

Effect of tumor necrosis factor- α on the metabolism of arachidonic acid in human neutrophils

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Abstract Although tumor necrosis factor- α (TNF- α) has been shown to induce marked changes in the physiology/pathophysiology of cells, little is known about the effects of this cytokine on cellular lipid metabolism. In this study we examined the effects of TNF- α on the metabolism of eicosatetraenoic acid (arachidonic acid, 20:4(n-6)) in human neutrophils. Pretreatment of neutrophils with TNF- α caused a rapid increase in the incorporation of [^{1-¹⁴C}]20:4(n-6) substrate into cellular phosphatidylinositol and phosphatidic acid and a slower rise in the incorporation into phosphatidylcholine and phosphatidylethanolamine. Radioactivity was exclusively associated with the *sn*-2 position of each molecule. The labeling pattern of other phospholipids, neutral lipids, and eicosanoids was unchanged. TNF- α had no effect on the distribution of radioactivity in 1-acyl, 1-alkyl, and 1-alk-1-enyl subclasses of phosphatidylcholine, phosphatidylethanolamine, and triglyceride. Chain elongation, β -oxidation, and desaturation of [^{1-¹⁴C}]20:4(n-6) were not modulated by the cytokine. TNF- α stimulated the release of [³H]20:4(n-6) from prelabeled neutrophils and also induced the production of endogenous unesterified 20:4(n-6). Concomitantly, treatment with the cytokine caused a decrease in the mass of cellular phosphatidylinositol, phosphatidylcholine, and phosphatidylethanolamine and an increase in the levels of corresponding lysophospholipids, but had no significant effect on sphingomyelin, phosphatidic acid, diglyceride, and other lipids. TNF- α did not evoke neutrophils prelabeled with [³H]lyso platelet activating factor to produce [³H]phosphatidylethanol, [³H]phosphatidic acid, or [³H]diglyceride in the presence of ethanol, indicating that phospholipases D and C were not activated. Treatment of the leukocytes with the cytokine had no effect on the activity of neutral and acidic sphingomyelinase. **■** These data collectively provide evidence that TNF- α specifically induces the turnover of neutrophil phosphatidylinositol, phosphatidylcholine, and phosphatidylethanolamine which are enriched with 20:4(n-6) by the activation of phospholipase A₂.—Robinson, B. S., C. S. T. Hii, A. Poulos, and A. Ferrante. Effect of tumor necrosis factor- α on the metabolism of arachidonic acid in human neutrophils. *J. Lipid Res.* 1996. **37**: 1234–1245.

Supplementary key words TNF- α • phospholipase A₂ • phospholipase D • phospholipase C • sphingomyelinase

TNF- α is a cytokine that has multiple biological activities (1–7). Its effects are mediated via cell surface recep-

tors which are present on numerous cell types (1, 2). TNF- α is one of the major factors responsible for changes in tissue and organ functions seen during infection, cancer, and autoimmune inflammatory diseases (1–3). Leukocytes are not only major producers of TNF- α but are also prime targets of the cytokine and consequently TNF- α is believed to play a significant role in the mediator-regulator network that operates during cellular inflammation (1–3). This cytokine has been shown to augment neutrophil responses to several agents including FMLP (8–11), TPA (8, 11), and opsonized micro-organisms (12–17).

During the course of the inflammatory response, a variety of mediators are generated that regulate the reaction (18). One important class of mediators is the polyenoic fatty acids, and in particular, 20:4(n-6) (18, 19). 20:4(n-6) has been shown to directly stimulate production of oxygen-derived reactive species (respiratory burst) (20–27), induce degranulation (28–30), alter cell migration/chemotaxis (31), modify cell adhesion to endothelial cells (32), and enhance expression of cell surface receptors on neutrophils (32). Apart from being an important structural component of membranes, 20:4(n-6) acts as a precursor of physiologically active oxygenated metabolites called eicosanoids (33, 34) and plays an obligatory step in platelet activating factor production (35). Because both TNF- α and 20:4(n-6) are present during the same time period of an inflammatory response, the metabolism of 20:4(n-6) in neutrophils could be altered by the presence of TNF- α . In the

Abbreviations: TNF- α , tumor necrosis factor- α ; 20:4(n-6), eicosatetraenoic acid (arachidonic acid); 22:4(n-6), docosatetraenoic acid (adrenic acid); 24:4(n-6), tetracosatetraenoic acid; 19:0, nonadecanoic acid (nonadecylic acid); FMLP, N-formyl-L-methionyl-L-leucyl-L-phenylalanine; TPA, 12-O-tetradecanoylphorbol 13-acetate; HBSS, Hank's balanced salt solution; GLC, gas-liquid chromatography; MS, mass spectrometry.

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present report we studied the effect of TNF- α pretreatment on the incorporation and release of 20:4(n-6) associated with lipids of human neutrophils.

MATERIALS AND METHODS

Materials

Human recombinant TNF- α derived from *E. coli* (6×10^7 U/mg, purity > 99%) was donated by Dr. G. R. Adolf from Boehringer Ingelheim, Vienna, Austria. [$1\text{-}^{14}\text{C}$]20:4(n-6) (55 Ci/mol), [5,6,8,9,11,12,14,15- ^3H]20:4(n-6) (211 Ci/mmol), [^3H]lyso platelet activating factor (1- O -[^3H]octadecyl-*sn*-glycero-3-phosphocholine, 154 Ci/mmol), [*methyl*- ^3H]choline chloride (80 Ci/mmol), and high performance autoradiography film (Hyperfilm- ^3H) were purchased from Amersham Australia Pty. Ltd., North Ryde, NSW, Australia. 20:4(n-6), [$^2\text{H}_8$]20:4(n-6), 19:0, phospholipase A₂ (from

