RESTING MACROPHAGES PRODUCE DISTINCT
METABOLITES FROM EXOGENOUS ARACHIDONIC ACID*

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Prior studies have shown that mouse peritoneal macrophages contain unusually high levels of arachidonic acid (20:4)[1] in their phospholipids. Under resting conditions of in vitro cultivation, 20:4 is retained in cell phospholipids and insignificant amounts are metabolized via lipoxygenase and cyclo-oxygenase pathways (1, 2). However, when stimulated by the appropriate membrane-perturbing agents of both soluble and particulate nature (1–3), up to 50% of the 20:4 is released in the form of oxygenated metabolites. Release occurs under conditions in which tritiated 20:4 has been previously incorporated into phospholipids (1) and leads to the formation of radiolabeled prostaglandins, hydroxyeicosatetraenoic acids (HETEs), and leukotriene C, a slow-reacting substance (1–2, 4, 5).

In this paper we report the unique response of resting macrophages to exogenously supplied 20:4 and its rapid metabolism in the absence of phagocytic or pharmacologic stimuli. We show that within 5 min approximately one-third of the radiolabeled 20:4 supplied in serum-free medium is incorporated into cell phospholipids and approximately two-thirds is converted to a spectrum of oxygenated products—primarily prostacyclin and HETEs. Qualitatively different results are obtained when *Corynebacterium parvum*-elicited macrophages are employed.

Materials and Methods

Macrophage Cultures. Primary cultures of peritoneal macrophages were established from resident cells of female and male Swiss Webster (Taconic Farms, Germantown, N. Y.) or CD-1 (The Trudeau Institute, Saranac Lake, N. Y.) mice, as previously described (1). Approximately 6×10^6 peritoneal cells suspended in 1 ml of minimum essential α medium (α-MEM, Grand Island Biological Co., Grand Island, N. Y.) containing 10% fetal calf serum (FCS) were added to 35-mm Diam plastic culture dishes. After 2 h at 37°C in 5% CO_2/95% air, cultures were washed three times in calcium and magnesium-free phosphate buffered saline (PD) to remove nonadherent cells and incubated overnight (16 h) in fresh α-MEM plus 10% FCS.

C. parvum-elicited Macrophages. *C. parvum*-elicited peritoneal macrophages were obtained from

mice injected intraperitoneally 11–14 d before harvest with 1.4 mg of formalin-killed *C. parvum* (Coparvax; a generous gift of Dr. R. Tuttle, Burroughs Wellcome Co., Greenville, N. C.).

Synthesis of 20:4 Oxygenated Products.   At the end of the overnight incubation period, macrophage cultures were washed three times with cold PD and overlaid with 1 ml of fresh α-MEM (no serum) containing 0.5 μCi of [5,6,8,9,11,12,14,15-^{3}H]20:4 ([^{3}H]20:4) (62.2 Ci/mmol sp act; New England Nuclear, Boston, Mass.) or [^{3}H]20:4 and the indicated concentration of unlabeled 20:4 (Nu-Chek-Prep, Inc., Elysian, Minn.). Media were removed after incubation for the appropriate periods under 5% CO_2/95% air at 37°C. Cell monolayers were washed twice with PD, overlaid with 1 ml 0.05% Triton X-100 (Rohm and Haas Co., Philadelphia, Pa.), and the dishes were scraped. 50 μl aliquots of media and Triton X-100 cell lysates were removed for radioactivity determinations. The protein content of cell lysates was determined by the method of Lowry et al. (6) with bovine serum albumin as a standard. The protein content of cultures in 35-mm dishes was 94 ± 18 μg (*n* = 50).

20:4 metabolites were extracted from culture media following a modification of the procedure described by Unger et al. (7). In brief, 1 vol of absolute ethanol was added. After acidification with formic acid (85% wt/wt; 10 μl/ml of medium, final pH ~3), media were extracted twice with 1 vol each of chloroform. The chloroform phases were combined and taken to dryness under a stream of nitrogen. The 20:4 metabolites were resuspended in 1 ml of chloroform and again taken to dryness. This procedure was repeated twice before the 20:4 metabolites were dissolved in 0.5 ml of the appropriate starting buffer for reverse-phase high performance liquid chromatography (HPLC).

