Tumor necrosis factor-induced release of endogenous fatty acids analyzed by a highly sensitive high-performance liquid chromatography method

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Abstract A highly sensitive method to determine agonist-induced release of endogenous fatty acids from cells in culture was developed using high-performance liquid chromatography and fluorescence detection. Fatty acids were selectively derivatized with 1-pyrenyldiazomethane and separated on a LC_{18} reversed phase column using an acetonitrile–water gradient. The detection limit was approx. 20 fmol and the recovery of the complete method using oleic acid was 93–98%. Tumor necrosis factor alpha (TNF-α) increased the extracellular release of endogenous arachidonic acid (20:4n–6) from 21 to 153 pmol/well per 4 h using 2.7×10^6 WEHI fibrosarcoma cells. In cells preincubated with 50 μM 20:4n–6, the corresponding figures were 463 and 3379 pmol 20:4n–6/well. Simultaneously, nearly equimolar amounts of 22:4n–6 were released together with slightly lower amounts of 24:4n–6, 16:0, 16:1n–9, and 18:1n–9. Analysis of cell lipid fatty acids showed that phosphatidylcholine was the major source of the released fatty acids. TNF-α increased the intracellular concentration of unesterified 20:4n–6 and 22:4n–6 by 368% and 451%, respectively. This suggests that released 20:4n–6 is rapidly chain elongated to 22:4n–6. The results indicate that the present method facilitates studies on agonist-induced release of endogenous fatty acids, and that TNF-induced fatty acid release seems to be less selective for 20:4n–6 than previously reported.—**Brekke, O-L., E. Sagen, and K. S. Bjerve.** Tumor necrosis factor-induced release of endogenous fatty acids analyzed by a highly sensitive high-performance liquid chromatography method. *J. Lipid Res.* 1997. **38:** 1913–1922.

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The mononuclear cell-derived cytokine tumor necrosis factor (TNF) activates phospholipase A_2 (PLA_2) which plays a crucial role in TNF-induced cytotoxicity (1). PLA_2 hydrolyzes glycerophospholipids with a consequent release of lysophospholipids and fatty acids with potent biological functions. Arachidonic acid itself is probably a second messenger in signal transduction (2), and released fatty acids may therefore either have direct effects or be precursors for eicosanoids such as prostaglandins and leukotrienes. Prostaglandin E_2 has previously been shown to enhance TNF-induced cytotoxicity (3), and to modulate TNF-induced cell growth and TNF-induced gene expression (4). Enriching the target cells with specific, unsaturated fatty acids has also been shown to enhance TNF-induced cytotoxicity (5, 6).

We have previously shown that TNF-induced cytotoxicity in WEHI 164 fibrosarcoma cells is associated with enhanced release of 20:4n–6 (7), probably due to stimulation of PLA_2. Furthermore, several other unsaturated fatty acids increased the susceptibility to TNF similarly to 20:4n–6 (6). It was therefore of interest to study whether TNF also stimulated the release of these and other endogenous ω–6 and ω–3 fatty acids. Previous studies on TNF-induced extracellular release of fatty acids have only used radiolabeled fatty acids (1, 5, 7), as the mass of free fatty acid (FFA) released is small and therefore difficult to measure. Phorbol ester-induced release of endogenous fatty acids has also been examined in U937 cells using gas–liquid chromatography (GLC) (8). A luminometric method with sensitivity down to 10 pmol was used to study the total amount of fatty acids released in cultured cells (9), but such methods gave no information on individual fatty acid release. As highly sensitive GLC analysis using flame-ionization

Abbreviations: HAc, acetic acid; BSA, bovine serum albumin; DEE, diethyl ether; FAME, fatty acid methyl esters; FCS-M, RPMI-1640 containing 10% (v/v) fetal bovine serum, 2 mM L-glutamine, and 40 mg/l gentamicin sulfate; FFA, free fatty acids; GLC, gas–liquid chromatography; HPLC, high-performance liquid chromatography; I.S., internal standard; PC, glycerophosphatidylcholine; PDAM, 1-pyrenyldiazomethane; PE, glycerophosphatidylethanolamine; PI, glycerophosphatidylinositol; PLA_2, phospholipase A_2; rTNF-α, recombinant tumor necrosis factor-alpha; TG, triglyceride; TLC, thin-layer chromatography; WEHI, WEHI 164 clone 13 cells.

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detectors may be disturbed by carbon-containing contaminants, we looked for a sensitive high-performance liquid chromatography (HPLC) method to measure release of individual FFA in cultured cells.

Several reagents have been used to derivatize carboxylic acids before analysis by HPLC. Some agents such as 1-bromoacetylpyrene (10) require a base catalyst and heating, which may initiate oxidation of polyunsaturated fatty acids. Aryldiazoalkanes such as ADAM (11) react with carboxylic acids at room temperature, but are unstable in solution and in the solid state (11). Nimura et al. (12) therefore synthesized the stable diazoalkane 1-pyrenyldiazomethane (PDAM) and used it to derivatize pure mixtures of fatty acids. However, HPLC methods using PDAM derivatives of long chain fatty acids have, to our knowledge, not previously been applied for the quantitation of long chain fatty acids in biological samples. This paper reports an improved highly sensitive HPLC method using fluorescence detection of fatty acid PDAM derivatives designed to measure the release of fatty acids from a low number of cells. Using this method, rTNF-α-induced extracellular release of endogenous fatty acids was measured for the first time.

