UPTAKE AND METABOLISM OF MONOHYDROXY-EICOSATETRAENOIC ACIDS BY MACROPHAGES*

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Mouse resident macrophages are potent sources of arachidonic acid (20:4)[1] metabolites generated via both the cyclooxygenase and lipoxygenase pathways (1–5). The phospholipids of this cell are highly enriched with 20:4, and upon appropriate membrane stimulation, up to 50% of endogenous 20:4 is released and metabolized to a mixture of prostaglandins, hydroxy-eicosatetraenoic acids (HETEs), and leukotriene C, a slow-reacting substance of anaphylaxis (2–3). Resident macrophages also have the capacity to quantitatively convert exogenously supplied 20:4 in the absence of a stimulus to a qualitatively different mixture of oxygenated products (4). The overall proportion of lipoxygenase products is enhanced compared to the metabolites generated from endogenous 20:4, but the formation of leukotriene C is diminished. Finally, the metabolites produced from either endogenous or exogenous sources of 20:4 are altered by the activation state of the macrophage (4–5).

In this report, we show that resting macrophages have a considerable capacity to both incorporate mono-HETEs into cell phospholipid and metabolize these hydroxy acids. The major metabolite generated from 5-HETE is a conjugated triene, a structural characteristic of certain 20:4 derivatives with potent biologic activities. These two metabolic fates of mono-HETEs were altered in macrophages elicited with *Corynebacterium parvum*.

Materials and Methods

Mice. Male and female ICR (CD-1) mice (20–25 g) were purchased from the Trudeau Institute, Saranac Lake, NY.

Human Peripheral Blood Components. Leukocyte concentrates were obtained from The New

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[1] *Abbreviations used in this paper:* A23187, calcium ionophore A23187; α-MEM, minimal essential medium/alpha modified; 20:4, arachidonic acid; [^{3}H]20:4, [5,6,8,9,11,12,13,15-^{3}H]20:4; EDTA, ethylenediaminetetraacetic acid; FCS, fetal calf serum; HETE, hydroxy-eicosatetraenoic acid; 15-HETE, 15-hydroxy-5,8,-11,13-eicosatetraenoic acid; 12-HETE, 12-hydroxy-5,8,10,14-eicosatetraenoic acid; 5-HETE, 5-hydroxy-6,8,11,14-eicosatetraenoic acid; 5(S), 12(S)-di-HETE, 5(S), 12(S)-dihydroxy-6,8,10,14-eicosatetraenoic acid; HPLC, high pressure liquid chromatography; leukotriene B_4, 5(S), 12(R)-dihydroxy-6,8,10,14-*cis*, *trans*, *trans*, *cis*-eicosatetraenoic acid; leukotriene C, 5(S)-hydroxy-6(R)-S-γ-glutamylcysteinylglycyl-7,9,11,14-eicosatetraenoic acid; PBS, phosphate-buffered saline; PD, calcium- and magnesium-free phosphate-buffered saline; PGE_2, prostaglandin E_2; $PGF_{2\alpha}$, prostaglandin $F_{2\alpha}$; 6-keto $PGF_{1\alpha}$, 6-keto prostaglandin $F_{1\alpha}$; TXB_2, thromboxane B_2.

York Blood Center, New York, NY. Platelets were isolated from whole blood obtained from healthy human volunteers after informed consent and collected into a final concentration of 7% (vol/vol) of 77 mM EDTA as anticoagulant.

Materials. Minimal essential medium/alpha modified (α-MEM, KC Biological Inc., Lenexa, KS) supplemented with 100 U/ml penicillin G and 100 μg/ml streptomycin (Gibco Laboratories, Grand Island Biological Co., Grand Island, NY) was used throughout for culture of mouse peritoneal cells. Formalin-killed *C. parvum* (Coparvax) was obtained from Burroughs Wellcome Co., Research Triangle Park, NC. [5,6,8,9,11,12,14,15-^{3}H]20:4 ([^{3}H]20:4) (78.2 Ci/mmol sp act) was purchased from New England Nuclear, Boston, MA, and unlabeled 20:4 was obtained from Nu-Chek-Prep, Inc., Elysian, MN. [5,8,9,11,12,14,15-^{3}H] 6-keto prostaglandin $F_{1\alpha}$, [5,6,8,9,11,12,14,15-^{3}H] thromboxane B_2, [5,6,8,9,11,12,14,15-^{3}H] prostaglandin $F_{2\alpha}$, and [5,6,8,11,12,14,15-^{3}H] prostaglandin E_2 were all purchased from New England Nuclear (all 120–200 Ci/mmol sp act). Hydrofluor was obtained from National Diagnostics, Inc., Advanced Applications Institute Inc., Somerville, NJ. Dextran 6% (wt/vol) in 0.9% NaCl was obtained from Abbott Diagnostics, Diagnostic Products, North Chicago, IL, and Ficoll-Paque was purchased from Pharmacia Fine Chemicals, Div. of Pharmacia Inc., Piscataway, NJ. Calcium ionophore A23187 was obtained from Calbiochem-Behring Corp., San Diego, CA. Soybean lipoxidase type IV was obtained from Sigma Chemical Co., St. Louis, MO. Silica gel H plates were purchased from Analtech, Inc., Newark, DE, and Redi-Coat-2D plates were purchased from Supelco, Inc., Bellefonte, PA. High pressure liquid chromatography (HPLC) columns 4.6-mm × 25-cm of ultrasphere C-18 were obtained from Beckman Instruments, Inc., Fullerton, CA. HPLC solvents were either HPLC grade or distilled before use. Zymosan was purchased from ICN K&K Laboratories Inc., Plainview, NY, and trypan blue 0.4% in 0.9% NaCl (wt/vol) was purchased from Gibco Laboratories.