bee venom, *Apis mellifera*), phospholipase C (Type III: from *Bacillus cereus*), calcium ionophore A23187, TPA, BSA (essentially fatty acid-free), 2',7'-dichlorofluorescein, *p*-nitrophenyl phosphate, β -glycerophosphate, phenylmethylsulfonyl fluoride, Na₃VO₄, Na₂MoO₄, ATP, leupeptin, aprotinin, benzamidine, and butylated hydroxytoluene were purchased from Sigma Chemical Co., St. Louis, MO. Silica gel 60 TLC plates (20 cm \times 20 cm \times 0.25 mm) were obtained from E. Merck, Darmstadt, Germany and scintillation cocktail (Opti Phase "Hi Safe" 3) was supplied by LKB Wallac, Turku, Finland. Phosphatidylethanol was prepared according to Kobayashi and Kanfer (36). To prepare [*choline-methyl*- ^3H]sphingomyelin (6 mCi/mmol), HeLa cells (seeded at 0.5×10^6 cells/25 ml Dulbecco's modified Eagle's medium/dish; 10 \times 15 cm dishes) were incubated with [*methyl*- ^3H]choline chloride (25 μCi /dish) at 37°C in air/CO₂ (19:1, v/v) for 8 days. After harvesting the cells, lipids were extracted by the method of Bligh and Dyer (37). All other chemicals were

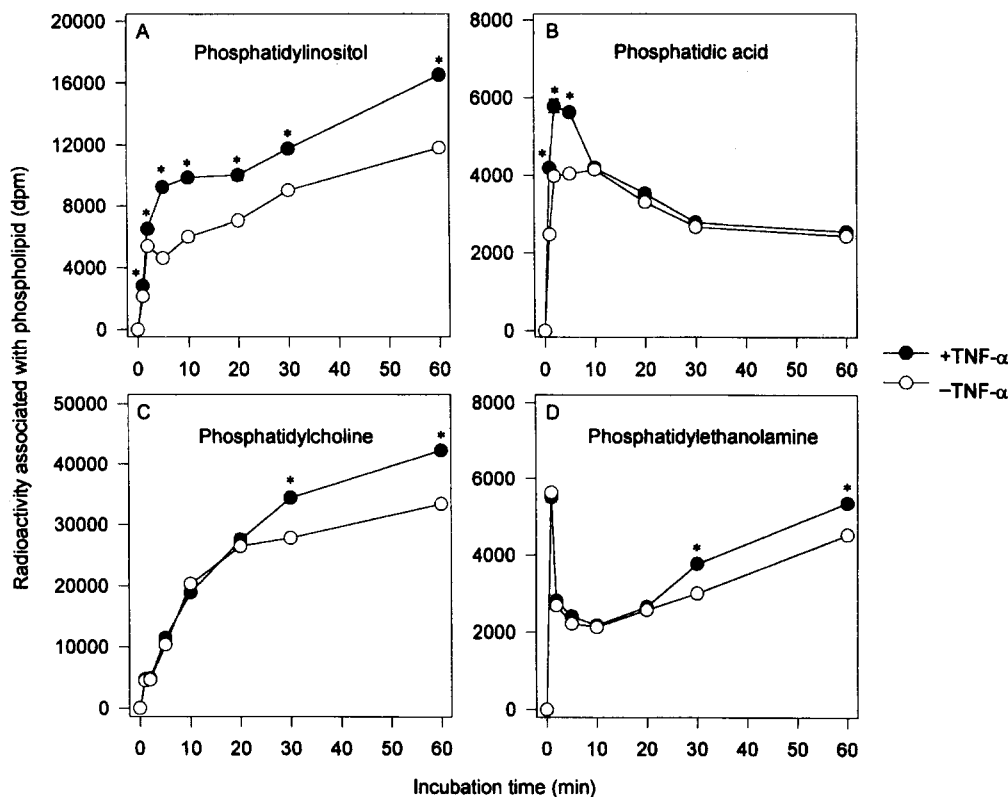


Fig. 1. Effect of TNF- α pretreatment on the incorporation of [$1\text{-}^{14}\text{C}$]20:4(n-6) into neutrophil phosphatidylinositol, phosphatidic acid, phosphatidylcholine, and phosphatidylethanolamine. Neutrophils (10^6) were preincubated with and without 1,000 U TNF- α in 0.4 ml of HBSS at 37°C for 30 min. The cells were then incubated in 1 ml final vol. of HBSS containing [$1\text{-}^{14}\text{C}$]20:4(n-6) (1.1 μCi , 20 μM) at 37°C for up to 60 min. The radioactivity associated with phosphatidylinositol, phosphatidic acid, phosphatidylcholine, and phosphatidylethanolamine of neutrophils was determined as described in the Materials and Methods section. Each point represents the mean \pm SEM for three determinations. This experiment was conducted three times with similar results. * $P < 0.001$, for significant differences between treatments.

of reagent grade. Solvents were distilled prior to use and contained the antioxidant butylated hydroxytoluene (0.005%, w/v).

Preparation of substrates and agonists

TNF- α (concentrated stock in saline stored at -70°C), radiolabeled substrates, calcium ionophore A23187, 20:4(n-6) (concentrated stocks in ethanol stored at -20°C) and TPA (concentrated stock in DMSO stored at -20°C) were diluted with HBSS immediately prior to use. Ethanol or DMSO alone appropriately diluted in HBSS were used as controls. The final concentration of ethanol or DMSO in neutrophil incubations was less than 0.2% (v/v) unless stated otherwise. In the majority of cases, TNF- α was used at a dose of 1,000 U/ 10^6 cells and was scaled up proportionally. TLC/autoradiography indicated that the radiolabeled lipids were of high purity. Agonists and buffer were shown to be free of endotoxin contamination using the Limulus Amebocyte Lysate assay.

Isolation of neutrophils

Human neutrophils were isolated from the peripheral blood of healthy volunteers by the rapid single-step method of Ferrante and Thong (38). The preparation of neutrophils was of >99% purity and >99% viability as judged by morphological examination of cytopsin preparations and the ability of viable cells to exclude Trypan Blue stain. Neutrophils were suspended in HBSS (3.33×10^6 cells/ml) and used within 30 min of preparation. Trypan Blue exclusion indicated that the cells remained viable throughout subsequent incubations.

