Biosynthesis of 5-Oxo-6,8,11,14-eicosatetraenoic Acid from 5-Hydroperoxyeicosatetraenoic Acid in the Murine Macrophage*

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5-Oxo-6,8,11,14-eicosatetraenoic acid (5-oxo-ETE) is a metabolite of arachidonic acid shown to possess important biological activities within different cell types. In the neutrophil, a specific NADPH-dependent dehydrogenase utilizes 5-lipoxygenase-derived 5-hydroxy-6,8,11,14-eicosatetraenoic acid (5(S)-HETE) as the required substrate. In the present study, 5-hydroperoxy-6,8,11,14-eicosatetraenoic acid (5(HpETE)), rather than 5-HETE, was found to be the biosynthetic precursor of 5-oxo-ETE in the murine macrophage. The macrophage was not able to convert 5-HETE into 5-oxo-ETE even when preincubated with phorbol ester or with other lipid hydroperoxides. The factor responsible for the conversion of 5-HpETE into 5-oxo-ETE was found predominantly in the cytosolic fraction of the macrophage, with an approximate molecular weight of 50,000–60,000, as assessed by size exclusion chromatography. Formation of 5-oxo-ETE was rapid and the catalytic protein was found to have an apparent $K_m$ of 5.3 μM for the eicosanoid. Furthermore, the protein could efficiently utilize 5(R,S)-HpETE as substrate and was heat and protease labile. This novel pathway of 5-oxo-ETE biosynthesis in the murine macrophage was consistent with reduction of a 5-hydroperoxy group to an intermediary alkoxy radical that could be subsequently oxidized to the 5-oxo product. Such a mechanism would enable racemic 5-HpETE, derived from free radical oxidation of arachidonic acid, to be efficiently converted into this potent chemotactic eicosanoid.

Arachidonic acid is the precursor of a number of lipid mediators of diverse activity as well as chemical structure and whose formation is controlled by the action of several enzymatic systems. One biosynthetic pathway involves 5-lipoxygenase, which initiates a cascade of arachidonic acid metabolism leading ultimately to the formation of a group of biologically active compounds, including the leukotrienes (1). The molecular events directed by 5-lipoxygenase involve insertion of molecular oxygen at carbon-5 of the arachidonate chain with formation of 5(S)-hydroperoxy-6,8,11,14-eicosatetraenoic acid (5(S)-HpETE),¹ which can be reduced by peroxidases to the hydroxy analog 5(S)-hydroxy-6,8,11,14-eicosatetraenoic acid (5(S)-HETE) or stereospecifically dehydrated to leukotriene A₄ (LTA₄) by a second 5-lipoxygenase-catalyzed step (2). LTA₄ can be further converted into LTB₄ by the enzyme LTA₄ hydrolyase (3) or into the cysteinyl-leukotrienes LTC₄, LTD₄, and LTE₄, by the enzyme LTC₄ synthase (4). LTB₄ is a potent chemokinetic and chemotactic agent for the human polymorphonuclear leukocyte (5), whereas LTC₄ and LTD₄ are among the most potent mediators of bronchoconstriction in man (6).

More recently, another 5-lipoxygenase metabolite, 5-oxo-6,8,11,14-eicosatetraenoic acid (5-oxo-ETE), has been identified as being made within human polymorphonuclear leukocytes (7, 8), eosinophils (9), monocytes, and lymphocytes (10). This eicosanoid elicits a different set of important biological activities as a potent agonist increasing cytosolic calcium levels, chemotaxis, and degranulation by a mechanism independent of the LTB₄ receptor in the human neutrophil (11, 12). Low concentrations have been shown to increase the surface expression of the β₂ integrin CD11b, actin polymerization and adherence (13). It is the most active lipid-derived chemoattrant factor for human eosinophils (14, 15), with a potency in the range of the CC chemokines, eotaxin and RANTES (regulated on activation normal T cell expressed and secreted), both of which also enhance 5-oxo-ETE-induced chemotaxis (16). This keto eicosanoid also causes L-selectin shedding, surface expression of CD11b, calcium mobilization, and actin polymerization (17). It can also activate directional migration and actin polymerization within the human monocyte (18), and lead to volume reduction of guinea pig intestinal epithelial cells (19). Furthermore, 5-oxo-ETE has been shown to promote eosinophil transmigration through basement membranes (20), suggesting an important role for this eicosanoid in the recruitment of these potent inflammatory cells in pathologic conditions such as asthma and allergy. Recently, a specific G-protein-linked receptor with high affinity for 5-oxo-ETE has been reported (21).