Macrophage Cultures. Primary cultures of peritoneal macrophages were established from resident cells of male and female ICR (CD-1) mice, as previously described (1). Approximately 8×10^6 peritoneal lavage cells suspended in 1 ml of α-MEM 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 containing 10% FCS.

C. parvum-elicited peritoneal macrophages were obtained from mice injected intraperitoneally with 1.4 mg of formalin-killed *C. parvum* 11–14 d before harvest. Primary cultures were established as described for resident macrophages.

Metabolism of Exogenously Supplied 20:4 or 20:4 Oxygenated Metabolites. At the end of the initial 16-h incubation period, macrophage cultures were placed on ice, washed three times with cold PD, and overlaid with 1 ml of fresh α-MEM without serum containing the appropriate quantity of 20:4 or oxygenated 20:4 metabolite to be tested, including (*a*) 0.5 μCi of [^{3}H]20:4 (6.4 nM) or 0.5 μCi of [^{3}H]20:4 and the indicated concentration of unlabeled 20:4; (*b*) mono-HETEs (15-, 12-, or 5-HETE); and (*c*) cyclooxygenase products of 20:4 [6-keto prostaglandin $F_{1\alpha}$ (6-keto $PGF_{1\alpha}$), thromboxane B_2 (TXB_2), prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$), and prostaglandin E_2 (PGE_2)]. 20:4 or each 20:4-derived product was dissolved in a quantity of ethanol sufficient to make the final ethanol concentration in the incubation medium 0.5%. Control incubations always contained equal amounts of ethanol.

After incubation for the indicated times at 37°C under 5% CO_2/95% air, media were removed and maintained on ice under an atmosphere of nitrogen. 20:4 metabolites were extracted from culture media after a modification of the procedure described by Unger et al. (6), as previously described (4). In brief, 1 vol of absolute ethanol was added, the mixture acidified with formic acid (final pH ~3), and the media extracted twice with 1 vol each of chloroform. The combined chloroform phases were dried completely under a stream of nitrogen and finally suspended in 400 μl of the appropriate starting buffer for HPLC.

Routinely, the kinetics of uptake for each ^{3}H-labeled compound were measured in medium aliquots taken from triplicate or quadruplicate cultures. At the end of the incubation period, the cultures were washed twice with PD and scraped into 1 ml of PD. The amount of cell-associated compound was calculated as the percent radioactivity found in cell lysates of the total recovered in the sum of radiolabel in medium plus cell lysates.

Cell lipids were immediately extracted at 4°C, following the procedure of Bligh and Dyer (7). Individual phospholipids were separated by two-dimensional thin layer chromatography (1) on plates of silica gel. 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/acetic acid/water (30:40:10:10:5, vol/vol/vol/vol/vol) (8). 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).

Determination of Cell Viability and Phagocytic Capacity. After an initial 16-h cultivation in α-MEM with 10% FCS, macrophage monolayers on glass coverslips were incubated for 2 h with each mono-HETE in α-MEM or α-MEM alone under serumless conditions. Duplicate coverslips were immediately assessed for viability by trypan blue exclusion and phagocytic capacity by ingestion of zymosan, as previously described (1).

HPLC. All HPLC analyses were performed using columns (4.6-mm × 25-cm) of ultrasphere C-18 at a flow rate of 1 ml/min. Fractions of 1 ml were collected. Fraction contents or aliquots were dried under a stream of air, and radioactivity was measured by liquid scintillation counting. The mutual separation of cyclooxygenase products and di- and tri-HETEs, mono-HETEs, and unreacted 20:4 was achieved using the buffer system described by Borgeat and Samuelsson (9–10). The columns were eluted isocratically with 60–80 ml of methanol/water/acetic acid (75:25:0.01, vol/vol/vol) followed by 40 ml of methanol/acetic acid (100:0.01, vol/vol). This combination of eluting solvents is referred to as solvent system 1.