Studies on the incorporation of [$1\text{-}^{14}\text{C}$]20:4(n-6) into cellular lipids

Neutrophils (10^6) were preincubated with and without 1,000 U TNF- α in 0.4 ml of HBSS at 37°C for 30 min. The cells were then incubated in 1 ml final volume of HBSS containing [$1\text{-}^{14}\text{C}$]20:4(n-6) (1.1 μCi , 20 μM) at 37°C for up to 60 min. In some experiments, [^3H]20:4(n-6) (1.1 μCi , 1 μM) was used instead of [$1\text{-}^{14}\text{C}$]20:4(n-6). The reaction was terminated by the addition of 3.75 ml of chloroform-methanol-acetic acid 1:2:0.02 (by vol) (39). The mixture was left at 4°C overnight and subsequently partitioned by the addition of 1.25 ml of chloroform and 1.25 ml of water (37).

Radioactivity associated with lipid classes was determined as follows. A portion of the lipid phase was applied to a silica gel 60 TLC plate impregnated with magnesium acetate (1 g/plate) and developed in the first dimension in chloroform-methanol-28% (w/w) ammonia 13:7:1 (by vol) and then in the second dimension in

chloroform-acetone-methanol-acetic acid-water 10:4:2:2:1 (by vol) to separate phospholipids (40). A fraction of the lipid phase was applied to a silica gel 60 TLC plate and developed half-way in hexane-diethyl ether-acetic acid 60:40:1 (by vol) and then to the top in hexane-diethyl ether-acetic acid 90:10:1 (by vol) to resolve neutral lipids. In some analyses, eicosanoids (oxygenated 20:4(n-6) derivatives), phospholipids, and neutral lipids were separated on silica gel TLC plates in diethyl ether-hexane-acetic acid 60:40:1 (by vol) or the organic phase of ethyl acetate-iso-octane-acetic acid-water 11:5:2:10 (by vol) (41). The lipid zones were located with I_2 vapor or by preparing autoradiographs, scraped into scintillation vials, and the radioactivity was measured after adding water (0.5 ml) and scintillation cocktail (8 ml) with a liquid scintillation counter (Model 1409 set with external standardization and automatic efficiency control to correct for quenching; Wallac, Turku, Finland). Identification of the lipids was based on a comparison of their TLC mobility with that of authentic unlabeled standards.

Phosphatidylinositol, phosphatidic acid, phosphatidylcholine, phosphatidylethanolamine, and a total neutral lipid fraction were resolved by two-dimensional TLC as outlined above. The lipid areas were located under ultraviolet light after spraying the plates with 0.2% (w/v) dichlorofluorescein in 95% (v/v) ethanol and eluted from the silica gel with two 4-ml volumes of

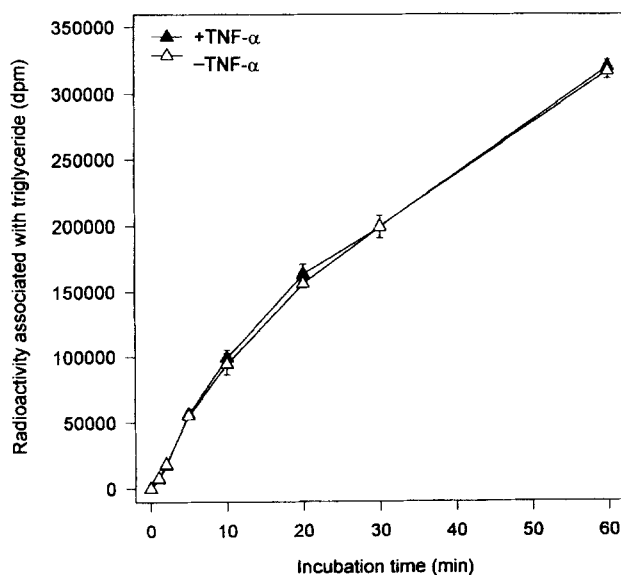


Fig. 2. Effect of TNF- α pretreatment on the incorporation of [$1\text{-}^{14}\text{C}$]20:4(n-6) into neutrophil triglyceride. Neutrophils were incubated as described for Fig. 1. The radioactivity associated with triglyceride of neutrophils was determined as described in the Materials and Methods section. Each point represents the mean \pm SEM for three determinations. This experiment was performed three times with similar results.

TABLE 1. Effect of TNF- α pretreatment on the incorporation of [^3H]20:4(n-6) into neutrophil phosphatidylinositol, phosphatidic acid, phosphatidylcholine, phosphatidylethanolamine, and triglyceride

Lipid	Incubation Time <i>min</i>	Treatment	
		-TNF- α <i>radioactivity associated with lipid (dpm)</i>	+TNF- α
PI	5	2,919 \pm 93	5,575 \pm 40 ^c
	30	4,142 \pm 90	6,327 \pm 28 ^c
PA	5	3,028 \pm 97	4,686 \pm 33 ^c
	30	1,771 \pm 38	1,824 \pm 12
PC	5	6,779 \pm 216	7,770 \pm 56 ^a
	30	16,915 \pm 368	21,029 \pm 93 ^b
PE	5	1,919 \pm 93	2,020 \pm 65
	30	5,450 \pm 118	5,961 \pm 24 ^a
TG	5	35,008 \pm 1,117	34,251 \pm 244
	30	154,228 \pm 3,356	155,467 \pm 2,784

Neutrophils (10^6) were preincubated with and without 1,000 U TNF- α in 0.4 ml of HBSS at 37°C for 30 min. The cells were then incubated in 1 ml final vol of HBSS containing [^3H]20:4(n-6) (1.1 μCi , 1 μM) at 37°C for 5 or 30 min. The radioactivity associated with phosphatidylinositol (PI), phosphatidic acid (PA), phosphatidylcholine (PC), phosphatidylethanolamine (PE), and triglyceride (TG) of neutrophils was determined as described in Materials and Methods. Each point represents the mean \pm SEM for three determinations. This experiment was repeated once with similar results.