The biosynthesis of 5-oxo-ETE has been extensively examined in human neutrophils with the identification of a specific microsomal NADPH-dependent dehydrogenase responsible for the conversion of 5(S)-HETE, but not 5(R)-HETE (7). In intact cells, significant amounts of this metabolite are synthesized only when neutrophils are preincubated with phorbol myristate acetate (PMA), a protein kinase C activator that elevates NADPH (22). The same biosynthetic pathway for 5-oxo-ETE has been also identified in human monocytes and lymphocytes (10).

An alternative pathway for 5-oxo-ETE synthesis could proceed from 5-HpETE, because unsaturated fatty acid hydroperoxides have been shown to be direct precursors of oxo-fatty acids catalyzed by hematin and heme-containing proteins such as hemoglobin (23, 24). In a similar manner, platelet 12-lipoxygenase and soybean 15-lipoxygenase under anaerobic conditions has been shown to convert 12-HpETE and 15-HpETE,
respectively, into the corresponding 12- and 15-oxo derivatives (25).

Recently, 5-oxo-ETE was found to be an eicosanoid synthesized within the elicited murine peritoneal macrophage (26), but the pathway responsible for the production of 5-oxo-ETE was not investigated. The objective of the present study was to define the biosynthetic pathway that leads to the synthesis of 5-oxo-ETE in the macrophage and elucidate the mechanism responsible for the formation of this keto eicosanoid in this cell type.

**EXPERIMENTAL PROCEDURES**

**Materials—**5-Oxo-ETE, 5(S)-HETE, 5(S)-HpETE, 5(R,S)-HpETE, 15(S)-hydroperoxyeicosatetraenoic acid (15(S)-HpETE), 13(S)-hydroperoxyoctadecadienoic acid (13(S)-HpODE), d5-HETE, and d5-oxo-ETE were purchased from Cayman Chemical Company (Ann Arbor, MI). All solvents were HPLC grade and obtained from Fisher. Type I-bovine serum albumin was purchased from Millipore Corp., Bedford, MA) fed with deionized water. CompleteTM protease inhibitor mixture tablets were obtained from Roche Molecular Diagnostics. Dulbecco's modified Eagle’s medium and Hanks’ balanced salt solution were purchased from Cellgro by Mediatech Inc. (Herndon, VA). Stanton chloride anhydrous (SnCl2), PMA, trypsin, hematin, and phosphate-buffered saline were purchased from Sigma. Thiglycollate was purchased from BD Difco (Franklin Lakes, NJ).

**Preparation of Murine Elicited Peritoneal Macrophages—**Elicited macrophages were obtained by injecting 1 ml of 4% thiglycollate into the peritoneum of ICR mice. After 3 days, the mice were euthanized in a CO2 atmosphere. For the experiments with intact cells, the peritoneum was lavaged once with 10 ml of Dulbecco’s modified Eagle’s medium, 10% fetal bovine serum, 100 units/ml penicillin, and 100 g/ml streptomycin, and peritoneal macrophages were plated onto polystyrene 6-well culture dishes (Corning Inc., Corning, NY) at a concentration of 3 x 105 cells/well. For some experiments, the peritoneum was lavaged once with 10 ml of cold Hanks’ balanced salt solution and cells were kept in suspension at the concentration of 20-30 x 106 cells/ml. The resulting supernatant was subjected to centrifugation at 100,000 x g for 60 min at 4 °C. The cytosolic fraction (supernatant) and the pellet (microsomal fraction), resuspended in lysis buffer, were kept on ice until used. For some experiments the cytosol fraction (100 μl) was resuspended in a boiling water bath (94 °C) for 20 min prior to testing for enzymatic activity.

**Cell and Cytosolic Fraction Incubations—**Cytosolic fractions were prepared according to the method of Haslett et al. (27). Additional experiments were performed in macrophages, where 5(S)-HpODE (1 μM) was coincubated with 5(S)-HpETE or 5(S)-HpHODE (1 μM) and 5(S)-HETE or 5(R,S)-HpETE (1 μM) for 30 min at 37 °C; supernatants were then collected in 1 volume of ice-cold methanol containing 5 μg of d5-HETE and 10 ng of d5-oxo-ETE as internal standards. Identical incubations were conducted using human neutrophils (3 x 106/ml) and with cytosol fraction (100 μl) was resuspended in a boiling water bath (94 °C) for 20 min prior to testing for enzymatic activity.