Appropriate fractions of the polar metabolites of the mono-HETEs compounds separated using solvent system 1 were pooled, dried under reduced pressure, and rechromatographed using solvent system 2 (60 ml of methanol/water/acetic acid [65:35:0.1, vol/vol/vol, pH 5.4, with NH_4OH] followed by 40 ml of methanol/acetic acid [100:0.01, vol/vol]). Ultraviolet absorption spectra of the major polar metabolites of each mono-HETE tested were recorded in the first eluting buffer for solvent system 2.

HPLC analyses of cyclooxygenase products recovered in medium extracts were performed using solvent system 3 under otherwise identical chromatographic conditions (4). The initial buffer consisted of water/acetonitrile/benzene/acetic acid (76.7:23.0:0.2:0.1, vol/vol/vol/vol) (11) for 100 min, followed by methanol/acetic acid (100:0.01, vol/vol) for 40 min.

Preparation of 5-, 12-, and 15-HETEs. 5- and 12-HETEs were prepared as described by Borgeat and Samuelsson (9, 12–13). Human neutrophils used for the preparation of 5-HETE were isolated from leukocyte concentrates by sequential dextran sedimentation and gradient separation on Ficoll-Paque according to established methods (14). Contaminating erythrocytes were eliminated by hypotonic lysis. Suspensions of neutrophils thus prepared were 95–98% pure. 12-HETE was generated using human platelets isolated from platelet-rich plasma obtained by centrifuging anticoagulated whole blood at 120 *g* at room temperature for 15 min. Platelets were then isolated from platelet-rich plasma by centrifuging at 1,100 *g* for 15 min, washing twice with PD, and final resuspension in phosphate-buffered saline (PBS) containing calcium and magnesium at 37°C.

Human neutrophils (30–40 × 10^6/ml) or platelets (the derivative of 100 ml whole blood in 25 ml final buffer) suspended in PBS were incubated with 1 μCi/ml [^{3}H]20:4 and 10 μg/ml of A23187 at 37°C under an atmosphere of 5% CO_2/95% air for 15 min. The reaction was terminated by the addition of 1.5 vol of cold methanol, and extraction using diethyl ether was performed as described by Borgeat and Samuelsson (9). Whole extracts were dried to nil under nitrogen and subjected to silicic acid chromatography, also as described (12). Respective silicic acid column fractions containing mono-HETEs derived from either the neutrophil or platelet preparations were subjected to HPLC using solvent system 1 for final purification of 5- and 12-HETEs.

15-HETE was generated by incubating an appropriate mixture of [^{3}H]20:4 and unlabeled 20:4 with soybean lipoxidase under the conditions described by Funk et al. (15). In brief, 0.9 × 10^{-3} M 20:4 in 0.047 M borate buffer (pH 9) containing 3,000 U/ml of lipoxidase was incubated under an atmosphere of 95% O_2 at 20°C for 5 min. The reaction products were

extracted as described for the extraction of oxygenated metabolites of 20:4 from incubation media described above. After reduction with triphenylphosphine (16), the reaction products were partially purified by thin layer chromatography on heat-activated (120°C, 2 h) silica gel H plates in hexane/ethyl acetate/acetic acid (1:1:0.005, vol/vol/vol). The major band of radioactivity was eluted from the plates and further purified by HPLC in solvent system 1.

Each of the three mono-HETEs generated by these methods was separable by HPLC using solvent system 1. Typical elution times under the specified conditions were 34–35, 38–39, and 45–47 min for 15-, 12-, and 5-HETEs, respectively. The δ-lactone of 5-HETE was found to elute at 55–65 min.

All purified mono-HETE preparations were quantified by measuring the optical density in methanol at 235 nm (ϵ = 30,500 [9]) and were stored in chloroform/methanol (2:1, vol/vol) at −20°C under nitrogen until used.