^aP < 0.05; ^bP < 0.001; ^cP < 0.0001 for significant differences between treatments.

chloroform-methanol-acetic acid-water 50:39:1:10 (by vol) (42). The resulting extracts were partitioned with 2 ml of 1 M NH₄OH to remove the dye and then washed with 2 ml of methanol-water 1:1 (v/v). The positional distribution of [^{14}C]20:4(n-6) in purified phosphatidylinositol, phosphatidic acid, phosphatidylcholine, and phosphatidylethanolamine was assessed by degrading the compounds with phospholipase A₂ and measuring the radioactivity of the reaction products (unesterified fatty acids and lysophospholipids) after isolation by TLC (43). Radioactivity incorporated into 1-acyl, 1-alkyl, and 1-alk-1-enyl subclasses of phosphatidylcholine and phosphatidylethanolamine was measured as described previously (44, 45). In brief, these phospholipids were hydrolyzed with phospholipase C and the resulting diglycerides were acetylated. 1-Acyl, 1-alkyl, and 1-alk-1-enyl subclasses of the acetylated diglycerides were then separated by TLC and radioactivity was determined. The purified total neutral lipid fraction was applied to a silica gel 60 TLC plate and developed twice in the same direction in hexane-diethyl ether 9:1 (v/v) to resolve

triglyceride subclasses (46). Authentic standards were co-chromatographed to assist identification of the triglyceride species. Only 1-acyl and 1-alkyl triglycerides were detected and their radioactivity was quantitated. The identity of the 1-acyl and 1-alkyl triglyceride subclasses was confirmed by mild alkaline and acid hydrolysis of fractions and analysis of the products (47).

Radioactivity associated with various types of esterified and unesterified fatty acids was determined by the procedure of Robinson, Johnson, and Poulos (48) to provide information about the elongation, desaturation, and β -oxidation of [^{14}C]20:4(n-6). Briefly, a portion of the lipid phase was transesterified with H₂SO₄ in methanol and the radioactivity of the resulting fatty acid methyl esters was determined after separation according to degree of unsaturation and carbon chain length by argentation and reversed-phase TLC.

Studies on the cellular uptake of [^{14}C]20:4(n-6)

Neutrophils were incubated as described for studies on the incorporation of [^{14}C]20:4(n-6) into cellular lipids. The incubate was immediately centrifuged at 14,000 *g* for 10 sec at 4°C and the medium was removed. The cell pellet was resuspended in 0.5 ml of HBSS and recentrifuged. The cells were taken up in 1 ml of HBSS and sonicated for 30 sec using a Ystrom Systems ultrasonicator (Westwood, NJ; power setting 8, tune setting 4). The cell sonicate and combined medium and cell wash were placed in scintillation vials along with 8 ml of scintillation cocktail, and the radioactivity was measured.

Determination of cellular lipid mass

Neutrophils (5×10^7) were incubated with and without 50,000 U TNF- α in 20 ml of HBSS at 37°C for 60 min. The incubate was centrifuged at 600 *g* for 5 min at room temperature and the medium was discarded. The cell pellet was resuspended in 1 ml of water and the lipids were extracted and resolved by TLC as outlined above. Phospholipids were located with I₂ vapor, digested with HClO₄ (in the presence of silica gel), and the phosphorus content was measured (49). Neutral lipids were located under ultraviolet light after spraying the plates with dichlorofluorescein and eluted from the silica gel as described above. The neutral lipid samples were transesterified and the resulting fatty acid methyl esters were quantitated by GLC using 19:0 (50 nmol) as a reference standard (43).

Measurement of phospholipase A₂ activity

Phospholipase A₂ activity in intact neutrophils was assessed by examining the release of [^3H]20:4(n-6) from prelabeled cells (50-52). Neutrophils (6×10^7) were

TABLE 2. Effect of TNF- α pretreatment on the incorporation of [^{14}C]20:4(n-6) into phosphatidylcholine, phosphatidylethanolamine, and triglyceride subclasses of neutrophils.

Lipid Subclass	Recovered Radioactivity (% of total)					
	-TNF- α			+TNF- α		
	PC	PE	TG	PC	PE	TG
1-Acyl	87.8 \pm 0.9	46.2 \pm 1.3	96.9 \pm 0.2	86.5 \pm 1.6	48.3 \pm 0.7	96.4 \pm 0.2
1-Alkyl	10.9 \pm 0.7	12.8 \pm 0.5	3.1 \pm 0.2	12.0 \pm 1.3	12.5 \pm 0.4	3.6 \pm 0.2
1-Alk-1-enyl	1.3 \pm 0.1	41.0 \pm 0.9	ND	1.5 \pm 0.2	39.2 \pm 1.1	ND

Neutrophils were incubated as described for Fig. 1. The radioactivity associated with phosphatidylcholine (PC), phosphatidylethanolamine (PE), and triglyceride (TG) subclasses of neutrophils was determined as described in Materials and Methods. 1-Acyl, 1-alkyl, and 1-alk-1-enyl represent an acyl, alkyl or alk-1-enyl linkage, respectively, at the *m*-1 position of the lipid. Each value is the mean \pm SEM of three analyses. This experiment was conducted twice with similar results. ND, not detectable.

incubated with [^3H]20:4(n-6) (10 μCi , 24 nM) in 2 ml of HBSS at 37°C in air-CO₂ 19:1 (v/v) for 60 min. The cells were washed three times with 10 ml of HBSS containing BSA (0.001%, w/v), once with 10 ml of HBSS, and finally resuspended in HBSS (3.33 \times 10⁶ cells/ml). Approximately 80% of the radiolabeled fatty acid substrate was taken up by the cells of which >98% was esterified in lipids. [^3H]20:4(n-6) labeled neutrophils (10⁶) were incubated with and without agonists in 1 ml of HBSS at 37°C for up to 60 min. The incubation mixture was immediately centrifuged at 14,000 *g* for 10 sec at 4°C and the radioactivity of the medium was measured. In some experiments lipids were extracted from the medium and cell pellet and subjected to TLC/autoradiography as described above for studies on the incorporation of [^{14}C]20:4(n-6) into cellular lipids.