20:4 metabolites were separated and identified by HPLC on columns (4.6 mm × 25 cm) of ultrasphere C-18 (Altex Scientific, Inc., Beckman Instruments, Inc., Berkeley, Calif.) at a flow rate of 1 ml/min. Fractions of 1 ml were collected. The contents of fractions were dried under a stream of air and radioactivity was measured by liquid scintillation counting in Hydrofluor (National Diagnostics, Inc., Advanced Applications Institute, Inc., Sommerville, N. J.). Prostaglandins, HETEs, and 20:4 were resolved using the conditions described by Borgeat and Samuelsson (8) for the resolution of HETEs. Columns were eluted isocratically with 80 ml of solvent 1 (methanol/water/acetic acid, 75:25:0.01, vol/vol/vol) followed by 40 ml of solvent 2 (methanol/acetic acid, 100:0.01, vol/vol). For the identification of prostaglandins, fractions 4–10 were pooled, dried under a steam of nitrogen, and the residue dissolved in solvent 3 (water/acetonitrile/benzene/acetic acid, 76.7:23.0:0.2:0.1, vol/vol/vol/vol). Separation of prostaglandins was achieved by HPLC in the same solvent. Leukotriene C was separated from other 20:4 metabolites by HPLC of medium extracts using solvent 4 (methanol/water/acetic acid, 65:35:0.1, pH 5.4, vol/vol/vol). Prior to chromatography, the chloroform extracts were taken to dryness under a stream of nitrogen (above), however, the vessel was washed with ethanol/water (80:20, vol/vol). The 20:4 metabolites were finally dissolved in 0.5 ml of solvent 4 before application to the column.

^{3}*H-labeled Standards.* ^{3}H-labeled standards were subjected to the same HPLC procedures for purposes of identification. ^{3}H-labeled prostaglandins including prostaglandin E_2 (PGE$_2$), prostaglandin F$_{2α}$ (PGF$_{2α}$), 6-keto prostaglandin F$_{1α}$ (6-ketoPGF$_{1α}$), and thromboxane B$_2$ (TXB$_2$) were purchased from New England Nuclear. ^{3}H-labeled 5-hydroxy-6,8,11,14-eicosatetraenoic acid (5-HETE), 12-hydroxy-5,8,10,14-eicosatetraenoic acid (12-HETE), and 15-hydroxy-5,8,11,13-eicosatetraenoic acid (15-HETE) were generated by published procedures using [^{3}H]20:4 as the substrate. 5-HETE was obtained from human neutrophils (8), 12-HETE from human platelets (10), and 15-HETE after incubation of [^{3}H]20:4 with soybean lipoxygenase (11). Each HETE was extracted from culture medium or the reaction mixture as described above, and purified by HPLC in solvent 1.

Inhibitors of Cyclo-oxygenase and Lipoxygenase.   Nordihydroguaiaretic acid (NDGA) and indomethacin were obtained from Sigma Chemical Co., St. Louis, Mo. 5,8,11,14 eicosatetraynoic acid (ETYA) was kindly provided by Dr. W. E. Scott, Hoffman-LaRoche, Inc., Nutley, N. J. Stock solutions of NDGA (3 mg/ml) and indomethacin (1 mg/ml) were prepared in absolute ethanol and diluted into α-MEM. ETYA stocks (0.87 mg/ml) in hexane-ethanol mixture (10:1) were diluted in hexane before use.

Kinetics of [^{3}H]20:4 Uptake by Macrophage Cultures.   Explanted macrophages were cultured overnight in α-MEM plus 10% FCS as described above. Cultures were placed on ice, washed

twice with PD, and overlaid with 1 ml of α-MEM containing 0.5 μCi of [^{3}H]20:4. The PD and α-MEM were precooled to 4°C before use. Cultures were transferred to 37°C and incubated under an atmosphere of 5% CO_2/95% air. At the indicated times, duplicate 35-mm cultures were placed on ice, and the medium was removed. Cells were washed twice and scraped into PD precooled to 4°C. Duplicate aliquots of media and cells suspended in PD were removed for radioactivity determinations.

Cell lipids were immediately extracted at 4°C, following the procedure of Bligh and Dyer (12). Individual phospholipids were separated by two-dimensional thin-layer chromatography (1) on plates of silica gel (2-D Redi Coats, Supelco, Inc., Bellefonte, Pa.). The organic phase of cell extracts was concentrated under nitrogen, spotted on plates, and overlaid with 0.1 μmol of carrier lipid (nonradioactive) extracted from the macrophage-like cell line J774. Chromatograms were developed in the first dimension with chloroform/methanol/ammonium hydroxide (65: 35:5, vol/vol/vol) and in the second dimension with chloroform/acetone/methanol/acetone acid/water (30:40:10:10:5, vol/vol/vol/vol/vol) (13). Lipid containing regions were visualized by a brief exposure of plates to iodine vapors and were scraped into scintillation vials. Radioactivity was determined in Hydrofluor after the addition of water (1 ml).