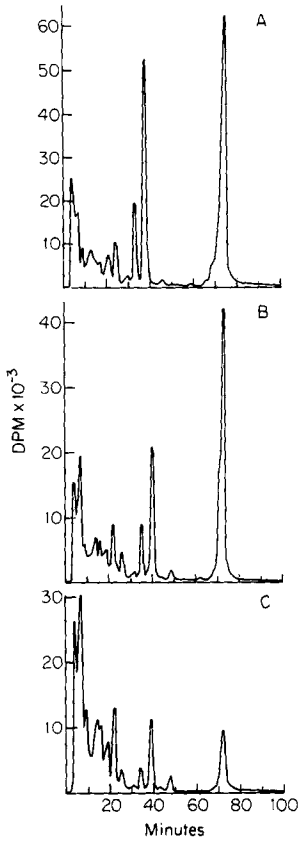
Results

Time-Course of Exogenous 20:4 Metabolism. In a previous report (4), we found that, in the absence of a stimulus, macrophages maintained in serum-free medium both rapidly incorporate exogenously supplied 20:4 into cellular lipids and metabolize the fatty acid to more polar metabolites. Over a wide concentration range (10 nM–1 μM), uptake was found to plateau after 10 min. Thereafter, ~30% of the added [^{3}H]20:4 remained cell-associated, and 70% was found in the medium (4). Fig. 1 shows the kinetics of exogenously supplied 20:4 conversion to polar metabolites. After 5 min of incubation, 70% of the radiolabel remaining in the culture medium was metabolized. Greater than 90% conversion was evident by 60 min. Similar kinetics were observed when the 20:4 concentration was varied from 6.4 nM to 1 μM. These observations suggest that the production of oxygenated metabolites from exogenously supplied 20:4 is significantly more rapid than that from endogenous 20:4 released in response to specific stimuli (1).

Fig. 1 also shows that under these conditions, mono-HETEs represent a major class of metabolites whose proportions are found to decrease as a function of incubation time. Mono-HETEs were 36.7%, 26.1%, and 11.9% of the total 20:4 metabolites recovered from the incubation medium at 5, 10, and 60 min, respectively. In experiments such as that illustrated in Fig. 1, the mono-HETE proportion of the total metabolites decreased threefold to fourfold over the 5–60 min incubation interval. Concomitant with this decrease in mono-HETEs was an increase in more polar metabolites eluting in fractions 4–25 in solvent system 1 (Fig. 1).

The decrease in the proportion of mono-HETEs recovered from the medium over time was postulated to result from additional metabolic events.

Metabolism of 15-, 12-, and 5-HETEs. The metabolic fate of exogenously supplied mono-HETEs was directly examined by incubating resting macrophage cultures with radiolabeled 15-, 12-, and 5-HETEs (final concentrations, 1 μM). As shown in Fig. 2, each compound was extensively metabolized. A number of polar products, including one major peak with a retention time of 13–16 min, was produced from each mono-HETE. HPLC analyses of the radiolabel recovered from the medium showed that after 2 h of incubation, 87.8 ± 6.0% of 15-HETE, 79.2 ± 0.5% of 12-HETE, and 100% of 5-HETE (n = 2) were converted to more polar metabolites. Greater than 98% of each HETE was recovered in unmodified form after incubation in the absence of cells (Fig. 2). Cell viabilities of cultures exposed to 1-μM concentrations of mono-HETEs for 2 h always remained comparable to control cultures exposed to α-MEM alone



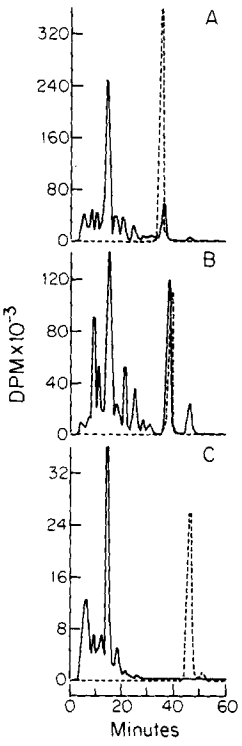


FIG. 1. (left) HPLC chromatograms of ^{3}H-labeled products in culture medium after incubation of resident macrophages with 0.5 μCi of [^{3}H]20:4 (6.4 nM). A, 5 min; B, 10 min; and C, 60 min. Macrophages cultured for 16 h were washed with cold PD, overlaid with cold α-MEM containing [^{3}H]20:4, and then incubated at 37°C for the indicated periods of time. Media were extracted and subjected to HPLC using solvent system 1, as described in Materials and Methods. HPLC profiles of [^{3}H]20:4 incubated in medium without cells for 60 min showed that at least 90% of the recovered radiolabel was unreacted 20:4. ^{3}H-labeled 6-keto $PGF_{1\alpha}$, $PGF_{2\alpha}$, PGE_2, and TXB_2 elute at 4–11 min; mono-HETE standards elute at 30–50 min, and 20:4 elutes at 70–75 min. Macrophages incubated with 1 μM 20:4 generated polar metabolites with a similar percentage of conversion with time.

FIG. 2. (right) HPLC chromatograms (——) of polar metabolites produced by resident macrophages after 2-h incubation with 1-μM concentrations of 15-(A), 12-(B), and 5-HETEs (C). (- - -) represent the chromatograms obtained after incubation of each respective mono-HETE in medium without cells for 2 h. Resident macrophages in culture for 16 h were washed with PD and incubated in α-MEM containing each mono-HETE. After 2 h, the media were extracted and subjected to HPLC using solvent system 1, as described in Materials and Methods. Mono-HETE elution times vary with the HPLC column age, but the separation of individual mono-HETEs is not affected. The major polar metabolites of each HETE eluted at 13–16 min.