Phospholipase A₂ activity was also evaluated by quantitating the mass of unesterified 20:4(n-6) produced by the cells. Neutrophils (4 \times 10⁶) were incubated in the presence and absence of agonists in 4 ml of HBSS at 37°C for 15 min. [$^2\text{H}_8$]20:4(n-6) (2.5 nmol) was added to the incubate as a reference standard and lipids were extracted by the addition of 15 ml of chloroform-methanol-acetic acid 1:2:0.02 (by vol) followed by 5 ml of chloroform and 5 ml of water. Lipids were separated by TLC using the solvent system diethyl ether-hexane-acetic acid 60:40:1 (by vol). The free fatty acid zone was located by reference to co-chromatographed standards exposed to I₂ vapor and eluted from the silica gel with four 3-ml volumes of chloroform-methanol 2:1 (v/v). The recovered fatty acids were converted to methyl esters with ethereal diazomethane. GLC-MS of fatty acid methyl esters was performed on a JEOL JMS-DX303 mass spectrometer operating in electron-impact mode and coupled with a Hewlett-Packard 5890A gas chromatograph and a JEOL JMA-DA5000 data system. Chromatographic separation was achieved with a 25 m \times 0.22 mm internal diameter fused-silica BP-1 column of 0.25 μm phase thickness (Scientific Glass Engineering, Ringwood, Victoria, Australia) temperature programmed from 160-320°C at 10°C/min. The injector

and ion-source temperatures were 270 and 250°C, respectively. He gas (1 ml/min) was the carrier and the ionization voltage was 70 eV. Molecular ions were monitored for 20:4(n-6) methyl ester (*m/z* 318) and [$^2\text{H}_8$]20:4(n-6) methyl ester (*m/z* 326). The absolute amount of 20:4(n-6) was determined by comparison of the signal intensity for 20:4(n-6) methyl ester relative to that of the known amount of [$^2\text{H}_8$]20:4(n-6) methyl ester reference standard. Corrections were made for the reference standard background and for the relative response ratio by analyzing [$^2\text{H}_8$]20:4(n-6) methyl ester alone and a 1:1 molar mixture of 20:4(n-6) and [$^2\text{H}_8$]20:4(n-6) methyl esters, respectively, under the same conditions.

Assessment of phospholipase D and phospholipase C activity

Phospholipase D and phospholipase C activity was assayed by measuring the formation of [^3H]phosphatidylethanol, [^3H]phosphatidic acid, and [^3H]diglyceride in cells prelabeled with [^3H]lyso platelet activating factor (based on the procedure of P \acute{e} rianin et al. (53)). Neutrophils (5 \times 10⁷) were incubated with [^3H]lyso platelet activating factor (15 μCi , 49 nM) in 2 ml of Ca²⁺- and Mg²⁺-free HBSS at 37°C in air-CO₂ 19:1 (v/v) for 75 min. The cells were washed four times with 10 ml of Ca²⁺- and Mg²⁺-free HBSS and finally resuspended in

TABLE 3. Effect of TNF- α pretreatment on the elongation of [^{14}C]20:4(n-6) by neutrophils

Fatty Acid	Recovered Radioactivity (% of total)	
	-TNF- α	+TNF- α
20:4(n-6)	95.3 \pm 0.1	95.4 \pm 0.1
22:4(n-6)	4.4 \pm 0.2	4.3 \pm 0.2
24:4(n-6)	0.3 \pm 0.1	0.3 \pm 0.1

Neutrophils were incubated as described for Fig. 1. The radioactivity associated with neutrophil tetraenoic fatty acids was determined as described in the Materials and Methods section. Each value is the mean \pm SEM of three analyses. This experiment was repeated twice with similar results.

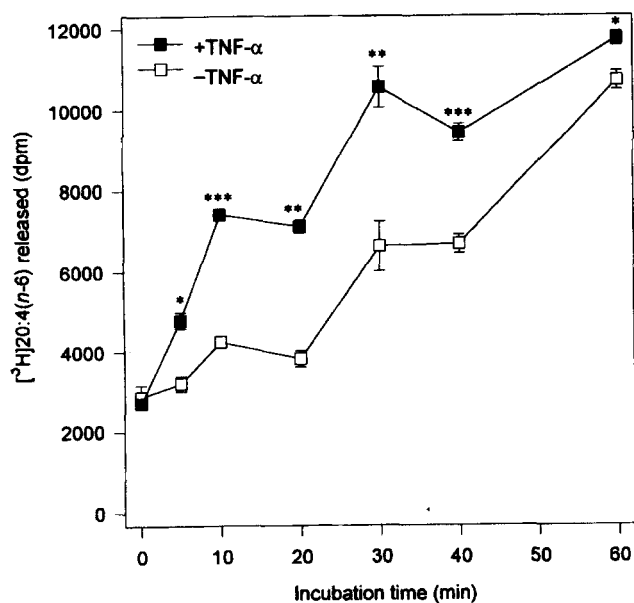


Fig. 3. Effect of TNF- α on the release of [^3H]20:4(n-6) from pre-labeled neutrophils. Neutrophils (10^6 , pre-labeled with [^3H]20:4(n-6)) were incubated with and without 1,000 U TNF- α in 1 ml of HBSS at 37°C for up to 60 min and the radioactivity of the medium was measured as described in the Materials and Methods section. Each point represents the mean \pm SEM for three determinations. This experiment was repeated four times with similar results. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, for significant differences between treatments.