Results

Incorporation of 20:4 by Macrophages in Serum-free Medium. Fig. 1 A shows the kinetics of [^{3}H]20:4 incorporation by macrophages incubated in serum-free α-MEM. The uptake of fatty acid increased linearly for 5–10 min. After this time, the amount of radiolabel in the culture medium remained constant or decreased at a much reduced rate. Maximal levels of 20:4 incorporation reached 34.8 ± 10.0% (mean ± SD, n = 10) of the fatty acid supplied to the medium and this value was independent of the exogenous fatty acid concentration over the range of 10 nM to 1 μM. Exposure of macrophages to 20:4 concentrations >1 μM for periods of 1–2 h led to cell death, as determined by trypan blue exclusion. Although we were unable to determine saturating levels of 20:4, it is evident that macrophages have a considerable capacity for 20:4 uptake.

Figure 1 B shows the distribution of radiolabel in macrophage phospholipids at various times following exposure of serum-free cultures to [^{3}H]20:4. At all time points, 90–95% of the incorporated fatty acid was recovered in phospholipid. In the initial portion of the labeling period, the highest percentage of radiolabel was incorporated into phosphatidylinositol and the major macrophage phospholipid, phosphatidylcholine. Subsequently, the relative amounts of radiolabel in phosphatidylinositol decreased with a concomitant increase in the radiolabel content of phosphatidylcholine. The amount of ^{3}H in phosphatidylethanolamine, in contrast, remained constant. Overall, this labeling pattern is similar to that seen during the longer time course (24 h) of 20:4 uptake by macrophages maintained in serum containing medium, which was linear over a 8 h period (1). Two exceptions, however, are notable. In serum cultures, a higher percentage (30%) of radiolabel was recovered in neutral lipid. Furthermore, the ^{3}H content of phosphatidylethanolamine increased to 40% of the total radiolabel in phospholipid with decreases in ^{3}H levels of both phosphatidylcholine and phosphatidylinositol as a function of labeling time. These distinctions, however, may result from differences in the exposure time of cultures to [^{3}H]20:4 in addition to effects of serum.

Conversion of Exogenously Supplied 20:4 to Oxygenated Metabolites. The abrupt cessation of 20:4 incorporation into phospholipid illustrated in Fig. 1 A was explained when the culture medium was extracted and subjected to HPLC under conditions that allowed mutual separation of prostaglandins, HETEs, and unreacted 20:4 (Fig. 2). Recovery
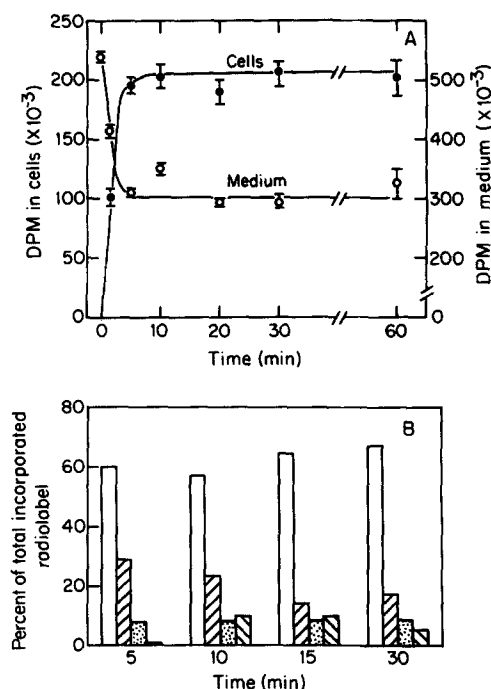
Fig. 1.   Uptake of [³H]20:4 by macrophage cultures in serum-free α-MEM and distribution of [³H]20:4 among macrophage lipids. Cultures were incubated overnight, washed with cold PD, and overlaid with cold α-MEM containing 0.5 μCi of [³H]20:4. At time = 0, the cultures were transferred to 37°C. (A) Time-course of [³H]20:4 uptake by macrophages and ³H content of medium. The medium was removed from duplicate cultures at the indicated times. Cell monolayers were rinsed in the cold and scraped into 0.05% Triton X-100. Radioactivity was determined in aliquots of medium (○) and Triton cell lysates (●). Values are means ± range. (B) Percentage of incorporated radiolabel in macrophage phospholipids and neutral lipid as a function of exposure time to [³H]-20:4. The protocol was identical to that in (A) except that cells were scraped into PD and the lipids extracted. The lipid extracts were subjected to two-dimensional thin layer chromatography. Areas of the chromatograms containing lipid were scraped and the radioactivity of each was determined. Data are expressed as the percentage of recovered radiolabel. □, phosphatidylcholine; ▨, phospha-tidylethanolamine; ▩, phosphatidylinositol; ▧, phosphatidylinositol; ▢, neutral lipid. Less than 1% of the radiolabel in lipid extracts of cells was present as free fatty acid. The total percentage of radiolabel in other macrophage phospholipids, including phosphatidylserine, sphingomyelin, and cardiolipin was low (<2%) and did not vary during the labeling period. The SEM was <5%.