($\leq$99%). Likewise, the phagocytic capacities of cultures incubated with each mono-HETE (1 μM) for 2 h remained comparable to controls.

Time-Course of Mono-HETE Metabolism. The time-course of mono-HETEs metabolism was examined using 15-HETE (Fig. 3). The rate of conversion to polar metabolites is concentration dependent. Approximately 50% conversion was reached at 30 min and 60 min, respectively, for 0.33- and 1-μM HETE. Similar kinetics were observed

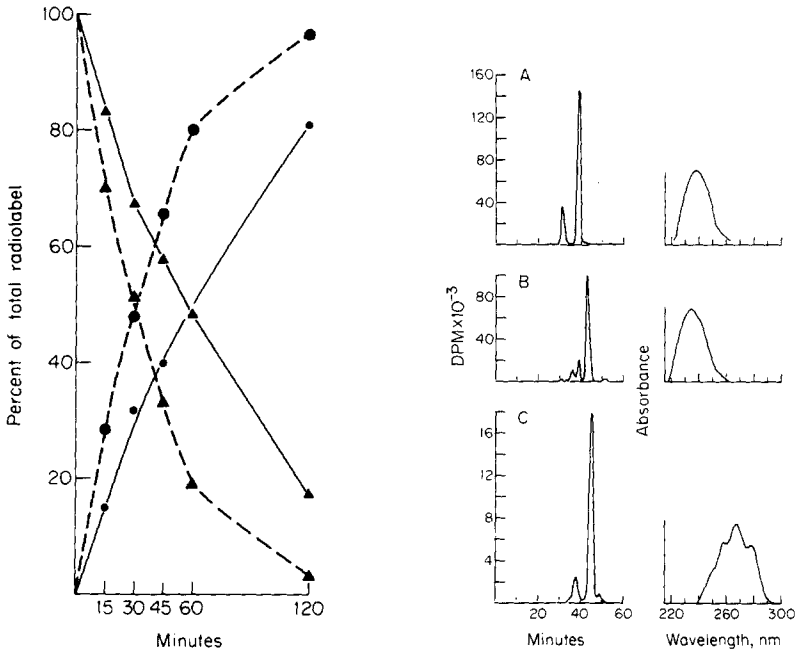


FIG. 3. (left) Time-course of 15-HETE metabolism by resident macrophages. 16-h cultures of resident macrophages were incubated with either 1 μM (——) or 0.33 μM (- - -) concentrations of 15-HETE in α-MEM. At the indicated times, the incubation medium was extracted and subjected to HPLC using solvent system 1, as described in Materials and Methods. Chromatographic data were analyzed for percentage conversion of radiolabel remaining in the medium to metabolites more polar than the native compound. The percent decrease in 15-HETE (▲) and the percent increase in total polar metabolites (●) recovered from the medium over time at each concentration are presented. A similar time-course for metabolism of 5- and 12-HETEs (1 μM) was found after incubation with resident macrophages.

FIG. 4. (right) HPLC chromatograms and ultraviolet absorption spectra of major mono-HETE metabolites generated by resident macrophages. A, 15-HETE; B, 12-HETE; and C, 5-HETE metabolites. The major metabolites of 15-, 12-, and 5-HETEs isolated by HPLC using solvent system 1 (retention time 13–16 min, Fig. 2) were dried under reduced pressure and rechromatographed using solvent system 2, as described in Materials and Methods. The insets to the right show the ultraviolet absorption spectra recorded in HPLC buffer for the major peak in each profile.

for the conversion of 1-μM 12- and 5-HETEs. Resting macrophages, therefore, metabolize each mono-HETE with a time-course comparable to that for the production of oxygenated metabolites from endogenous 20:4 (1).

Rechromatography of the major HETE metabolites generated from each mono-HETE (retention times, 13–16 min, Fig. 2), using solvent system 2, yielded a single major peak of radioactivity, as shown in Fig. 4. Retention times of the products derived from each mono-HETE differed slightly, and their elution order followed that of the three mono-HETEs using solvent system 1 (Fig. 2). Additional smaller peaks of radioactivity separated from each major peak after rechromatography, but the major peak accounted for >75% of the total in each instance.

Ultraviolet absorption spectra were obtained for each of the major metabolites derived from mono-HETEs (Fig. 4). The 15- and 12-HETE products retained the spectral characteristics of the original HETEs (single maxima at 235–237 nm). In contrast, the major 5-HETE metabolite had an ultraviolet spectrum with a maximum

at 268 nm and shoulders at 258 and 278 nm, indicating the presence of three conjugated double bonds (conjugated triene) (12).

Rechromatography of the other more polar metabolites (fractions 4–12, Fig. 2) of 15-, 12-, and 5-HETEs, using solvent system 2, indicated that in total, 10 or more individual metabolites are produced from each mono-HETE after incubation with macrophages.