**Intact Cell Incubations—**Elicited peritoneal macrophages (3 x 106 cells/ml) were incubated for 30 min at 37 °C with 5(S)-HETE (1 μM) and the formation of specific products was analyzed by combined liquid chromatography-mass spectrometry. Specific ion transitions formed by collisional activation were monitored to detect the elution of 5-oxo-ETE (m/z 317 → 203) and 5-HETE (m/z 319 → 115) as well as internal standards added in this experiment for quantitative analysis, d5-HETE (m/z 327 → 116) and d5-oxo-ETE (m/z 324 → 210). There was no significant production of 5-oxo-ETE derived from the exogenous added 5-HETE in the murine macrophage (Fig. 1); in contrast, the incubation of 5-HETE with human polymorphonuclear leukocytes resulted in the formation of a small, but
The process involved in the conversion of 5(S)-HpETE into 5-oxo-ETE for which there was only a small quantity observed at this retention time. Additional experiments were carried out by incubating 5(S)-HpETE (1 μM) in the presence of d$_3$-5(S)-HETE (2 μM) to assess whether or not there was any direct precursor role for 5-HpETE. In this experiment the formation of 5-oxo-ETE from the d$_3$-5(S)-HETE would be revealed by a product eluting at the expected retention time at 27.5 min only having a molecular anion at m/z 324 (d$_3$-product). Collision-induced decomposition of this molecular anion would result in a product ion at m/z 210 and this transition could be used to detect the formation of any d$_3$-5-oxo-ETE from d$_3$-5-HETE. In this experiment there was a robust formation of unlabeled 5-oxo-ETE as indicated by the component eluting at 27.5 min, having the transition m/z 317 → 203 for unlabeled 5-oxo-ETE, and yet very little conversion of d$_3$-5-HETE into d$_3$-5-oxo-ETE (Fig. 4). These studies further supported a direct conversion of 5-HpETE into 5-oxo-ETE without intermediate formation of 5-HETE.

Subcellular Localization—Cytosolic and microsomal fractions from murine peritoneal macrophages were prepared after sonication and successive centrifugations at 12,000 and 100,000 × g. Each of these subcellular fractions were incubated with 5(S)-HpETE (1 μM) for 10 min at 37 °C. The biosynthetic activity for 5-oxo-ETE was found predominantly within the cytosolic fractions (Table I) with 17.2 ± 14 and 6.5 ± 0.5 pmol/10⁶ cells of 5-oxo-ETE formed in the cytosolic and microsomal fractions, respectively. Various cofactors were also added to each of these subcellular fractions, including NADP$^+$, NADPH, and NADH (each at 1 mM) to examine whether or not they had any effect on 5-oxo-ETE biosynthesis from either 5(S)-HpETE or 5(S)-HETE (1 μM). After pretreatment of each of the subcellular fractions with the cofactors for 5 min at 37 °C, the production of 5-oxo-ETE was assessed by LC/MS/MS analysis. There was no significant increase in 5-oxo-ETE biosynthesis when NADP$^+$ and NADH were added; however, there was a significant decrease in 5-oxo-ETE production (p < 0.01) when NADPH was added. The production of 5-oxo-ETE was 10–15 times higher when 5-HpETE was used as substrate relative to 5-HETE (Table II).
5-oxo-ETE was found not to be greatly altered by boiling for 20 min, because the rate of 5-oxo-ETE production dropped only 20% after heating. Furthermore, this treatment caused the formation of substantial denatured proteins observed as an abundant precipitate that had no biochemical activity (data not shown).

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Incubation of racemic 5-HpETE with the cytosolic fraction prepared from separate macrophages was found to yield substantial 5-oxo-ETE (16.2 pmol/10^6 macrophages), virtually identical to that observed when the same preparation of macrophage cytosol was incubated with the same concentration of 5(S)-HpETE (17.2 pmol/10^6 macrophages) (Table II). Thus, the cytosolic fraction displayed the same lack of stereospecificity observed with intact cell incubations in the formation of 5-oxo-ETE. The cytosolic fraction also catalyzed conversion of 13-HpODE and 15-HpETE into their respective oxo-lipids, but to a reduced extent with 6.6 pmol/10^6 cells of 13-oxo-ODE and 6.0 pmol/10^6 cells of 15-oxo-ETE under conditions identical to those used for Table I.

