sum of the peak areas (total fatty acids) was taken as 100 for the control, above, for comparison with the total fatty acids from the stimulated membranes. The results represent means and SEM of five experiments.

[a]Significant by the two-tailed, paired *t*-test, $P < 0.05$.

DISCUSSION

We have previously presented evidence that cytoplasmic lipid droplet numbers alone are not sufficient to account for the NMR lipid signal in LPS-stimulated human neutrophils (5). The data presented in Fig. 1 confirm that the lipid signal in arises from the plasma membrane. The lipid signal is also stable, persisting in the membranes throughout cytoplast formation, during which cytochalasin B induces the expulsion of the nucleus and granules and the loss of half the cytoplasm and some of the plasma membrane from the intact cells (10). As the plasma membranes of cytoplasts are functionally and physically intact after nuclear extrusion (10) it is highly unlikely that select plasma membrane domains are lost during this process. The signal also persisted after the hypotonic lysis of the cytoplasts and two ultracentrifugation steps. It is unlikely that lipid droplets loosely "stuck" onto the cytoplasmic side of the membranes would survive this process; some mechanism for the attachment of TAG-containing droplets would be required.

Crosspeak G (Fig. 1b) is common to both TAG and phospholipids and arises from the methine to methylene coupling of glycerol. It is difficult to discount some contribution to the lipid spectrum from phospholipid, which increases 2-fold in plasma membranes from LPS-stimulated cells (Table 2 and ref. 6). Previously it has been established that the F crosspeak from scalar coupling between the protons of the α and β carbons of fatty acyl chains arises predominantly from triglyceride (4) rather than from phospholipid (17). The volume of crosspeak F is almost seven times higher in membranes from stimulated cells than in membranes from resting cells. Because the intensity of NMR signals depends on the mobility as well as the number of protons present, the TAG giving rise to the spectrum in Fig. 1b must be more mobile as well as more abundant (cf the 2-fold increase in TAG with stimulation in Table 2). The G′ crosspeak in the COSY spectrum arises from the coupling between the geminal protons of carbons 1 and 3 of the glycerol backbone. This crosspeak is produced only from carbon 1 of phospholipids, thus the greater prominence of G′ in stimulated membranes is consistent with a spectrum arising predominantly from TAG

(Fig. 1b). The crosspeaks C and D both arise from protons adjacent to double bonds, and their low intensity or absence in the stimulated membranes is consistent with the presence of more saturated TAG, or alternatively, with more restricted motion of these protons.

Our analytical data show that both TAG and DRG increase in the plasma membranes of stimulated cells. While DRG is also measured by our TAG assay, it is only a minor component compared with TAG and would contribute little to the lipid spectrum. The increase in DRG observed under our conditions in whole cells is much smaller than that observed after exposure to other stimuli, e.g., N-formylmethionyl-leucyl-phenylalanine, phorbol 12-myristate 13-acetate diester, A23187 (18).

Only very few neutral lipid-containing droplets are present in unstimulated neutrophils (5, 19). A comparison of the fatty acid composition of resting neutrophil polar and neutral lipids (Tables 3 and 4) suggests that the cytoplasmic neutral lipids (predominantly TAG and cholesterol ester) are repositories of ether-linked (alkenyl or plasmalogen) fatty acids (16:0 dma) and myristic acid (14:0). Myristic acid could be used for the post-translational modification of proteins. On stimulation with LPS, the plasmalogen-linked fatty acid in the neutral fraction possibly becomes incorporated into phosphatidylethanolamine, as this phospholipid class increases during stimulation with LPS (6). Arachidonate also is mobilized from the neutral lipids in response to LPS (Table 4). TAG has been shown to act as a store of arachidonate after certain stimuli (8, 9), which may be used for phospholipid remodelling or eicosanoid production. LPS stimulation resulted in the apparent transfer of saturated fatty acids 16:0 and 18:0 from the polar fraction to the neutral lipids, whilst 18:1 fatty acid was transferred from the neutral lipids to the polar lipids (Tables 3 and 4).

Although we have shown that the phospholipid content of the plasma membrane is doubled after stimulation with LPS (Table 2) and exhibits an increase in phosphatidylserine and a decrease in 1-alkyl, 2-arachidonyl phosphatidylcholine (6), the fatty acid composition of the polar fraction was, surprisingly, unaltered by stimulation (Table 5). The increased phospholipid probably arises from cholesterol-poor vesicles originating from internal membranes such as the endoplasmic reticulum (6). When neutrophils are stimulated, there is translocation and fusion of intracellular phospholipid-containing vesicles and granules with the plasma membrane (20), and it is possible that the increased TAG, protein, and phospholipid content of the plasma membranes occurs this way in response to LPS. There is a lower content of the saturated fatty acids 16:0 and 18:0 in the plasma membrane polar lipids, when compared with whole cell polar lipids, in the resting cells (Tables 3 and 5) and a lower content of 18:0 in the plasma membrane polar lipids from the stimulated cells.