Incorporation of Mono-HETEs into Cell Lipids. Separate resting macrophage cultures were incubated with 1-μM concentrations of 15-, 12-, or 5-HETEs. After 2 h of incubation, ~12–30% of the recovered radiolabel was cell associated ($n = 3$). The uptake of 5-HETE tended to be greater than that of the other mono-HETEs tested (Table I). Kinetic studies indicated that maximum uptake occurred by ~30 min for each HETE.

Two-dimensional thin-layer chromatography of cell extracts indicated that 76–89% of the cell-associated radiolabel was incorporated into cellular lipid ($n = 2$). Fig. 5 A shows the distribution of radiolabel among the various cell lipids. Each mono-HETE was found incorporated primarily into phospholipid (15-HETE, 83%; 12-HETE, 71.6%; and 5-HETE, 50%), with the remainder recovered in neutral lipid or as free fatty acid. 74.4% and 80.8% of the total phospholipid-associated radiolabel was found in phosphatidylcholine, the major macrophage phospholipid, after incubation with 5- and 12-HETEs, respectively. Similar data for 15-HETE indicated that 73% of the radiolabel in phospholipids was distributed nearly equally between phosphatidylcholine and phosphatidylinositol. In spite of these variations, this general pattern of incorporation into phospholipids (particularly into phosphatidylcholine) is similar to that after incubation of macrophages with 20:4 in serum-free or in serum-containing medium (1, 4).

The uptake of the polar HETEs metabolites by macrophages was also examined. Cultures were incubated with the total metabolites generated from 12-HETE after a 2-h incubation with macrophages (fractions 4–30, Fig. 2 B). Only 3% of the total radiolabel recovered was cell associated. HPLC of the medium extracts revealed an elution profile identical to that before incubation. These results indicate that the polar

TABLE I

Comparison of Resident and Activated Macrophage Uptake and Metabolism of Mono-HETEs

	15-HETE		12-HETE		5-HETE	
I. Uptake*						
A. % in cells	12.4 ± 7.8	(11.2 ± 2.2)	14.3 ± 3.4	(19.1 ± 0.2)	30.5 ± 0.5	(69.0 ± 3.0)
B. % in phospholipid	83.3	(27.5)	71.6	(37.7)	50.1	(15.8)
II. Metabolism‡	88	(17)	79	(27)	100	(65)

All data are expressed as percent of total recovered. Data outside parentheses are derived from experiments with resident macrophages, and data inside are from *C. parvum*-elicited macrophages.

* Macrophages were incubated with each mono-HETE (1 μM) in α-MEM for 2 h, as described in Materials and Methods. A, percent cell-associated radiolabel of the total recovered from medium and cells. Results are given for the percent uptake (mean ± SEM) determined from three to four replicate culture plates per experiment. The uptake of each mono-HETE was tested in three separate experiments with resident macrophages and one experiment with activated macrophages. B, percent cell-associated radiolabel recovered in phospholipids after incubation with resident ($n = 2$) or activated ($n = 1$) macrophages, as described in Fig. 5.

‡ Percent conversion of radiolabeled HETE (1 μM) recovered from incubation medium to polar metabolites after incubation with resident ($n = 2$–3) and activated macrophages ($n = 1$), as described in Fig. 2.

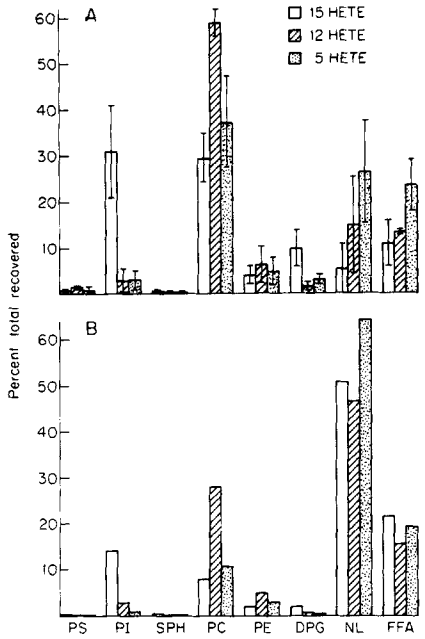


FIG. 5. Incorporation of mono-HETEs into macrophage lipids. 15-, 12-, and 5-HETEs (1 μM) in α-MEM were incubated with resident (A) or *C. parvum*-elicited macrophages (B) for 2 h, as described in Fig. 2. Cell monolayers were then washed, scraped into PD, and the lipids extracted. The extracts were then subjected to two-dimensional thin layer chromatography, as described in Materials and Methods. Areas of chromatograms containing lipid were scraped and the radioactivity of each was determined. Data are expressed as the percentage of the total recovered radiolabel. Each mono-HETE was tested in two experiments with resident macrophages and one experiment with activated macrophages. PS, phosphatidylserine; PI, phosphatidylinositol; SPH, sphingomyelin; PC, phosphatidylcholine; PE, phosphatidylethanolamine; DPG, diphosphatidyl glycerol; NL, neutral lipids; FFA, free fatty acids.

metabolites, once formed, are neither incorporated nor metabolized further by macrophages.