of the radiolabel on extraction was quantitative (>95%). The major percentage of the radiolabel (80%) was resolved by HPLC into a number of compounds considerably more polar than 20:4 (Fig. 2). This suggested extensive metabolism of 20:4 by macrophages. Few or none of these products were evident in controls that consisted of incubating [³H]20:4-containing medium in the absence of cells (Fig. 2). The predominant 20:4 metabolites in peak 1 (Fig. 2, fractions 4–10) contained cyclo-oxygenase products. Considerable radioactivity was also recovered in the region of the chromatogram corresponding to the elution times (8) of di- and tri-HETEs (fractions 11–25) in addition to mono-HETEs (fractions 28–40). Several criteria in addition to elution characteristics on HPLC are consistent with this radiolabeled material being HETEs and include: (a) elution times of 5-HETE, 12-HETE, and 15-HETE standards (25–40 min), (b) the similar R_f values of these macrophage products and HETEs standards on thin layer chromatography (1), and (c) the reduced
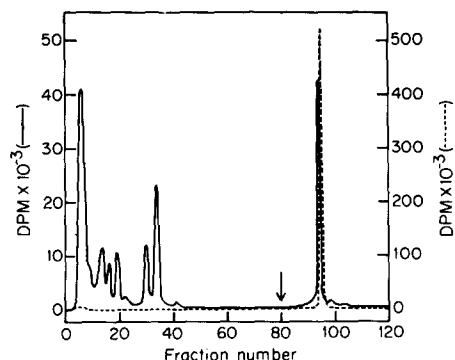
Fig. 2. HPLC chromatograms of ^{3}H-labeled products in culture medium after a 20 min incubation with 0.5 μCi of [^{3}H]20:4. (———), Products formed by resident peritoneal macrophages. Cultures were incubated overnight in α-MEM plus 10% FCS, rinsed, and overlaid with α-MEM containing [^{3}H]20:4. Incubation with [^{3}H]20:4 was for 20 min at 37°C. (- - -), No cell control. Medium plus [^{3}H]20:4 was incubated for 20 min in culture dishes without macrophages. Media samples were extracted as described under Materials and Methods. Prostaglandins and HETEs were separated on an ultrasphere C-18 column in solvent 1. Arrow indicates the point at which the column was treated with solvent 2 to elute unreacted 20:4. Prostaglandin standards (6-ketoPGF$_{1\alpha}$, PGF$_{2\alpha}$, PGE$_2$, and thromboxane B$_2$) were eluted in fractions 4-11; 5-HETE, 12-HETE, and 15-HETE standards in fractions 30-40; and 20:4 in fractions 94-96.

quantities of these 20:4 products formed by macrophages pre-treated with NDGA, a lipoxygenase inhibitor (below). HPLC of medium extracts in solvent 4, which resolves leukotriene C from other 20:4 metabolites, indicated that <3% of the radiolabel was converted to slow reacting substance (C. Rouzer, personal communication).

The cyclo-oxygenase products formed from exogenously supplied 20:4 were identified by subjecting the pooled material in peak 1 (Fig. 2) to HPLC in solvent 3. Peak 1 resolved into some very polar material that eluted at the solvent front (Fig. 3). According to Bokoch and Reed (14) phospholipid may be found in this peak. Peak 1 further resolved into a major peak of radiolabel that coeluted with 6-keto PGF$_{1\alpha}$ (fractions 19-23), a peak of PGE$_2$ (fractions 82-89), and a small peak of unidentified material (fractions 31-36). The ratio of 6-keto PGF$_{1\alpha}$ to PGE$_2$ ranged from 2.7 to 4.0 (n = 4). This was considerably different from a ratio of 0.66 for the same products synthesized from endogenous 20:4 released by cultures exposed to a phagocytic stimulus (W. A. Scott, unpublished results). Approximately 22 and 45% of the radiolabel was recovered as cyclo-oxygenase and lipoxygenase products, respectively. Of the remaining radiolabel, 20% was recovered as unreacted 20:4 and 14% as unidentified 20:4 metabolites.