The fatty acid composition of TAG isolated from the plasma membranes of resting neutrophils (Table 6) differs from the whole cell neutral lipid (Table 4) in that it, like the polar lipids, is lower in saturated fatty acids 14:0, 16:0, and 18:0. It is also higher in arachidonic acid and 18:2, and contains a small quantity of a number of long chain unsaturated fatty acids, e.g., 22:1 which were below the level of detection when whole cell neutral lipids were analyzed. After stimulation by LPS, there was a 1.7-fold increase in the percentage of 16:0 in the plasma membrane TAG. As total fatty acid content of TAG increased by 2.7-fold (Table 6), the increase in the absolute amount of 16:0 was 4.7-fold. Increases in the absolute amounts of 18:1 and 18:2 were also highly significant. There was a decrease, on stimulation, in percentage composition, but not absolute amounts, of arachidonate and minor fatty acids such as 22:1, which did not reach statistical significance (Table 6).

We have documented an increased fluidity of the plasma membrane of LPS-stimulated neutrophils as measured by increased merocyanine binding. Increased fluidity has been recorded using this method for neutrophils stimulated with fMLP (N-formyl-L-methionyl-L-leucyl-L-phenylalanine), PMA (phorbol 12-myristate 13-acetate) and ionomycin (13) and low concentrations (10 ng/ml) of LPS (21). It is well established that the lipid structural order in membranes at physiological temperatures is largely determined by the cholesterol and sphingomyelin content and by the degree of saturation of the phospholipid acyl chains (22). The decrease in cholesterol/phospholipid ratio (Table 2) is probably the main contributing factor to the increased fluidity of the plasma membranes from LPS-stimulated cells, as we have previously shown that there is no change in sphingomyelin content (6) and there is no change in the fatty acid saturation of the membrane polar lipids (Table 5). The contribution of the changes in fatty acid composition of TAG (Table 6) to the overall fluidity of the plasma membrane is unknown.

The increased 16:0 in plasma membrane TAG after stimulation by LPS does not appear to come from phospholipase degradation of the membrane polar lipid fraction, as there is no change in fatty acid composition of this fraction with stimulation, despite a 2-fold increase in phospholipid content of the plasma membrane (6); thus it is probable that 16:0 is released during intracellular polar lipid remodelling (Table 3). Whether the 16:0 is translocated from the cytosol in the form of TAG in vesicles or droplets or whether the 16:

0 is incorporated into TAG in situ in the plasma membrane is unknown. Extracellular fatty acids incorporated into the cells from the serum that is present during stimulation could also contribute to the plasma membrane TAG.

Increased 16:0 in plasma membrane TAG could also arise from the reversible palmitoylation of proteins. Palmitoyl-CoA:protein S-palmitoyltransferase activity is located in the plasma membrane (23). Stimulation with LPS caused a 2.6-fold increase in membrane protein/lipid ratio. Many of these proteins are inserted in the plasma membrane by their 16:0 lipid tails, e.g., the α subunit of G proteins (23). There is then a turnover of 16:0 in the membrane as tails are attached or detached according to the stimulus (24). While acyl carnitine can act as a fatty acid store in some cell types, e.g., erythrocytes (25), we postulate that plasma membrane TAG could also be a store for 16:0 fatty acid for the reversible acylation–deacylation of proteins and also for 16:0 displaced from phospholipids during lipid remodelling.

Studies with artificial vesicles have shown that TAG molecules can spread and incorporate into phospholipid monolayers at an air–water interface to a finite solubility of about 2–5 mol %. Such TAG molecules are then accessible to lipases in the aqueous environment of plasma and cells (26). It is of interest that the amount of TAG in membranes from both resting and stimulated cells remains constant at 10.6 and 10.7 mol % of the phospholipids, respectively (Table 2), because both TAG and phospholipid increase about 2-fold in membranes from stimulated cells. This ratio is comparable with values obtained for other cell types giving rise to the high resolution lipid NMR signal (1), e.g., leukemic lymphoblasts (9.6, 7.9 mol %), CHO cells (14.0 mol %), and some mammary adenocarcinoma lines (12.5, 9.5 mol %). This suggests that solubility of TAG in the phospholipid bilayers is exceeded, even in the control neutrophils. The excess TAG results in the formation of emulsion particles (27). The presence of higher amounts of the saturated palmitic acid in TAG from stimulated cell membranes then becomes important in accounting for the differences in intensity of the lipid signal observed between the resting and LPS-stimulated neutrophils. First, saturated fatty acids have more methylene protons to contribute to an NMR-visible lipid signal than highly unsaturated fatty acids. In addition, lipoproteins with a high proportion of saturated fatty acids in the TAG have been shown to undergo a crystalline–liquid phase transition at around body temperature, resulting in narrow intense proton NMR resonances from the highly mobile molecules (28). If there was preferential separation of the more saturated TAG into the isotropic liquid phase, this could explain why the membrane TAG from the LPS-stimulated cells does not appear to be more soluble in a bilayer with a decreased cholesterol/phospholipid ratio, as suggested by Hamilton et al, (26). It has been proposed that the excess TAG that phase-separates can form pockets within the interior of the bilayer (27, 29).

Previously we found that in CHO cells increasing ^{1}H-NMR lipid signals coincided with increasing saturation of the acyl chain of plasma membrane esterified cholesterol and decreasing membrane free cholesterol (30). We conclude that stimulation of human neutrophils with LPS results in increased plasma membrane TAG, enriched in 16:0 fatty acid, and that both of these factors contribute to the increased ^{1}H-NMR lipid signal.

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