HBSS at 3.33×10^6 cells/ml. Under these conditions, approximately 75% of the radiolabeled substrate became cell associated, of which >95% was present in phosphatidylcholine (1-alkyl). Pre-labeled neutrophils (10^6) were incubated with and without agonists in 1 ml

of HBSS containing ethanol (0.5%, v/v) at 37°C for 15 min. Lipids were extracted (as outlined above for studies on the incorporation of [^{14}C]20:4(n-6) into cellular lipids), separated by TLC (organic phase of the solvent system ethyl acetate-*iso*-octane-acetic acid-water 13:2:3:10 (by vol) for phosphatidylethanol and phosphatidic acid; solvent system hexane-diethyl ether-methanol-acetic acid 65:35:4.5:2 (by vol) for diglyceride), and the radioactivity was determined. Identification of the lipids was achieved by reference to co-chromatographed standards.

Measurement of sphingomyelinase activity

The micellar sphingomyelinase assay using exogenous [*choline-methyl*- ^3H]sphingomyelin was conducted according to Wiegmann et al. (54) with some modifications. Neutrophils (3×10^6) were incubated with and without 300–3,000 U TNF- α in 3 ml of HBSS at 37°C for 10 min. The treatment was terminated by immersing the incubate in a methanol/dry ice bath for 5 sec. After centrifugation at 600 *g* for 5 min at 4°C, the cell pellet was washed twice with 3 ml of ice-cold HBSS. To measure neutral sphingomyelinase activity, cell pellets were resuspended in 0.2 ml of lysis buffer containing 20 mM HEPES (pH 7.4), 10 mM MgCl₂, 2 mM EDTA, 5 mM dithiothreitol, 38 mM *p*-nitrophenyl phosphate, 10 mM β -glycerophosphate, 1 mM phenylmethylsulfonyl fluoride, 100 μM Na₃VO₄, 100 μM Na₂MoO₄, 750 μM ATP, 20 μM leupeptin, 5 μM aprotinin, 64 μM benzamide, and 0.2% (v/v) Triton X-100. After 15 min at 4°C, the cells were homogenized by repeated aspiration using a 0.2-ml pipette and then centrifuged at 800 *g* for 7 min

TABLE 4. Effect of TNF- α on neutrophil phospholipid levels

Phospholipid	Treatment	
	-TNF- α	+TNF- α
	<i>amount of phospholipid (nmol/5 $\times 10^7$ cells)</i>	
Lysophosphatidylinositol	1.1 \pm 0.3	3.1 \pm 0.5 ^a
Lysophosphatidic acid	ND	ND
Lysophosphatidylcholine	ND	0.3 \pm 0.1 ^a
Lysophosphatidylethanolamine	ND	0.3 \pm 0.1 ^a
Phosphatidylinositol	19.8 \pm 0.9	16.5 \pm 0.7 ^a
Phosphatidic acid	1.3 \pm 0.5	1.8 \pm 0.3
Phosphatidylserine	39.3 \pm 0.9	38.3 \pm 1.2
Sphingomyelin	34.4 \pm 0.8	35.0 \pm 0.9
Phosphatidylcholine	132.5 \pm 2.3	119.8 \pm 0.7 ^b
Phosphatidylethanolamine	131.0 \pm 3.4	118.8 \pm 0.8 ^a
Phosphatidylglycerol	3.1 \pm 0.4	3.1 \pm 0.0

Neutrophils (5×10^7) were incubated with and without 50,000 U TNF- α in 20 ml of HBSS at 37°C for 60 min. Cellular phospholipid levels were determined as described in Materials and Methods. Each value is the mean \pm SEM of three analyses. This experiment was repeated once with similar results. ND, not detectable.

^a $P < 0.05$; ^b $P < 0.01$ for significant differences between treatments.

TABLE 5. Effect of TNF- α on the production of [^3H]phosphatidylethanol, [^3H]phosphatidic acid, and [^3H]diglyceride in neutrophils prelabeled with [^3H]lyso platelet activating factor

Lipid Product	Treatment		
	-Agonist	+TNF- α	+TPA
	<i>radioactivity (dpm/10⁶ cells)</i>		
Phosphatidylethanol	278 \pm 64	376 \pm 28	6,076 \pm 24 ^a
Phosphatidic acid	310 \pm 56	416 \pm 58	1,571 \pm 73 ^a
Diglyceride	2,651 \pm 28	2,588 \pm 55	6,688 \pm 89 ^a

Neutrophils (10⁶, prelabeled with [^3H]lyso platelet activating factor) were incubated with and without 1,000 U TNF- α or 0.1 μM TPA in 1 ml of HBSS containing ethanol (0.5%, v/v) at 37°C for 15 min. The radioactivity associated with phosphatidylethanol, phosphatidic acid, and diglyceride of neutrophils was measured as described in the Materials and Methods section. Each value is the mean \pm SEM of three determinations. This experiment was repeated three times with similar results.

^aP < 0.001, for significant differences between +TPA and -agonist.

at 4°C. Aliquots of the supernatants (10 μl , containing approximately 10 μg of protein) were incubated with [*choline-methyl*- ^3H]sphingomyelin (0.02 μCi , 67 μM) in 50 μl final volume of 20 mM HEPES, 1 mM MgCl₂ buffer (pH 7.4) at 37°C for 120 min. The amount of [^3H]phosphocholine liberated from the [^3H]sphingomyelin substrate was assessed by extracting with 0.8 ml of chloroform-methanol 2:1 (v/v) and 0.25 ml of water and measuring the radioactivity in the aqueous phase. In some cases the aqueous phase was subjected to TLC (solvent system methanol-1.2% (w/v) NaCl-28% (w/w) ammonia: 10:10:1, by vol) and [^3H]phosphocholine was found to account for >95% of the total recovered radioactivity. To measure acidic sphingomyelinase activity, washed cell pellets were resuspended in 0.2 ml of 0.2% (v/v) Triton X-100. After 15 min at 4°C, the cells were homogenized and centrifuged at 14,000 *g* for 2 min at 4°C. Supernatant aliquots were incubated with radiolabeled sphingomyelin in 50 μl final volume of 250 mM sodium acetate, 1 mM EDTA buffer (pH 5.0) and the amount of [^3H]phosphocholine produced was determined as described for the neutral sphingomyelinase assay.