MATERIALS AND METHODS

Materials

Acetonitrile of HPLC grade was obtained from Labscan Ltd. (Ireland). Methanol, diethyl ether (DEE), and acetic acid (HAc) of pro analysi quality, isooctane and n-hexane of Lichrosolv quality, and ethylacetate of Uvasol quality were obtained from Merck (Darmstadt, Germany). Ethanol was obtained from Vinmonopolet A/S (Oslo, Norway). BF_3/methanol was obtained from Supelco (Bellefonte, PA) and 1-pyrenyldiazomethane (PDAM) was purchased from Molecular Probes Inc. (Eugene, OR). Fatty acids were purchased from Sigma (St. Louis, MO), Larodan Fine Chemicals ab (Sweden) or from Nu-Chek Prep Inc. (Elysian, MN). Synthetic 24:4n–6 and 24:5n–6 fatty acids were generously provided by Prof. Howard Sprecher (Ohio, USA). Both [5,6,8,9,11,12,14,15-^{3}H]arachidonic acid and [methyl-^{3}H]thymidine were purchased from Amersham (Buckinghamshire, England). Dichloro(R)fluorescein was from B.D.H. Lab., Chemicals Division (Poole, England). Triolein, dipalmitoylglycerophosphatidylcholine, diheptadecanoylglycerophosphatidylcholine, diheptadecanoylglycerophosphatidylethanolamine, oleoyllysophosphatidylcholine, cholesteryl stearate, BHT, and fatty acid-free bovine serum albumin (BSA) were all obtained from Sigma. Adsorbosil plus-1 softlayer preadsorbent thin-layer chromatography (TLC) plates were purchased from Alltech (Deerfield, IL). RPMI-1640 was obtained from Gibco (Paisley, U.K.). Fetal bovine serum was from Hyclone (Cramlington, U.K.) and gentamicin sulfate was from Schering Corp. (Kenilworth, NJ). Bond-Elut C_{18} octadecyl columns were purchased from Varian (Harbor City, CA). Murine rTNF-α with a specific activity of 7.6×10^7 U/mg protein was generously provided by Genentech Inc. (South San Francisco, CA).

Extraction of fatty acids

Fatty acids were extracted according to Bligh and Dyer (13) using acidification with 2% (v/v) HAc, or by C_{18} solid phase extraction as previously described (14), but acidifying with HAc. The recovery of [^{3}H]arachidonic acid was 97 ± 4% (n = 3) using solid phase extraction. After the C_{18} column extraction, the ethylacetate received 1/2 vol water, and tubes were whirlmixed, centrifuged, and the ethylacetate was taken to dryness under N_2 to remove substances interfering with the PDAM derivatization. BHT was added to each sample to prevent oxidation of fatty acids. Solvents were finally evaporated at 30°C under a stream of N_2, and the lipids were dissolved in 100 μl methanol. Derivatized samples were stored under N_2 at −20°C for a few days or at −70°C for several weeks. Glassware was washed three times with methanol-HAc 98:2 (v/v) to remove contaminants.

Derivatization with PDAM

The isolated fatty acids dissolved in methanol received 100 μl PDAM (1 G/l) newly dissolved in ethylacetate (12). The reaction took place for at least 90 min (90–240 min) in Teflon-capped tubes filled with N_2 at room temperature (20–25°C) in the dark. Solvents were finally evaporated under N_2, and PDAM derivatives were dissolved in acetonitrile. Aliquots of 5–20 μl were subsequently injected on the HPLC.

HPLC of fatty acid PDAM derivatives

Two different HPLC instruments were used in this study. Instrument A was used when not otherwise indicated and consisted of one Waters 6000A and one Waters 510 pump controlled by a Waters model 680 automated gradient controller (Waters Associates Inc., Milford, MA). Samples were injected with a Waters 712 WISP autoinjector. Column temperature was stabilized at 30°C using Waters RCM-100 column heater controlled by Waters temperature control module as peak retention times were highly temperature dependent (data not shown). The LS 3 fluorescence detector from Perkin-Elmer Ltd. (Norwalk, CON) was equipped with a 25 μl flow cell and was connected to a C-R6A integrator from Shimadzu Corp. (Kyoto, Japan). When not otherwise stated, excitation and emission wavelengths

were set at 340 and 395 nm, respectively, using 10 nm bandpass. Samples were separated on a 5 μm LC_{18} Supelcosil column (250 × 4.6 mm I.D.) with a Pelliguard precolumn (4.6 × 20 mm) from Supelco. A gradient between water (solvent I) and acetonitrile (solvent II) was used on instrument A: 0–40 min, 90–100% solvent II and 40–70 min, isocratic 100% II. Flow rate was 1 ml/min.

Fluorescence and absorbance spectra of PDAM derivatives

Spectra were recorded on-line on HPLC system B consisting of a Hewlett-Packard 1050 gradient pump, connected to a 1046 fluorescence detector or a 1050 multiple wavelength diode-array absorbance detector controlled using Chem-station software, all from Hewlett-Packard (Avondale, PA). A gradient between water (solvent I) and acetonitrile (solvent II) was used on system B: 0–15 min, 85–100% solvent II; and 15–50 min, isocratic, 100% II. Flow rate was 1.5 ml/min, and this only slightly reduced separation. Column temperature and columns were the same as in HPLC system A.

We also checked the fluorescence spectra on HPLC system A as well as on a Shimadzu RF-5000 spectrofluorophotometer. In these cases, PDAM derivatives of heptadecanoic acid (17:0), 20:4n–6 and 22:4n–6 were purified by HPLC, dissolved in either 100% acetonitrile or acetonitrile–water 9:1 (v/v) and spectra were recorded using 1.0 cm light path (named "off-line" spectra).

Cell culture

The highly TNF-sensitive WEHI 164 clone 13 cells (15) were cultured in RPMI-1640 containing 10% (v/v) fetal bovine serum, 2 mM L-glutamine, and 40 mg/l gentamicin sulfate (FCS-M). Unless otherwise indicated, rTNF-α-induced release of endogenous fatty acids was assayed in cells seeded in 3 ml FCS-M at a density of 0.5 × 10^6 cells in 60-mm petri dishes from Costar (Cambridge, MA). Medium was changed after 4 h, and incubation continued for 44 h with or without 50 μM 20:4n–6. Cells were then washed four times with RPMI-1640 to remove extracellular fatty acids. Incubation was continued for another 4 h in 2 ml RPMI-1640 without phenolic red with or without rTNF-α (1 μg/l) containing fatty acid-free BSA (0.1 g/l) in order to bind the fatty acids released. The cell medium was then collected and centrifuged (800 *g*, 10 min) to remove loose cells and stored at −80°C.