Uptake and Metabolism of Cyclooxygenase Products. Resting macrophage cultures were incubated for 2 h with each of four radiolabeled cyclooxygenase products (PGE_2, $PGF_{2\alpha}$, TXB_2, and 6-keto $PGF_{1\alpha}$) under serum-free conditions. In each instance, at least 95% of the added radiolabel was recovered from the medium at the end of the incubation period. HPLC analyses of the radiolabel extracted from the medium revealed that these products were not altered by exposure to macrophages. In contrast to mono-HETEs, macrophages fail to either take up or metabolize cyclooxygenase products.

Metabolism and Uptake of Mono-HETEs by Activated Macrophages. Previous studies (4) showed that exogenous 20:4 metabolism was reduced and that uptake was increased in cultures of activated macrophages. Similarly, the extent of mono-HETE metabolism was markedly diminished in cultures of *C. parvum*-activated macrophages. 83%, 73%, and 45% of 15-, 12-, and 5-HETEs (1 μM) were recovered unmodified after a 2-h incubation with *C. parvum*-activated macrophages (Table I).

The uptake of 15- and 12-HETEs (11.2–19.2%) by *C. parvum*-activated macrophages was comparable to that by resident cells, whereas the incorporation of 5-HETE was

twofold greater (69%) than by resident macrophages (Table I). Most of the radiolabel recovered in lipid extracts of activated cells represented HETEs incorporated into cell lipids (71.3–80.5%). However, in contrast to resident cells, the cell-associated radiolabel in these activated cells was recovered predominantly (51%, 47%, and 65% for 15-, 12-, and 5-HETEs, respectively) in neutral lipid (Fig. 5 B).

Discussion

Mouse peritoneal macrophages have the capacity to metabolize exogenously supplied 20:4 in the absence of a discernible trigger (4). In this report, we show that substantial conversion of exogenous 20:4 to oxygenated metabolites occurs within 10 min (Fig. 1). This contrasts with the 60-min time-course for phospholipid-derived 20:4 release and metabolism in response to an inflammatory stimulus, a setting in which the rate-limiting step appears to be the release of fatty acid (1). The metabolism of exogenously supplied 20:4 thus provides an estimate of the rate at which macrophages convert 20:4 to oxygenated metabolites. Within the experimental limits of detection and within the lethal tolerance of macrophages for 20:4 (6.4 nM–1 μM), we were unable to either saturate the reaction or estimate a rate-limiting concentration of fatty acid substrate. It is therefore evident that these cells have a considerable capacity for the synthesis of cyclooxygenase and lipoxygenase products.

As noted previously (4), the proportion (70%) of exogenous 20:4 metabolized via the lipoxygenase pathway is considerably greater than from endogenous 20:4 stores (35%). Mono-HETEs and polar lipoxygenase products other than leukotriene C are the predominant metabolites generated from 20:4 in the culture medium. Mouse peritoneal macrophages synthesize 12-HETE (17) and also have the capacity to produce leukotriene C, the major lipoxygenase product formed from endogenous 20:4 after appropriate stimulation (2–3). This indicates the presence of 12- and 5- lipoxygenases. Although the individual mono-HETEs generated remain to be identified by mass spectrometric means, our results indicate the macrophage produces from exogenous 20:4 at least two major products with chromatographic properties compatible with mono-HETEs. Two of these products (elution times, 34–35 and 38–39 min; Fig. 1) co-chromatograph with 15- and 12-HETE standards on reverse-phase HPLC.

Macrophages are a potent source of 20:4 metabolites derived secondarily from mono-HETEs. 5-, 12-, and 15-HETEs are readily converted to more polar derivatives by macrophages. These reactions are specific in that neither cyclooxygenase products (prostaglandins and thromboxane) nor polar products of mono-HETEs are metabolically altered. A number of metabolic routes for related 20:4 metabolites have been described. Certain prostaglandins may undergo β- or ω-oxidation and ω-hydroxylation (18). Leukotriene B_4 (LTB_4; 5(S), 12(R)-dihydroxy-6,8,10,14-*cis*, *trans*, *trans*, *cis*-eicosatetraenoic acid), a mediator of neutrophil chemotaxis and adherence to endothelium (19–20), has recently been shown to undergo ω-hydroxylation and subsequent conversion to a dicarboxylic acid with a resulting change in its spectrum of biologic activity (21). Finally, sequential oxygenation reactions are possible, as have been shown for the generation of 5(S), 12(S)-dihydroxy-6,8,10,14-eicosatetraenoic acid [5(S), 12(S)-di-HETE] (22).