The effect of 5-HpETE substrate concentration on the total rate of 5-oxo-ETE revealed saturation behavior with approximate V_max of 92.2 pmol/mg of protein/min and apparent K_m of 5.3 μM 5-HpETE (Fig. 5B). Furthermore, the formation of 5-oxo-ETE from 5-HpETE was relatively rapid and incubations longer than 10 min led to a diminution of the quantity of 5-oxo-ETE present in these cells. Because it is known that 5-oxo-ETE can be rapidly metabolized to several metabolites, including a glutathione adduct termed FOG_7, it is likely that

| Fraction          | 5-Oxo-ETE pmol/10^6 cells |
|-------------------|--------------------------|
| Intact cells      | 20.4 ± 1.8               |
| Cytosol           | 17.2 ± 1.4               |
| Microsomal fraction | 6.5 ± 0.5               |

5-Oxo-ETE synthesis in the Macrophage

Table I

Macrophages (5 × 10^6/ml cells) and aliquots (1 ml) of cytosolic and microsomal fractions (equivalent to 5 × 10^6 cells/ml) were incubated for 10 min at 37 °C with 5(S)-HpETE (1 μM, final concentration). Samples were collected in 1 ml of ice-cold methanol containing the internal standards d_7-5-oxo-ETE (10 ng) and d_8-5-HETE (5 ng) and solid phase extraction was performed using C18 solid phase extraction cartridges. Metabolites were analyzed by RP-HPLC followed by electrospray ionization mass spectrometry and multiple reaction monitoring. Mean ± S.E. of three different cell preparations, with each sample run in duplicate.

FIG. 3. Mass spectrometric analysis by multiple reaction monitoring of supernatants from peritoneal macrophages incubated with 5(S)-HETE in the presence of hydroperoxides. Mass spectrometric analysis of extracts from the supernatant of murine peritoneal macrophages (3 × 10^6/ml) incubated for 30 min at 37 °C with 5(S)-HETE (1 μM) or (B) 13(S)-HpODE (1 μM). Elution of 5-oxo-ETE was detected by monitoring the specific transition m/z 317 → 203.

FIG. 4. Mass spectrometric analysis by multiple reaction monitoring of supernatants from peritoneal macrophages incubated with d_8-5(S)-HETE and 5(S)-HpETE. Mass spectrometric analysis of extracts from the supernatant of murine peritoneal macrophages (3 × 10^6/ml) incubated for 30 min at 37 °C with 5(S)-HpETE (1 μM) in the presence of d_8-5(S)-HETE (2 μM). A, elution of 5-oxo-ETE was detected by the ion transition m/z 317 → 203. B, elution of d_7-5-oxo-ETE was detected by the ion transition m/z 324 → 210.
5-Oxo-ETE synthesis by peritoneal macrophage cytosolic fractions in the presence of different cofactors and by boiled cytosol

Aliquots (1 ml) of cytosolic fractions from murine peritoneal macrophages (equivalent to 5 × 10⁶ cells/ml) were incubated for 10 min at 37 °C with 5(S)-HpETE, 5(R,S)-HETE, or 5(S)-HETE (final concentration 1 μM). Cytosolic fractions were also incubated for 10 min at 37 °C with 5(S)-HpETE or 5(S)-HETE in the presence of each cofactor at a final concentration of 1 mM. Cytosolic fractions were boiled for 20 min or treated with trypsin (2.5 mg/ml) for 10 min at 37 °C and incubated for 10 min at 37 °C with 5(S)-HpETE. Mean ± S.E. are from four different cell preparations with each sample run in duplicate.

| Cofactors | 5-oxo-ETE | pmol/10⁶ cells |
|-----------|-----------|----------------|
| Cytosol + 5(R,S)-HpETE (1 nmol) | 16.17 ± 2.2 |
| 5(S)-HpETE (1 nmol) | 17.2 ± 1.4 |
| NADP⁺ | 17.7 ± 1.6 |
| NADPH | 10.3 ± 1.7 |
| NADH | 17.4 ± 1.4 |
| 5(S)-HETE (1 nmol) | 0.34 ± 0.15 |
| NADP⁺ | 0.37 ± 0.22 |
| NADPH | 0.23 ± 0.11 |
| NADH | 0.37 ± 0.25 |
| Boiled cytosol + 5(S)-HpETE (1 nmol) | 13.9 ± 1.46 |
| Cytosol + trypsin (2.5 mg/ml) + 5(S)-HpETE (1 nmol) | 18.3 ± 0.5 |