Identification of fatty acid PDAM derivatives using GLC

The identity of fatty acids released after rTNF-α stimulation was verified partly by comparing relative retention times with standards, partly by checking coelution after spiking with fatty acid standards. Some fatty acid PDAM derivatives were also isolated from the HPLC and fractions were reanalyzed on GLC. To ensure enough fatty acids for GLC analysis, WEHl cells were seeded at a density of 4 × 10^6 cells/well in 1400-mm petri dishes from Nunc A/S (Roskilde, Denmark) and preincubated with 50 μM 20:4n–6 in 20 ml FCS-M. Stimulation with rTNF-α (1 μg/l) was performed as described above using 10 ml medium/well. Fatty acids were extracted using the Bligh and Dyer method (13) and derivatized with PDAM. Individual peaks were collected from the HPLC using pooled samples from 4 to 8 incubations. The isolated fractions were further purified on TLC using a mobile phase consisting of n-hexane–DEE–HAc 70:30:2 (v/v/v) containing BHT which separated fatty acid PDAM derivatives from other lipids. PDAM derivatives were visualized under UV light, and appropriate fractions were scraped into Teflon-capped tubes and 150 μl n-hexane containing BHT (10 μg) was added. Fatty acid PDAM derivatives were then transmethylated under N_2 using 0.5 ml BF_3/methanol at 100°C for 30 min; the reaction was stopped with 1.0 ml water and FAME were extracted twice using n-hexane. FAME were further purified on TLC using n-hexane–DEE 19:1 (v/v) containing BHT (1 g/l) and identified after spraying the standards with 0.001% (w/v) dichloro(R)fluorescein in ethanol and viewing in UV light. The appropriate spots were scraped and eluted from the gel using 2 × 5 ml DEE containing BHT (1 mg/l). The DEE was evaporated using N_2 and FAME were dissolved in isooctane before analysis using a Hewlett-Packard 5890A GLC equipped with a 30-m SP2330 capillary column from Supelco, and a Hewlett-Packard 7673A automatic injector programmed for splitless injection as previously described (6).

Fatty acid composition of cellular lipids

The fatty acid composition of cellular lipids was analyzed after Bligh and Dyer extraction as described above. Diheptadecanoylglycerophosphatidylcholine, diheptadecanoylglycerophosphatidylethanolamine, trinonadecanoin, and heptadecanoic acid were used as internal standards. Phospholipids were separated on Alltech TLC plates using a solvent of $CHCL_3$–MeOH–HAc–H_2O 81:10:45:3 (v/v/v/v). Plates were sprayed using dichloro(R)fluorescein and spots were visualized under UV light. PC, PE, PS, and PI were scraped, transferred to a glass tube containing BHT, and transmethylated using methanolic base for 20 min under N_2 at 80°C. The spots containing neutral lipids were scraped, transferred to glass sintered disks, and lipids were eluted using three volumes of n-hexane–DEE–HAc 70:30:2 (v/v/v) containing BHT. Neutral lipids were taken to dryness under N_2, dissolved in $CHCl_3$ MeOH 9:1 (v/v) and separated on Alltech TLC plates using

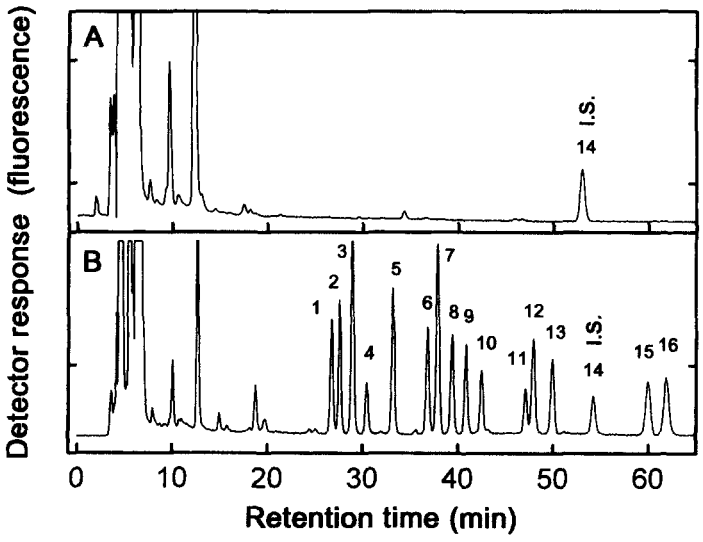

Fig. 1. Chromatograms of PDAM derivatized samples of reagent blank containing the I.S. 17:0 (panel A), and fatty acid standards (panel B). Peaks 1–16 correspond to PDAM esters of (1) 20:5n–3, (2) 14:1n–9, (3) 18:3n–3 and 18:3n–6, (4) 22:6n–3, (5) 20:4n–6, (6) 14:0 and 16:1n–9, (7) 18:2n–6, (8) 20:3n–6, (9) 22:4n–6, (10) 24:5n–6, (11) 18:1n–9, (12) 16:0, (13) 24:4n–6, (14) I.S. 17:0, (15) 20:1n–9, and (16) 18:0.

the solvent n-hexane–DEE–HAc 80:20:2 (v/v/v) containing BHT. FFA, TG, and CE fractions were identified under UV light after spraying with dichloro(R)fluorescein and comparison with standards. Spots were scraped into Teflon-capped glass tubes and dissolved in n-hexane. FFA were transmethylated using BF_3/MeOH for 10 min at 100°C, while TG and CE were transmethylated using methanolic base and analyzed using GLC as described above.

Thymidine incorporation assay

Cells were seeded in 96-well plates at a density of 8000 cells/well using 100 µl FCS-M. After 4 h incubation, cells received 100 µl FCS-M with or without arachidonic acid (50 µM final concentration). After 44 h further incubation, cell medium was removed and cells received control medium or TNF (1 µg/l). Simultanously, [^{3}H]thymidine (1 µCi/well) was added and the incubation continued for 4 h at 37°C. Cells were harvested and samples were analyzed as previously described (14).

Statistical analysis

The results are given as means ± SD if not otherwise indicated. Data were statistically analyzed using the SigmaStat version 1.0 program from Jandel Scientific GmbH (Erkrath, Germany) or the Statmed program obtained from Nycomed (Oslo, Norway).

RESULTS AND DISCUSSION

Separation of fatty acid PDAM-derivatives

Figure 1 shows a typical chromatogram of PDAM derivatives of major long chain fatty acid standards separated using HPLC. Separation was obtained between 18:2n–6, 20:3n–6, 20:4n–6, 22:4n–6, 24:4n–6, 24:5n–6, 18:3n–3, 20:5n–3, 22:6n–3, 16:0, 17:0, 18:0, 14:1n–9, 16:1n–9, 18:1n–9, and 20:1n–9. In this system, 16:0 and 18:1n–9 were partially resolved while 18:3n–3 and 16:1n–9 were not separated from 18:3n–6 and 14:0, respectively. The reagent blank contained only one small peak in the range where PDAM derivatives of long chain fatty acids elute (Fig. 1A), which did not interfere with the fatty acids of interest. The detection limit was approx. 20 fmol using HPLC system B (data not shown). Retention times were reproducible with standard deviations less than 1% when column temperature was stabilized (data not shown). The large peaks in the beginning of the chromatogram (Fig. 1) were due to the excess PDAM.