Interestingly, the major metabolite generated by macrophages from 5-HETE exhibits a triene spectrum with absorption maxima identical to that of 5(S), 12(S)-di-HETE (22). In addition, this compound has an elution time comparable to LTB_4 on

reverse-phase HPLC (N. A. Pawlowski, unpublished results) as does 5(S), 12(S)-di-HETE, as shown by others (22–23). Taken together, these findings suggest that the major metabolite of 5-HETE might arise from the introduction of a second hydroxyl group at position 12. The 5-HETE major metabolite, however, did not elicit a chemotactic response when tested with human neutrophils over a wide concentration range (maximum, 1 μM),[2] indicating nonidentity with LTB_4. The formation of 5(S), 12(S)-di-HETE has previously been shown with neutrophils incubated with 20:4 (100–150 μM) in the presence or absence of calcium ionophore A23187 (22–23). Although biologic activity has been reported for this compound (22), its physiologic role remains undetermined. It should be noted that the major metabolites of 12- and 15-HETEs generated by macrophages also have chromatographic properties of di-HETEs. However, neither of these derivatives have a triene spectrum. In the case of the 12-HETE derivative, this suggests that the second hydroxylation step does not occur at position 5. The structures and biological activities of these major metabolites of mono-HETEs are currently under investigation.

In addition to metabolic conversion, macrophages incorporate mono-HETEs into cell lipid. This reaction is specific for mono-HETEs in that macrophages fail to take up other oxygenated derivatives of 20:4. The uptake of mono-HETEs suggests a possible physiologic role for these compounds in regulating macrophage function. This is emphasized by their predominant incorporation into phospholipid and presumably cell membranes. Resting neutrophils likewise take up exogenously supplied mono-HETEs (24–25), but the extent of incorporation into phospholipids (22% and 12.5% of the cell-associated 5- and 12-HETE) contrasts with the high percentage of mono-HETEs incorporated into macrophage phospholipid (50–83%).

The reduced capacity of activated macrophages to metabolize each mono-HETE parallels the reduction in the generation of oxygenated metabolites from 20:4 in this cell population (5). This finding suggests that inhibition of lipoxygenase activity occurs as a result of macrophage activation in addition to the loss of prostacyclin synthetase (5) and specific enzyme(s) for leukotriene C biosynthesis (5). Because oxidative metabolism and esterification are distinct fates of exogenous 20:4 (4), the enhanced incorporation of 20:4 into cell phospholipid exhibited by activated macrophages might be a result of this diminished metabolic capacity. It is surprising, therefore, that only the uptake of 5-HETE was increased in activated cells and that incorporation was largely into neutral lipid rather than phospholipid. This might indicate that the mechanisms for esterification of 20:4 and HETEs are distinct, with the latter controlled by the activation state of the macrophage. Alternatively, the phospholipids of activated cells might be enriched with naturally occurring HETEs as a result of an in vivo phagocytic event causing the cell to shunt additional HETEs into neutral or storage lipid.

Summary

Within 5 min, resting macrophages metabolize μM quantities of exogenous arachidonic acid (20:4) to cyclooxygenase and lipoxygenase products. Mono-HETEs represent a major class of metabolites recovered from the medium. However, the quantity of mono-HETEs progressively decreases over a 60-min incubation period, with a concomitant increase in more polar lipoxygenase products, suggesting additional

[2] The chemotactic assay was kindly performed by Dr. Eva Cramer.

metabolic fates for these hydroxy acids. This was directly confirmed by exposing resident macrophage cultures to radiolabeled 15-, 12-, and 5-HETEs (1 μM). 12–30% of the recovered HETEs were cell-associated and predominantly esterified into phospholipid. High pressure liquid chromatography analyses of medium extracts indicated that 50% of each HETE was also converted to 10 or more metabolites over a 60-min time-course, a rate slower than for 20:4. The major metabolite generated from each mono-HETE had the elution characteristics of a di-HETE. The 5-HETE product has a triene spectrum similar to that of 5(S), 12(S)-di-HETE, whereas the 15- and 12-HETE products exhibited single ultraviolet absorption maxima, indicating a metabolic pathway for 5-HETE distinct from the other mono-HETEs. None of the stable cyclooxygenase products of 20:4 (6-keto $PGF_{1\alpha}$, $PGF_{2\alpha}$, PGE_2, TXB_2) nor polar metabolites of mono-HETEs are either incorporated or metabolized. The results indicate that macrophages have the capacity to specifically metabolize 20:4 and mono-HETEs to polar oxygenated products in the absence of a discernible trigger.

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