Cyclo-oxygenase and Lipoxygenase Inhibitors. The influence of lipoxygenase or cyclo-oxygenase inhibitors on the metabolism of exogenously supplied [^{3}H]20:4 was next examined. The percentage of metabolites formed was estimated from HPLC elution profiles. As shown in Table I, pre-exposure of cultures to indomethacin, an inhibitor of cyclo-oxygenase (15), led to a 64% decrease in prostaglandin synthesis with a compensatory increase in the percentage of 20:4 converted to HETEs. Treatment of macrophages with the lipoxygenase inhibitor NDGA (16) produced a 79% reduction in HETEs synthesis and a slight inhibition (17%) of prostaglandin synthesis. In contrast, ETYA, an acetylenic analog of 20:4 reported to block both cyclo-oxygenase
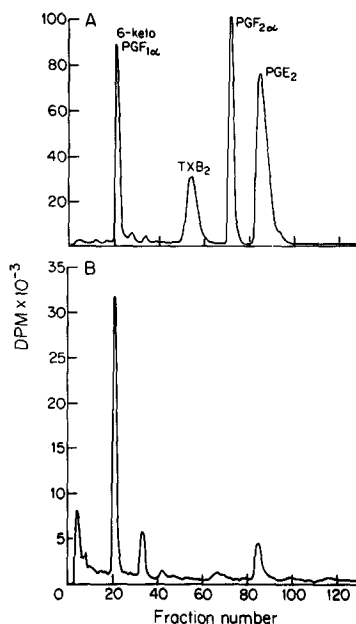
Fig. 3. HPLC chromatograms of prostaglandins. Separations were carried out on ultrasphere C18 columns eluted with solvent 3. (A) ^{3}H-labeled prostaglandin and TXB_2 standards. (B) Resident macrophage products. Cultures were incubated for 20 min in α-MEM containing 0.5 μCi of [^{3}H]-20:4. Medium was extracted and subjected to HPLC using solvent 1. Fractions 4–10 (Fig. 2) containing cyclo-oxygenase products were pooled and evaporated to dryness under a stream of nitrogen. The residue was dissolved in solvent 3 before chromatography in the same solvent.

TABLE I

Effect of Cyclo-oxygenase and Lipoxygenase Inhibitors on 20:4 Metabolism

Addition	Concentration	Percent of radiolabel in medium		
		Prostaglandin	HETEs	20:4
	$\mu g/ml$			
None	—	35.3	44.9	19.8
Indomethacin	5	12.6	81.6	5.8
NDGA	0.30	29.4	9.3	61.3
ETYA	2	16.4	20.1	63.5

Macrophages were isolated and incubated overnight in α-MEM plus 10% FCS. Cultures were washed three times with PD and overlaid with fresh α-MEM containing the indicated concentrations of inhibitors. After 60 min incubation at 37°C the medium was removed, and fresh α-MEM containing inhibitors and 0.5 μCi of [^{3}H]-20:4 was added. After an additional 20 min incubation at 37°C the medium was removed, and an aliquot counted. Macrophages were scraped into 1 ml of 0.05% Triton X-100 and the radiolabel content determined. The medium was extracted for 20:4 metabolites. Medium extracts were subjected to HPLC on ultrasphere C-18 columns eluted sequentially with 80 ml of solvent 1 and 40 ml of solvent 2. Medium extracts of duplicate 35-mm cultures were pooled before HPLC. Data are presented as the percent of the total ^{3}H recovered in HPLC effluents. The ^{3}H in fractions 3–10 is listed as prostaglandins, fractions 11–40 as HETEs, and fractions 90–105 as 20:4.

and lipoxygenase (17), inhibited prostaglandin (54%) and HETEs (55%) production to the same extent.

A higher percentage of radiolabel in the medium of cultures treated with NDGA or ETYA was recovered as unreacted 20:4 than in controls (no drug) or indomethacin-treated cultures. This suggested that NDGA and ETYA may also inhibit the acyl-transferase which catalyzes esterification of 20:4 into macrophage phospholipids. However, as shown in Table II, the percentage of exogenous 20:4 incorporated by drug-treated cultures was greater (20 ± 5%) than that of controls. Uptake of 20:4 therefore occurred in the presence of drugs.

20:4 Metabolism by C. parvum-elicited Macrophages. Macrophages were elicited by a single intraperitoneal injection of *C. parvum* and harvested after 11–14 d. These cells were cultured by the same methods used for resident macrophages. On exposure to [^{3}H]20:4 in serum-free medium, *C. parvum*-elicited macrophages incorporated over twofold (70 ± 3%, mean ± SD, $n = 3$) more fatty acid into cell phospholipids than did resident cells (34%). In addition, only 17% of the radiolabel remaining in the culture medium of elicited cells was recovered as 20:4 oxygenated metabolites. HPLC profiles of media extracts from elicited cells were characterized by a small peak of prostaglandins, the absence or near absence of HETEs, and a predominant peak of unreacted 20:4 (Fig. 4). Of the ^{3}H recovered in the medium, prostaglandins, HETEs, and 20:4 represented 12.5 ± 2.1, 4.1 ± 1.7, and 83.5 ± 0.5%, respectively, of the total ($n = 2$). Separation of the cyclo-oxygenase products formed by *C. parvum*-elicited cells showed (Fig. 5) that the small percentage of 20:4 metabolized via this pathway was recovered as 6-ketoPGF$_{1\alpha}$, TXB$_2$, PGF$_{2\alpha}$, and PGE$_2$ in proportions of 1:1.58:3.25:5.45 ($n = 3$). These profiles were strikingly different from those obtained from the cyclo-oxygenase products generated by resident macrophages (Fig. 3).