Linearity of detector response and molar response factors

A linear relationship was observed between the amount of fatty acid PDAM derivative injected and peak area, and PDAM had the same reaction rate with different fatty acids (data not shown).

When molar response factors were calculated relative to the internal standard (I.S.) 17:0, the response factors for PDAM derivatives of 16:0, 18:0, 18:1n–9, and 18:2n–6 were close to 1.0, compared to approx. 0.9 for 22:6n–3 and 18:3n–3 using the standard GLC-84 (**Table 1**). However, the molar response factor of 18:3n–3 relative to 18:1n–9 was 1.05 using the standard Larodan 32. As a standard of 16:0 prepared by weight had a response factor of 0.98 and as different commercial standards gave slightly different molar response factors (Table 1), we set the response factor to 1.0 for all fatty acids. The response factors in this report are comparable to

TABLE 1. Fatty acid molar response factors relative to the internal standard or 18:1n–9 measured using the fatty acid standard GLC-84 (standard 1), a mixture of 16:0 and 17:0 prepared by weight (standard 2), or relative to 18:1n–9 using the fatty acid standard Larodan 32 (standard 3)

	Relative Molar Response Factors		
Fatty Acid	Standard 1	Standard 2	Standard 3
16:0	0.97 ± 0.02	0.98 ± 0.004	1.02 ± 0.01
17:0 (internal standard)	1.00[a]	1.00[a]	
18:0	1.01 ± 0.03		1.11 ± 0.01
18:1n–9	1.04 ± 0.02		1.0[b]
20:1n–9	1.02 ± 0.02		
18:3n–3	0.93 ± 0.03		1.05 ± 0.03
22:6n–3	0.88 ± 0.04		
18:2n–6	0.98 ± 0.03		1.01 ± 0.01
20:4n–6	0.94 ± 0.04		

Fatty acid standards were dissolved in methanol, derivatized with PDAM, and analyzed using HPLC system A as described in Materials and Methods.

[a]Molar response factors relative to the I.S. 17:0 were calculated from peak areas measured using standards 1 and 2. Results using standard 1 are given as means ± SD from two separate experiments, each performed with five parallels (n = 10). The response factors of standard 2 are from one experiment (n = 4).

[b]Standard 3 did not contain 17:0 and molar response factors are therefore calculated relative to 18:1n–9 and given as means ± SD (n = 5) from one experiment.

other studies using ADAM (11). Nimura et al. (12) found an approximate 2-fold difference in response between PDAM derivatives of 18:1n–9 and 16:0. This discrepancy is most likely due to the different gradients as the fluorescence quantum yield depends on the solvents used (12).

Fluorescence and absorbance spectra of PDAM derivatives

Fluorescence spectra of PDAM derivatives are shown in **Fig. 2.** The emission spectrum showed one broad peak at approx. 390–395 nm, while the excitation spectrum had major peaks at 230 nm and 240 nm, and smaller peaks at 270, 330, and 340 nm. Excitation and emission spectra of purified PDAM derivatives of 17:0, 20:4n–6, and 22:4n–6 recorded "off-line" on HPLC system A and the Shimadzu fluorimeter, were similar to those observed by Nimura et al. (12), but different from those observed on HPLC system B. This discrepancy is most likely due to differences in excitation light intensities at the lower wavelengths. The differences in spectra also highly affected detector sensitivity. Using emission set at 390 nm on HPLC system B, the mean relative peak areas were 15, 24, and 100 (n = 3) using excitation at 340, 270, and 230 nm, respectively, when 50 pmol 17:0 was injected. In comparison, the HPLC system A showed relative peak areas of 332, 120, and 100 (n = 3) at the same wavelenghts. PDAM derivatives also have absorbance spectra with peaks at 230–240, 270, and 340 nm (Fig. 2), indicating that UV detection can be used but with lower sensitivity.

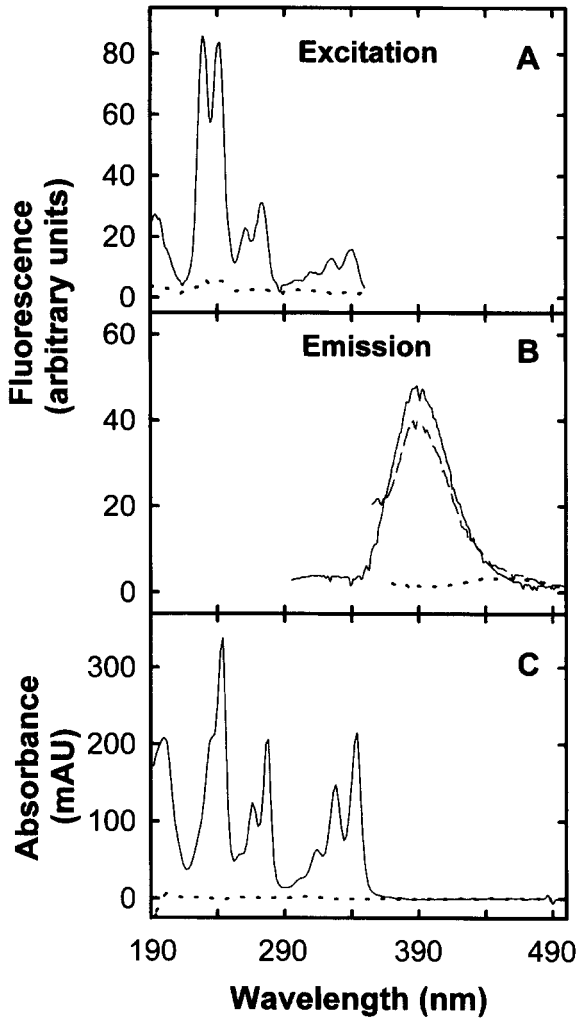

Fig. 2. Fluorescence excitation spectra (panel A), emission spectra (panel B) and UV absorbance spectra (panel C) of 17:0 PDAM derivative in 100% acetonitrile (——, – –) and the mobile phase (100% acetonitrile) alone (- - -). All spectra were recorded on HPLC system B. Excitation spectra were recorded when emission was set at 390 nm. Emission spectra of 17:0 PDAM derivative were recorded using excitation set at 340 nm (– –) and 230 nm (——), and emission spectrum of the mobile phase alone was recorded using excitation set at 340 nm. C: UV absorbance spectrum.