Statistical analyses

Results are expressed as mean \pm SEM. Statistical analyses were performed by a two-tailed Student's *t*-test for unpaired data. Values of *P* < 0.05 were considered significant.

RESULTS AND DISCUSSION

After neutrophils were incubated with [^{14}C]20:4 (n-6) for up to 60 min, radioactivity was predominantly recovered in triglyceride, with smaller amounts in other neutral lipids, phospholipids, and eicosanoids. Pretreatment of neutrophils with TNF- α for 30 min increased the subsequent incorporation of [^{14}C]20:4(n-6) into

phosphatidylinositol throughout a 60-min labeling period (Fig. 1A) and into phosphatidic acid during the first 10 min (Fig. 1B). The cytokine enhanced the incorporation of the radiolabeled fatty acid into phosphatidylcholine (Fig. 1C) and phosphatidylethanolamine (Fig. 1D) between 20 and 60 min. The radioactivity recovered in phosphatidylinositol, phosphatidic acid, phosphatidylcholine, and phosphatidylethanolamine was found to be almost exclusively associated with the *sn*-2 position of the molecule with and without cytokine pretreatment. TNF- α had no significant effect on the incorporation of [^{14}C]20:4(n-6) into other neutrophil phospholipids (sphingomyelin, phosphatidylserine, and phosphatidylglycerol) over 60 min (results not shown). In addition, TNF- α did not influence the labeling of triglyceride (Fig. 2), other neutral lipids (monoglyceride, diglyceride, and cholesterol esters), and eicosanoids (mainly monohydroxylated 20:4(n-6) derivatives) (results not shown) during this time. Preincubation of neutrophils with TNF- α for 30 min slightly inhibited the subsequent uptake of [^{14}C]20:4(n-6) over a 60-min period (results not shown). This observation eliminated the possibility that TNF- α enhancement of [^{14}C]20:4(n-6) incorporation into certain phospholipids was due to the cytokine increasing the amount of fatty acid associated with the cells. The effects of TNF- α on [^{14}C]20:4(n-6) incorporation into neutrophil lipids were obtained with a 20:4(n-6) concentration of 20 μM . When a lower concentration of 20:4(n-6) was used ([^3H]20:4(n-6) at 1 μM), TNF- α exerted effects similar to those observed above on the incorporation of label into neutrophil phosphatidylinositol, phosphatidic acid, phosphatidylcholine, phosphatidylethanolamine, and triglyceride (Table 1).

After the leukocytes were incubated with [^{14}C]20:4(n-6) for 60 min, the proportion of radioactivity associated with phosphatidylcholine and triglyceride subclasses was in the order 1-acyl > 1-alkyl > 1-alk-1-enyl and with phosphatidylethanolamine subclasses in the order 1-acyl > 1-alk-1-enyl > 1-alkyl. Preincubation of the

cells with TNF- α for 30 min did not modulate the distribution of radioactivity in these lipid subclasses (Table 2). Table 3 shows that the cells chain elongated a small portion of the [$1\text{-}^{14}\text{C}$]20:4(n-6) substrate to form radiolabeled 22:4(n-6) and 24:4(n-6) after 60 min that was not modified by TNF- α preincubation for 30 min. The neutrophils were found to have a very low capacity to convert [$1\text{-}^{14}\text{C}$]20:4(n-6) to labeled saturated, monoenoic, dienoic, and trienoic fatty acids by β -oxidation and to labeled pentaenoic fatty acids by desaturation, and again this was not affected by the cytokine (results not shown). It is evident that TNF- α only affects the incorporation of [$1\text{-}^{14}\text{C}$]20:4(n-6) into neutrophil phosphatidylinositol, phosphatidic acid, phosphatidylcholine, and phosphatidylethanolamine (phospholipids known to be enriched with 20:4(n-6) (45, 55)) and not other metabolic processes.

We investigated whether TNF- α enhances the degradation of neutrophil lipids. Figure 3 indicates that TNF- α promoted the release of [^3H]20:4(n-6) from prelabeled neutrophils over a 60-min period. Calcium ionophore A23187 was used as a positive control and released substantially more [^3H]20:4(n-6) from the cells after 20 min compared to TNF- α (data not shown). The calcium ionophore stimulated the production of [^3H]eicosanoids (predominantly monohydroxylated 20:4(n-6) and leukotriene B₄) whereas TNF- α had no effect (data not shown). After neutrophils were challenged with TNF- α or calcium ionophore A23187 for 15 min, there was a significant increase in the mass of unesterified 20:4(n-6) (6 ± 3 , $50 \pm 7^*$ and $720 \pm 80^{**}$ pmol 20:4(n-6)/ 4×10^6 cells incubated in 4 ml of HBSS at 37°C for 15 min with no agonist, 4,000 U TNF- α and 5 μM calcium ionophore A23187, respectively; mean \pm SEM of three analyses; $*P < 0.01$, $**P < 0.001$, for significant differences between +agonist and -agonist). The calcium ionophore induced a much greater increase in the level of 20:4(n-6) compared to TNF- α , which was consistent with the [^3H]20:4(n-6) release studies. It should be noted that the release of 20:4(n-6) from neutrophils treated with TNF- α was several-fold higher when measured by mass compared to when it was measured radiometrically. Ramesha and Taylor (56) reported that the radiometric method underestimates the production of 20:4(n-6) from stimulated human neutrophils and platelets. While the radiometric assay measures only the release of exogenously incorporated radioactive 20:4(n-6), the determination of 20:4(n-6) mass accounts for release from all of the endogenous pools. Table 4 shows that incubation of neutrophils with TNF- α for 60 min caused a decrease in the mass of cellular phosphatidylinositol, phosphatidylcholine, and phosphatidylethanolamine and an increase in the amount of cellular lysophosphatidylinositol, lysophos-