Relationship between 20:4 Esterification and Synthesis of 20:4 Oxygenated Products. The above experiments indicated that exogenous 20:4 supplied to macrophages maintained in serum-free medium is esterified into cell phospholipid and also converted to

TABLE II

Effect of Cyclo-oxygenase and Lipoxygenase Inhibitors on the Uptake of 20:4 by Macrophages Cultured in Serum-free α-MEM

Addition	Concentration	Percent 20:4 incorporated by macrophages	
		10 min	20 min
	μg/ml	%	
None	—	49.7 ± 2.2	48.5 ± 0.6
Indomethacin	5	56.9 ± 3.7	54.1 ± 0.8
NDGA	0.30	58.4 ± 1.7	61.2 ± 2.0
ETYA	2	58.3 ± 3.6	59.7 ± 1.2

Macrophages were isolated and incubated overnight in α-MEM plus 10% FCS. Cultures were washed three times in PD and overlaid with α-MEM containing the indicated concentrations of inhibitors. After 60 min incubation at 37°C the medium was removed and the cultures exposed to fresh α-MEM containing 0.5 μCi [^{3}H]20:4 and inhibitors. Medium was removed at the indicated times and the macrophages were scraped into 1 ml of 0.05% Triton X-100. The radiolabel content of medium and cell suspensions was determined in aliquots of each. Data are reported as duplicate determination on triplicate cultures (mean ± SD) of the total recovered ^{3}H.
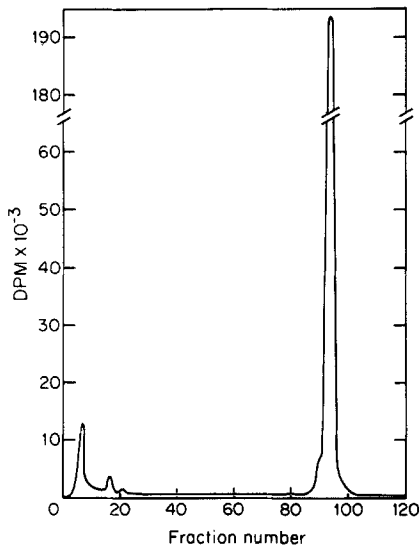
Fig. 4.   HPLC chromatogram of 20:4 products formed by *C. parvum*-elicited macrophages. The details of the experimental protocol are the same as those described under Fig. 2 for resident macrophages.
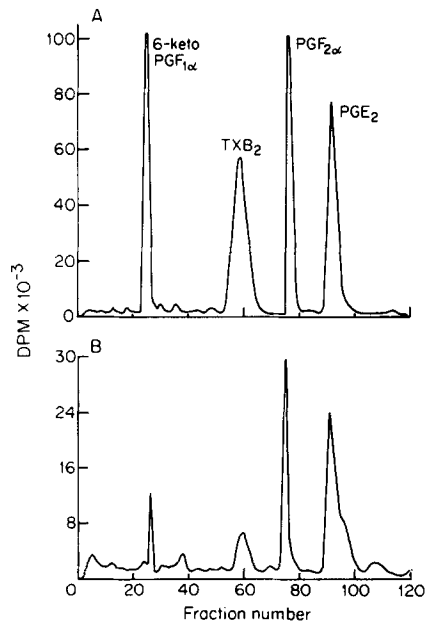

Fig. 5.   HPLC chromatogram of cyclo-oxygenase products formed by *C. parvum*-elicited macrophages. The experimental details are described under Fig. 3. (A) ^{3}H-labeled standards; (B) macrophage products.

cyclo-oxygenase and lipoxygenase products. We questioned whether esterification is an initial step in 20:4 metabolism and this is followed by de-esterification and conversion to a spectrum of products. To test this possibility, we carried out the following experiment. Resident macrophages were labeled in α-MEM (no serum) for