Reaction conditions and stability of PDAM derivatives

To optimize derivatization conditions, we next examined whether the yield of fatty acid PDAM derivative was dependent on PDAM concentration. The yield decreased at PDAM concentrations below 62.5 mg/l (**Fig. 3A**). However, the concentration of palmitic acid measured was not affected by PDAM concentration down to 7.8 mg/l (Fig. 3B), giving a molar ratio PDAM/total fatty acid of 0.645. This indicates that PDAM reacts with the same efficacy with 16:0 and 17:0.

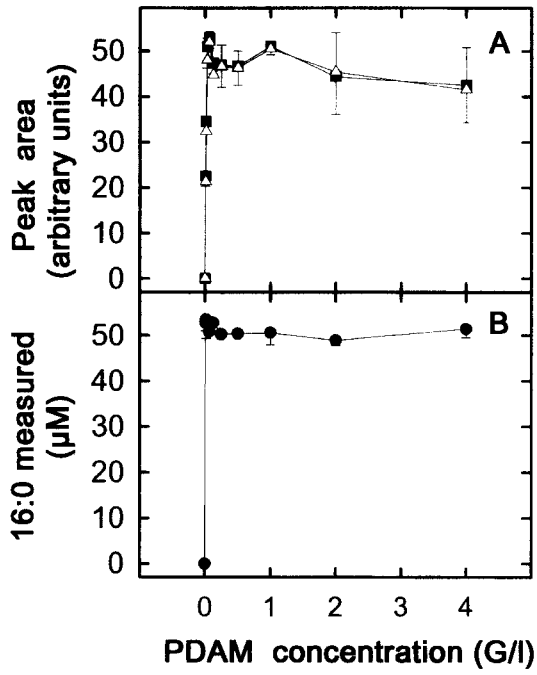

Fig. 3. Effect of PDAM concentration on yield of fatty acid PDAM derivative. Increasing concentrations of PDAM in 100 μl ethylacetate were added to 16:0 (50 μl, 50 μM) and 17:0 (50 μl, 50 μM) in methanol. Samples were analyzed by HPLC and results are expressed as peak area (panel A) of 16:0 (■) and 17:0 (△), or as concentration of 16:0 (●) measured using 17:0 as I.S. (panel B). The results are given as means and SD (n = 3) from one of two similar experiments.

The effect of different solvents and pH during derivatization was then examined, as PDAM reacts more efficiently with methylmalonic acid at alkaline pH (16). Results showed that PDAM reacted efficiently with FFA both in methanol and methanol–ethylacetate 1:1 (v/v) and the yield was not improved at pH 8.0 (data not shown).

The PDAM derivatives of 18:3n–3 and 17:0 were stable for at least 48 h at 25°C both in the presence and absence of BHT, and at least 26 days when stored at −20°C in the presence of BHT (data not shown). The results are similar to those showed by Nimura et al. (12), but the stability at −20°C was slightly better in this study, possibly due to the presence of BHT.

Specificity of PDAM reaction towards free fatty acids and method recovery

PDAM did not react significantly with triolein, oleoyl-lysophosphatidylcholine, cholesterylstearate, and dipalmitoylglycerophosphatidylcholine, but only with oleic and palmitic acid (**Table 2**). This suggests that PDAM reacts selectively with free but not esterified fatty acids. The reagent blank contained small amounts of oleic, palmitic, and stearic acid, probably due to contaminations in solvents and/or glassware. This, together with the possible traces of FFA in the esterified lipid preparations, could well explain the low amounts of PDAM de-

TABLE 2. PDAM specificity towards free fatty acids examined by derivatization of lipid standards and by comparing solid phase isolation of fatty acids and total lipid extracts

Sample Analyzed	Fatty Acid Added	Fatty Acid Analyzed					
		16:0	18:0	18:1n–9	20:4n–6	22:4n–6	24:4n–6
	nmol	*pmol fatty acid measured*[a]					
Lipid standards							
No addition	none	45 ± 41	4 ± 6	3 ± 8			
Dipalmitoyl-PC	100	64 ± 17					
Cholesteryl stearate	100		3 ± 5				
Triolein	100			14 ± 10[d]			
Oleoyl-lyso-PC	100			30 ± 2[d]			
Palmitic acid	5	4880 ± 240[d]					
Oleic acid	5			5310 ± 210[d]			
		nmol fatty acid measured[b]					
Cell medium							
Total lipid extract[c]		6.4 ± 0.32[c]	3.5 ± 0.24	5.5 ± 0.45[c]	28.5 ± 1.76[c]	22.3 ± 0.84	7.4 ± 0.22[c]
Isolated FFA[c]		8.0 ± 1.07	3.5 ± 0.93	6.8 ± 0.43	35.6 ± 4.45	24.0 ± 2.91	6.8 ± 0.13

Cell culture media were harvested after preincubating WEHI cells with 50 μM 20:4n–6 and stimulation with 1 μg/1 rTNF-α for 4 h in 1400-mm wells.

[a]Results are given as mean ± SD from two experiments, each performed in quadruplicate. Results were not corrected for reagent blank values.

[b]Results are given as mean ± SD from two experiments, each performed in triplicate and were corrected for blank values.

[c]Fatty acids were determined after PDAM derivatization either of a total lipid extract or of an isolated free fatty acid fraction from the same cell culture medium.

[d]$P < 0.05$ compared to no addition. Kruskal-Wallis one-way analysis on ranks was used for testing recovery of palmitic acid and oleic acid, while recovery of stearic acid was tested using Mann-Whitney rank sum test as data were not normally distributed.

[e]$P < 0.01$ compared with free fatty acids (FFA) isolated by solid phase extraction and analyzed using Student's *t*-test.