phatidylcholine, and lysophosphatidylethanolamine. The cytokine had no significant effect on the level of other neutrophil phospholipids (lysophosphatidic acid, phosphatidic acid, sphingomyelin, phosphatidylserine, and phosphatidylglycerol) (Table 4) and neutral lipids (triglyceride, monoglyceride, diglyceride, and cholesterol esters) (results not shown). Collectively, these results provide definitive evidence that TNF- α induces the breakdown of endogenous phospholipids enriched with 20:4(n-6) by the activation of phospholipase A₂. Several investigators previously reported that TNF- α does not activate phospholipase A₂ in neutrophils (8, 57, 58), while Atkinson et al. (10) observed stimulation of the enzyme. Interestingly, Bauldry et al. (57) found that TNF- α had negligible direct effect on neutrophil phospholipase A₂; however, it primed the cells for enhanced activation of the enzyme in response to FMLP. This discrepancy may be due to different cell isolation techniques and degree of assay sensitivity. It is likely that TNF- α enhancement of [$1\text{-}^{14}\text{C}$]20:4(n-6) (or [^3H]20:4(n-6)) incorporation into phosphatidylinositol, phosphatidic acid, phosphatidylcholine, and phosphatidylethanolamine of neutrophils (observed above) is due to the cytokine increasing the release of endogenous 20:4(n-6) from these phospholipids by phospholipase A₂ stimulation. As a consequence of TNF- α action, greater amounts of cellular lysophospholipids would be available for acylation with radiolabeled 20:4(n-6).

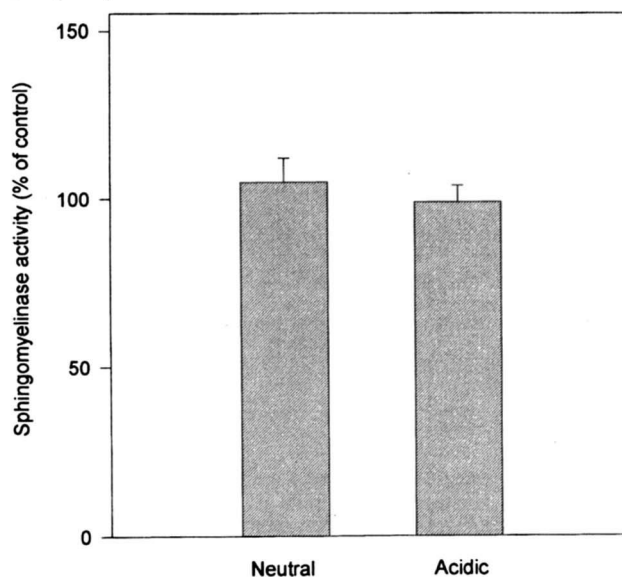


Fig. 4. Effect of TNF- α on sphingomyelinase activity in neutrophils. Neutrophils (3×10^6) were incubated with and without 3,000 U TNF- α in 3 ml of HBSS at 37°C for 10 min. Neutral and acidic sphingomyelinase activity was determined as described in the Materials and Methods section. Each point represents the mean \pm SEM for three determinations. This experiment was performed three times with similar results. Comparable data were obtained when the cells were incubated with lower levels of TNF- α (300 and 1,000 U).

It is important to note that the cytokine did not modulate the mass of phosphatidic acid or diglyceride in the cells (Table 4), which suggests that phospholipases D and C were not activated. TNF- α did not induce neutrophils prelabeled with [³H]lyso platelet activating factor to form [³H]phosphatidylethanol, [³H]phosphatidic acid, or [³H]diglyceride in the presence of ethanol (Table 5), providing additional evidence that phospholipases D and C were not activated. Conversely, TPA elicited substantial phospholipase D and possibly phospholipase C activation in these cells (Table 5). Our finding that TNF- α does not activate neutrophil phospholipase D and phospholipase C is consistent with previous reports (10, 59, 60). Schütze et al. (61) have demonstrated that TNF- α activates a phosphatidylcholine-specific phospholipase C in the human leukemia cell line U937, however this does not appear to exist in human neutrophils. Treatment of the leukocytes with TNF- α did not alter the mass of sphingomyelin (Table 4) or ceramide (results not shown), indicating that sphingomyelinase was not activated. This was confirmed by the finding that the cytokine had no significant effect on the activity of neutral and acidic sphingomyelinase (Fig. 4). TNF- α has been shown to activate sphingomyelinase to release C₂-ceramide in human leukemia HL-60 cells (62–64), but our results suggest that it does not operate in neutrophils. Yanaga and Watson (65) have also recently demonstrated that TNF- α does not evoke sphingomyelinase activation in human neutrophils. It is apparent that the action of TNF- α involves several signalling pathways that vary between cell types.

The products of phospholipase A₂ action on phospholipids (such as 20:4(n-6), lysophosphatidylcholine, and lysophosphatidylinositol) have been found to prime neutrophils for enhanced responses to FMLP and PMA (66–68). This raises the possibility that the mechanism of TNF- α priming of neutrophils may be related, at least in part, to its ability to activate phospholipase A₂. Ca²⁺-dependent and -independent forms of phospholipase A₂ have been identified in human neutrophils (69–74). In view of the fact that TNF- α does not mobilize Ca²⁺ in leukocytes (57, 75, 76), it may modulate phospholipase A₂ with low Ca²⁺ requirement. The signal transduction processes involved in the activation of leukocyte phospholipase A₂ by TNF- α and the characteristics of the enzyme(s) need to be elucidated. In addition, it remains to be determined how the products of phospholipase A₂ activation prime neutrophils at the molecular level. There is evidence that 20:4(n-6) modulates the action of GTP-binding proteins in human neutrophils and may thereby act as a second messenger in cellular priming and activation (77–79). ■

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