20 min with 0.5 μCi (8.3 nM) of [³H]20:4 and washed with PD. As shown in Fig. 1, the remaining cell-associated radiolabel (35% of the original) represented fatty acid incorporated into cell phospholipid. Subsequent exposure of these cultures to a higher concentration (10⁻⁶ M) of unlabeled 20:4 would be expected to promote release of the esterified radiolabeled fatty acid in the form of oxygenated metabolites if the above construct were true. However, the quantities of tritium recovered in the medium of cultures exposed to unlabeled 20:4 were comparable to the low levels obtained from controls similarly labeled and incubated in α-MEM alone (Fig. 6). Other groups of cultures were labeled with [³H]20:4, washed, and again overlaid with 0.5 μCi of [³H]20:4. The results indicated that macrophages readily esterified and metabolized the additional radiolabeled fatty acid to the same extent seen with the initial exposure. In contrast, release of cell associated radiolabel occurred when labeled cells were challenged with a maximal dose of 160 μg zymosan. The extent of release (35%) and the spectrum of products formed were identical to those reported previously (1) for phagocytizing cultures prelabeled with [³H]20:4 in serum-containing medium.

We infer the following from these results. First, 20:4 incorporated into cell phospholipid from serum-free medium represents a metabolically stable pool of fatty acid in resting macrophages. Thus, esterification of 20:4 and synthesis of 20:4 oxygenated metabolites appear to be mutually exclusive reactions. Second, we are unable to distinguish in functional terms between the cell phospholipid pools labeled by 20:4 supplied in serum-free medium and those labeled in the presence of serum.

Discussion

It is becoming increasingly clear that macrophages are a major source of 20:4 metabolites. After exposure to a particulate or soluble trigger, endogenous 20:4 is released from membrane phospholipids and converted to a complex mixture of cyclo-oxygenase and lipoxygenase products (1–3). In this report, we show that resident peritoneal macrophages also metabolize a large proportion of exogenously supplied 20:4 in the absence of a discernible trigger. This pathway yields qualitatively similar products to those generated in response to a phagocytic stimulus, but relative proportions of metabolites differ considerably (Table III). It is apparent that a fourfold greater percentage of exogenously supplied 20:4 is converted to HETEs together with three- to fivefold decrease in the ratio of PGE₂ to prostacyclin (recovered
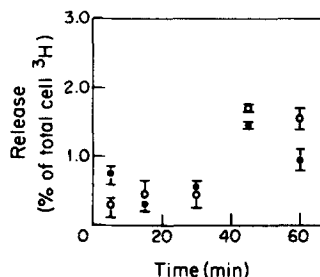
Fig. 6. Release of ³H by macrophages prelabeled with [³H]20:4 and exposed to 10⁻⁶ M unlabeled 20:4. Cultures in 35-mm diameter plastic dishes were incubated for 20 min at 37°C in α-MEM containing 0.5 μCi of [³H]20:4. After washing, the cultures were overlaid at time = 0 with α-MEM or α-MEM containing 10⁻⁶ M unlabeled 20:4. Aliquots of medium were removed at the indicated times and the radioactivity determined. ●, α-MEM plus 10⁻⁶ M 20:4; ○, α-MEM alone.

TABLE III

Cyclo-oxygenase and Lipoxygenase Products Formed by Resident Macrophages
from Exogenous and Endogenous Pathways

Product	Proportions of 20:4 metabolites*	
	Exogenous pathway‡	Endogenous pathway§
PGE$_2$	9	40
6-ketoPGF$_{1\alpha}$	24	25
HETEs	67	15
Leukotriene C	<3	15–20

* The proportions of metabolites rather than molar quantities are given, because the absolute amounts of products generated by the exogenous pathway is variable and dependent on the available arachidonic acid in extracellular fluid.

‡ Resting macrophages in α-MEM (no serum) were exposed to 0.5 μCi of [^{3}H]20:4 for 20 min at 37°C. The isolation and identification of 20:4 oxygenated metabolites is described in Materials and Methods.

§ Macrophages were labeled overnight with 0.5 μCi of [^{3}H]20:4 in α-MEM plus 10% FCS. Cultures were washed and exposed to maximal stimulus of 160 μg zymosan for 90 min (1). The isolation and identification of products was carried out as described in Materials and Methods.

as 6-ketoPGF$_{1\alpha}$).[2] In contrast, macrophages appear unable to utilize fatty acid in the medium for leukotriene C (a slow-reacting substance) synthesis, although slow-reacting substance represents up to 15–20% of the metabolites derived from endogenous 20:4 (4, 5).