TABLE 3. rTNF-α-induced release of endogenous fatty acids to the cell culture medium after 4 h incubation analyzed using Bligh and Dyer extraction and PDAM

Fatty Acid Released	Preincubation and Stimulation[a]					
	Control Medium			Arachidonic Acid		
	−rTNF-α	+rTNF-α	P^b	−rTNF-α	+rTNF-α	P^b
	pmol/well/4 h					
16:0	66 ± 72	2313 ± 210	<0.0286	145 ± 37	1795 ± 31	<0.0001
18:0	0 ± 30	287 ± 93	0.0008	0 ± 31	925 ± 29	0.0286
16:1n−9/14:0	43 ± 45	1029 ± 46	<0.0001	112 ± 14	519 ± 11	<0.0001
18:1n−9	390 ± 51	3708 ± 282	<0.0001	129 ± 15	1151 ± 53	<0.0001
20:4n−6	21 ± 8	153 ± 25	0.0286	463 ± 19	3379 ± 148	<0.0001
22:4n−6	ND	ND		570 ± 31	2152 ± 97	<0.0001
24:4n−6	ND	ND		378 ± 12	850 ± 25	<0.0001
Total FFA	671 ± 230	8147 ± 590	<0.0001	2241 ± 239	11608 ± 360	<0.0001

[a]WEHI cells were preincubated with or without 50 μM arachidonic acid for 44 h and stimulated 4 h with rTNF-α (1 μg/l). Arachidonic acid had no effect on cell growth or mortality (data not shown) and the wells contained 2.6 and 2.7 × 10^6 cells/well (means) after 44 h preincubation with or without 20:4n−6 (50 μM), respectively. Results are given as mean ± SD (n = 4) and were corrected for reagent blanks which contained 16:0, 18:1n−9, and 18:0. One of two similar experiments is shown. Minor fatty acids are not listed. ND, not detected.

[b]*P* value compared to without rTNF-α when tested using Student's *t*-test or Mann-Whitney rank sum test when the normality test failed.

rivatives from dipalmitoylglycerophosphatidylcholine and triolein.

To further confirm that PDAM does not react with esterified fatty acids, we compared derivatization of a total lipid extract from cell culture media with derivatization of isolated FFA (Table 2). The recovery of 18:0 and 22:4n−6 was similar with the two methods. Solid phase extraction gave 25% higher amount of 20:4n−6, while the amount of 24:4n−6 was 9% lower than Bligh and Dyer extraction. Fatty acid isolation using total lipid extraction thus did not result in higher amounts of fatty acids detected compared to isolation of the FFA prior to PDAM derivatization. This suggests again that PDAM does not react with esterified fatty acids.

We then compared the recovery of oleic acid added to RPMI-1640 containing BSA using either the Bligh and Dyer method (13) or C_{18} solid phase extraction. A linear relationship was observed between pmol oleic acid added and pmol oleic acid recovered both in a low (50 pmol) and high (5 nmol) concentration range (data not shown). The total recovery of oleic acid was in the range 93–98% (mean, n = 3) and the within-series coefficient of variation was typically less than 4% (data not shown).

rTNF-α-induced release of endogenous free fatty acids

We then examined whether rTNF-α-induced release of endogenous fatty acids could be measured with the present method. rTNF-α (1 μg/l) enhances the release of [^{3}H]arachidonic acid after 1–2 h in WEHI cells, while cell death is only detectable after 4–5 h (data not shown). **Table 3** shows that rTNF-α enhanced the release of endogenous 20:4n−6 from 21 to 153 pmol/well/4 h, i.e., a net release of 132 pmol. In cells preincubated with 20:4n−6, rTNF-α increased the release from 463 to 3379 pmol/well/4 h, i.e., a net release of 2916 pmol. This shows that rTNF-α-induced release of fatty acids can be significantly changed by enriching with 20:4n−6 (**Fig. 4**). Interestingly, the spontaneous release of 20:4n−6 also increased 22-fold after enrichment with 20:4n−6. Cell counting showed that 20:4n−6 had no effect on cell growth (Table 3). Cell growth in non-enriched and arachidonic acid-enriched cells after 4 h incubation in the presence or absence of TNF was not different using [^{3}H]thymidine incorporation assay. This indicates that the difference in fatty acid release was not due to different cell numbers. Furthermore, the data indicate that rTNF-α-induced release of major endogenous fatty acids in WEHI cells can be measured in as few as 20,000 cells as only 10% of the sample volume was injected on the HPLC. The non-enriched cells used in this study release only 0.5–1% of [^{3}H]arachidonic acid incorporated after stimulation for 4 h with 1 μg/l rTNF-α (data not shown) indicating the sensitivity of the present method.

We then confirmed the identity of two new fatty acid peaks found in WEHI cells preincubated with 20:4n−6 (peaks no. 9 and 13 in Fig. 4C and 4D). They were identified partly by comparing their retention times in two different HPLC systems with authentic standards, partly by isolating the pure peaks from the HPLC chromatogram, and then reanalyzing them as methyl esters on capillary GLC. The 20:4n−6 peak was included as a control (**Fig. 5**). Peaks no. 5, 9, and 13 in Fig. 4D showed one single peak on GLC corresponding to synthetic

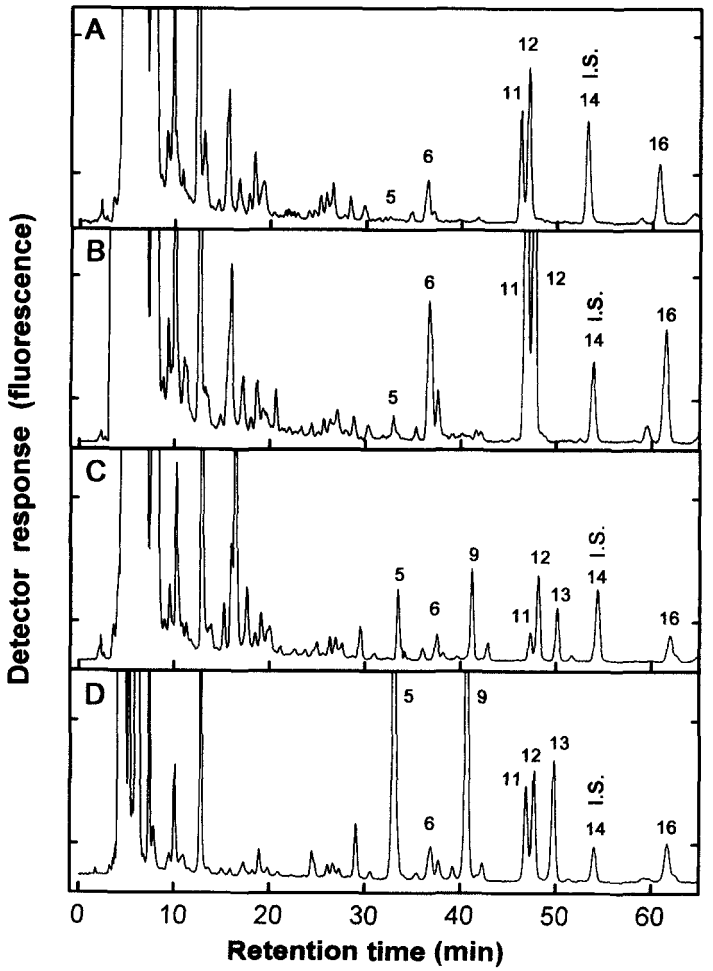

Fig. 4. rTNF-α-induced release of endogenous fatty acids in WEHI cells. Cells were preincubated 44 h in control medium (panels A and B) or in medium containing 50 μM 20:4n–6 (panels C and D). Thereafter, the cell medium was changed and cells were incubated 4 h with (panels B and D) or without (panels A and C) rTNF-α (1 μg/l) in RPMI-1640 containing fatty acid-free BSA. Fatty acids were isolated using Bligh and Dyer extraction, derivatized with PDAM, and analyzed using HPLC as described in Materials and Methods. The I.S. peak in panel D represents 6 pmol 17:0 PDAM derivative injected. Fatty acids are numbered as in the legend to Fig. 1.

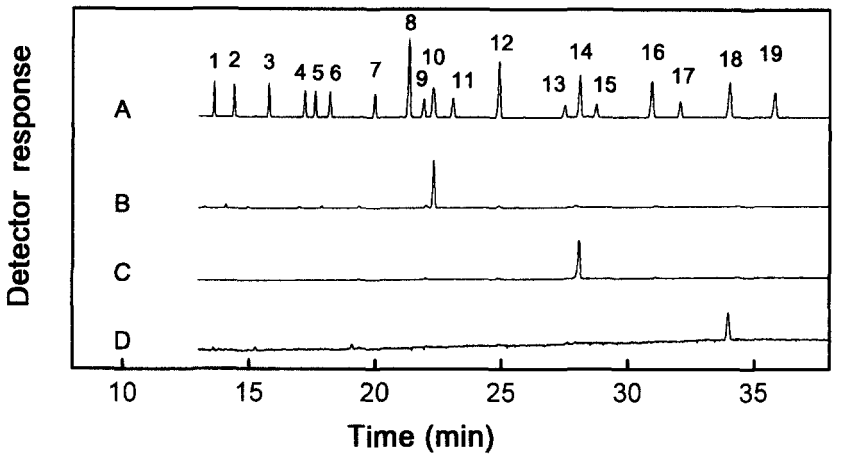

Fig. 5. Identification using capillary GLC of 20:4n–6, 22:4n–6, and 24:4n–6 released from WEHI cells. Cells were preincubated 44 h with 20:4n–6 (50 μM) and stimulated 4 h with rTNF-α. The unknown PDAM derivatives were isolated from pooled samples and then transmethylated, extracted, and further purified by TLC as described in Methods. FAME were analyzed by capillary GLC and the chromatograms shown are; A: FAME standards; (1) 18:0, (2) 18:1n–9, (3) 18:2n–6, (4) 20:0, (5) 18:3n–3, (6) 20:1n–9, (7) 20:2n–6, (8) 20:3n–6, (9) 22:0, (10) 20:4n–6, (11) 22:1n–9, (12) 20:5n–3, (13) 24:0, (14) 22:4n–6, (15) 24:1n–9, (16) 22:5n–3, (17) 22:6n–3, (18) 24:4n–6, (19) 24:5n–6. B: GLC of peak #5 in Fig. 4D, C: GLC of peak #9 in Fig. 4D, and D: GLC of peak #13 in Fig. 4D.

TABLE 4. Effect of rTNF-α on fatty acid composition of cellular lipids in WEHI clone 13 cells