Within 5 min, approximately one-third of the exogenously supplied 20:4 is found esterified into cell phospholipid and two-thirds is metabolized to oxygenated products and recovered in the extracellular medium. We questioned whether esterification is obligatory for the localization and transfer of 20:4 to the site of metabolite synthesis. This seems not to be the case. Rather, incorporation into cell phospholipids and synthesis of 20:4 oxygenated products represent distinct metabolic fates of exogenous 20:4. This situation contrasts with our previous studies which used macrophages exposed to a particulate, zymosan stimulus (1). Under these circumstances, [^{3}H]20:4 was incubated with cells in the presence of serum for 16 h. Over 70% of the fatty acid taken up was incorporated into phospholipids. Subsequent production of cyclo-oxygenase and lipoxygenase products was contingent on 20:4 release, which ensures only upon exposure to soluble stimuli (3) or upon interaction of particulate ligands with specific macrophage surface receptors (18). The different proportions of 20:4 oxygenated metabolites produced in the two experimental situations may be explained by the existence of separate phospholipid 20:4 and cyclo-oxygenase, and lipoxygenase compartments. In the exogenous pathway, plasma membrane-associated activities may metabolize fatty acid in the medium, whereas phospholipid 20:4 released by phagocytizing cells may be derived from microsomal and other intracytoplasmic pools and metabolized at these sites. Obviously, cell fractionation studies are required and should provide insights into these questions.

Distinct mixtures of products with proinflammatory activities accumulate from the "exogenous" and "endogenous" pathways. This together with the modulation evident

[2] Prostacyclin is unstable in aqueous solutions and is recoverd as 6-ketoPGF$_{1\alpha}$.

with elicited macrophages suggests a mediator role of macrophage-derived cyclo-oxygenase and lipoxygenase products in the control of inflammation. For example, one might expect exogenous metabolites to be formed during an acute inflammatory response when 20:4 is released from granulocytes and other tissue products. The resident cell cyclo-oxygenase products, PGE_2 and prostacyclin, would be expected to promote the response. Subsequent macrophage activation leads to down regulation of synthetic capacity and the production of the vasoconstrictors, $PGF_{2\alpha}$ and TXB_2, which may be factors in controlling the exacerbation of both cellular and humoral infiltrates. Too little is known concerning the physiologic activities of HETEs to include them in such a construct. One exception is leukotriene B (5(S), 12(R)-dihydroxy-6,8,10,114-(cis/trans/trans/cis)-eicosatetraenoic acid), which has been reported to be a chemotactic factor for neutrophils with activity comparable to that of the anaphylatoxin C5a (19, 20). However, it remains to be determined whether macrophages synthesize leukotriene B.

In summary, resting macrophages quantitatively metabolize exogenously supplied 20:4 in concentrations of up to 10^{-6} M without loss of cell viability. Other cell types, including neutrophils (8) and endothelial cells (21) are also reported to convert exogenous 20:4 to oxygenated products in the absence of a stimulus, however the quantity of fatty acid metabolized in comparison is small. Thus, macrophages contain high levels of cyclo-oxygenase and lipoxygenase activities, a contributing factor in the effectiveness of these cells as a source of 20:4 oxygenated metabolites.

Summary

Resident mouse peritoneal macrophages rapidly metabolize free arachidonic acid (20:4) in the absence of a discernible trigger. After a 20-min incubation in serumless medium, one-third of the fatty acid was found esterified in cell phospholipid and two-thirds was metabolized to oxygenated products which were recovered in the culture medium.

The 20:4 oxygenated metabolites were identified by reverse-phase high performance liquid chromatography as hydroxyeicosatetraenoic acids (HETEs) and 6-keto prostaglandin $F_{1\alpha}$ (6-ketoPGF$_{1\alpha}$), the stable form of prostacyclin, together with prostaglandin E_2 (PGE_2) in proportions of 67:24:9. Inhibitor studies using indomethacin, nordihydroguaiaretic acid, and 5,8,11,14-eicosatetraenoic acid confirmed these metabolites to be lipoxygenase and cyclo-oxygenase products. The proportion of products differs considerably from those generated from phospholipid 20:4 in response to a phagocytic stimulus (HETEs:6-ketoPGF$_{1\alpha}$:PGE$_2$:leukotriene C, 15:25:40:15–20). Corynebacterium parvum-elicited macrophages incorporated a higher percentage (70%) of exogenously supplied 20:4 and converted <20% of the fatty acid to oxygenated metabolites. Cyclo-oxygenase products (PGE_2, $PGF_{2\alpha}$, TXB_2, and 6-ketoPGF$_{1\alpha}$) represented the major 20:4 metabolites (74%) synthesized by these activated macrophages.

Esterification of 20:4 into cell phospholipids appeared not to be an initial obligatory step for synthesis of 20:4 oxygenated products by this route. To the contrary, incorporation of 20:4 into cell lipids and metabolism via the cyclo-oxygenase and lipoxygenase pathways represent distinct metabolic fates of exogenously supplied 20:4.

These observations establish that resting macrophages contain high levels of cyclo-

oxygenase and lipoxygenase activity and suggest macrophages can synthesize lipid mediators of inflammation in the absence of an inflammatory stimulus.

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