Fatty Acid Measured	Lipid Fraction[a]							
	PC		PE		FFA		TG	
	−TNF	+TNF	−TNF	+TNF	−TNF	+TNF	−TNF	+TNF
	mol percent of total fatty acids							
16:0	37.9 ± 0.78	42.1 ± 1.01[c]	18.5 ± 1.36	17.9 ± 0.38	16.3 ± 2.88	14.9 ± 1.05	13.7 ± 0.87	17.6 ± 0.76[c]
18:0	9.7 ± 0.08	7.9 ± 0.39[c]	20.2 ± 0.83	19.9 ± 0.46	7.7 ± 2.95	5.3 ± 0.75	4.3 ± 0.17	5.2 ± 0.70[b]
16:1n−9	2.0 ± 0.58	1.7 ± 0.02	1.2 ± 0.21	1.0 ± 0.09	8.7 ± 5.51	1.3 ± 0.03[b]	0.9 ± 0.06	1.2 ± 0.14[c]
18:1n−9	11.4 ± 0.20	11.7 ± 0.32	11.0 ± 0.23	10.3 ± 0.30[b]	5.5 ± 0.81	4.3 ± 0.65	8.0 ± 0.45	10.5 ± 0.49[c]
18:3n−3	0.08 ± 0.005	0.07 ± 0.01	0.07 ± 0.02	0.07 ± 0.02	ND	ND	0.08 ± 0.02	0.01 ± 0.03[c]
20:5n−3	0.06 ± 0.01	0.04 ± 0.03	0.09 ± 0.02	0.06 ± 0.06	0.08 ± 0.17	0.06 ± 0.08	0.05 ± 0.004	ND
22:5n−3	0.03 ± 0.004	0.01 ± 0.01[c]	0.03 ± 0.06	0.09 ± 0.10	ND	0.1 ± 0.08	0.07 ± 0.02	0.07 ± 0.01
22:6n−3	0.64 ± 0.004	0.61 ± 0.03	1.6 ± 0.06	1.5 ± 0.04[b]	1.1 ± 0.31	1.3 ± 0.06	1.0 ± 0.08	0.61 ± 0.02[c]
18:2n−6	1.1 ± 0.03	1.1 ± 0.02	0.5 ± 0.02	0.5 ± 0.03	1.2 ± 0.21	1.6 ± 0.11[b]	1.6 ± 0.08	1.4 ± 0.08[b]
20:2n−6	0.2 ± 0.01	0.2 ± 0.01	0.1 ± 0.01	0.1 ± 0.02	ND	ND	0.15 ± 0.01	0.16 ± 0.01
20:3n−6	0.5 ± 0.03	0.6 ± 0.03[c]	0.9 ± 0.21	1.1 ± 0.62	0.5 ± 0.36	1.0 ± 0.05[b]	1.1 ± 0.04	1.0 ± 0.12
20:4n−6	14.2 ± 0.29	11.3 ± 0.23[c]	21.0 ± 0.40	19.2 ± 0.59[c]	12.5 ± 2.09	16.7 ± 0.48[c]	9.4 ± 1.00	10.3 ± 1.07
22:4n−6	13.8 ± 0.23	14.2 ± 0.25	20.4 ± 0.90	23.6 ± 0.55[c]	23.4 ± 4.43	38.3 ± 3.53[c]	46.4 ± 0.89	39.8 ± 0.95[c]
22:5n−6	0.8 ± 0.002	0.8 ± 0.04	1.5 ± 0.06	1.6 ± 0.07	1.2 ± 0.34	2.5 ± 0.15[c]	2.0 ± 0.14	0.9 ± 0.08[c]
24:4n−6	3.1 ± 0.21	3.1 ± 0.12	1.4 ± 0.20	1.5 ± 0.08	2.8 ± 0.48	3.7 ± 0.10[c]	5.8 ± 0.04	6.5 ± 0.50[b]
24:5n−6	0.35 ± 0.01	0.32 ± 0.02[b]	0.3 ± 0.04	0.3 ± 0.03	0.8 ± 0.11	0.9 ± 0.09	1.2 ± 0.07	0.9 ± 0.05[c]
Total fatty acids	134.9 ± 11.6	109.9 ± 6.24[c]	25.9 ± 4.31	28.5 ± 4.05	23.0 ± 4.45	63.4 ± 10.4[c]	82.1 ± 7.75	106.2 ± 18.96

[a]WEHI clone 13 cells were seeded in 1400-mm wells and, after 4 h incubation, cells received medium containing 50 μM arachidonic acid. After 44 h, cells were washed four times and incubated for another 4 h in RPMI-1640 containing BSA with or without rTNF-α (1 μg/1) as indicated. Cell lipids were extracted, separated using TLC, and fatty acid composition was analyzed using capillary GLC as described in Methods. Results are given as means ± SD of quadruplicates. Minor fatty acids are not listed. ND, not detected.

[b]$P < 0.05$; [c]$P < 0.01$, significantly different from no TNF in each lipid fraction using Student's *t*-test.

20:4n−6, 22:4n−6, and 24:4n−6, respectively. Peaks no. 9 and 13 also coeluted in HPLC system A after spiking with synthetic 22:4n−6 and 24:4n−6, respectively (data not shown).

The present study shows for the first time that rTNF-α enhances the extracellular release of 22:4n−6 and 24:4n−6. It also shows that TNF in addition to 20:4n−6 enhances the release of several other fatty acids including 18:1n−9, 16:0, 20:4n−6, 22:4n−6, and 24:4n−6. A previous report shows that TNF induces the release of radioactively labeled 16:0 and 20:4n−6 in BALB/c 3T3 cells (17). It has also been shown that TNF activates a mitochondrial PLA_2 in WEHI 164 parental cells releasing 20:4n−6, 22:4n−6, 20:3n−6, and 22:6n−3 in the mitochondrial membranes (18). However, the GLC method required as much as 300×10^6 cells per incubation (18). The lack of fatty acid specificity in TNF-induced fatty acid release observed in this and other reports, may suggest that the low molecular weight non-pancreatic PLA_2 or some other enzyme(s) rather than the high molecular weight cytosolic PLA_2 are responsible for a major part of the rTNF-α-induced fatty acid release. This does not exclude the involvement of the cytosolic PLA_2, as it may participate in the activation of the non-pancreatic (group II) PLA_2 (19). Jayadev, Linardic, and Hannun (20) showed that both 20:4n−6 and 18:1n−9 may mediate sphingomyelin hydrolysis in HL-60 cells in response to TNF, indicating that fatty acids other than 20:4n−6 may be involved in TNF signal transduction.

To identify the source of the released fatty acids, fatty acid composition of cellular lipid fractions was analyzed (**Table 4**). TNF decreased total cell PC by 19%, increased total FFA by 276%, while no change was seen in PE and TG. The reduction in PC alone could explain the release reported in cells preincubated with 20:4n−6 (Table 3), and is in agreement with the activation of one or more phospholipases (1). Although there was a relative increase in 16:0, the absolute amount decreased by 4.9 nmol/mg protein in PC. With the exception of 16:1n−9, all FFA increased intracellularly (Table 4). The total increase in cell FFA was 40.4 nmol/mg protein of which 22:4n−6 and 20:4n−6 contributed 18.9 and 7.7 nmol/mg protein, respectively. This is similar to the release pattern from PC as well as that observed extracellularly. The ratio 22:4n−6/20:4n−6 was much higher in FFA intracellularly than found extracellularly. Furthermore, 22:4n−6 increased significantly in PE. One possible explanation could be that released 20:4n−6 is rapidly chain elongated to 22:4n−6, which again seems to be reincorporated into lipids more rapidly than 20:4n−6 itself (Table 4). Although the conversion of 20:4n−6 to 22:4n−6 is well documented, it has previously been reported that 22:4n−6 is preferentially retroconverted to 20:4n−6 (21, 22). This may suggest that the cell supply of 20:4n−6 regulates the balance

between chain elongation and retroconversion in WEHI cells.

In summary, the present method makes it possible to study agonist-induced release of all major endogenous fatty acids, which is difficult when using radiolabeled fatty acids. The data also indicate that TNF-induced release of fatty acids is more complex and less selective for 20:4n–6 than previously observed.

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