Fig. 5. Time course of 5-oxo-ETE production by peritoneal macrophage cytosolic fractions incubated with 5(S)-HpETE. Murine peritoneal macrophage cytosolic fractions (from 5 × 10⁶ cells) were incubated for 1, 3, 10, and 30 min at 37 °C with 5(S)-HpETE (1 μM). A, 5-oxo-ETE was analyzed by RP-HPLC followed by electrospray ionization-mass spectrometry. Mean ± S.E. are of three different cell preparations, with each sample run in duplicate. B, an aliquot of macrophage cytosol (equivalent to 5 × 10⁶ cells) was incubated with 5(S)-HpETE (0.1, 0.3, 1, 3, 10, 30, and 100 μM) for 1 min at 37 °C. The apparent kinetic parameters were calculated after curve fitting to the Michaelis-Menten equation.

FIG. 6. 5-Oxo-ETE synthesis in fractions eluted from size exclusion column. Aliquots (10 μl) of (A) cytosolic fractions (from 5 × 10⁶ cells), (B) boiled cytosol fractions, and (C) from cytosolic fractions pretreated with trypsin (2.5 mg/ml) were loaded on a size exclusion column and 1-min fractions (1 ml) were collected for 14 min. From each fraction, 10 μl was incubated for 10 min at 37 °C with 5(S)-HpETE (1 μM). 5-Oxo-ETE was analyzed by RP-HPLC followed by electrospray ionization-mass spectrometry. Inset, standard curve obtained running on the size exclusion column (50 μg) of (a) IgG, (b) bovine serum albumin, (c) glutathione S-transferase, (d) insulin, and (e) vitamin B₁₂. For these measurements UV absorbance was monitored at 214 and 280 nm.

Fig. 5. Time course of 5-oxo-ETE production by peritoneal macrophage cytosolic fractions incubated with 5(S)-HpETE. Murine peritoneal macrophage cytosolic fractions (from 5 × 10⁶ cells) were incubated for 1, 3, 10, and 30 min at 37 °C with 5(S)-HpETE (1 μM). A, 5-oxo-ETE was analyzed by RP-HPLC followed by electrospray ionization-mass spectrometry. Mean ± S.E. are of three different cell preparations, with each sample run in duplicate. B, an aliquot of macrophage cytosol (equivalent to 5 × 10⁶ cells) was incubated with 5(S)-HpETE (0.1, 0.3, 1, 3, 10, 30, and 100 μM) for 1 min at 37 °C. The apparent kinetic parameters were calculated after curve fitting to the Michaelis-Menten equation.

The conversion of 5-HpETE into 5-oxo-ETE was evaluated using size exclusion chromatography. Aliquots (10 μl) of macrophage cytosol and boiled cytosol (20 min) were injected onto an HPLC size exclusion chromatographic column and 1-min fractions collected for 14 min. Fractions were then tested for the presence of substances that would catalyze the conversion of 5-HpETE to 5-oxo-ETE under the standard conditions of 1 μM substrate concentration for 10 min at 37 °C. Only fractions eluting at 7 and 8 min had the capacity to convert 5-HpETE to 5-oxo-ETE (Fig. 6). Calibration of the HPLC size exclusion column with a number of proteins of different molecular weight (Fig. 6, inset) suggested that the factor(s) present in the cytosol had an apparent molecular weight between the 60,000 and 25,000 markers, calculated to be 55,000. This molecular weight suggested that a reasonably large protein was responsible for the catalytic activity rather than a low molecular weight substance such as inorganic iron or a small prosthetic group such as hematin. After boiling the cytosol (Fig. 6B) there was a substantial loss of catalytic activity in the size exclusion fractions (7–8 min), in sharp contrast to the results obtained from the analysis of the crude cytosol and boiled cytosol (Table I), where only a slight drop in catalytic activity was observed. This suggested that perhaps the high molecular weight protein released a factor such as hematin, which could carry out this conversion of 5-HpETE into 5-oxo-ETE as had been previously described (23). Attempts to chromatograph hematin either with the indicated mobile phase or at pH 9 to increase hematin solubility were unsuccessful with this column. Even injecting

metabolic reactions decreased the apparent level of 5-oxo-ETE at these longer incubation times (Fig. 5A).

Size Exclusion Chromatography—An evaluation of the molecular size of the factor present in the cytosol responsible for
larger amounts of hematin (8 nmol) did not result in elution from the column of detectable quantities of this porphyrin. Hematin was also added to the cytosol before as well as after boiling to final concentrations of 0.5 and 5 μM. With untreated cytosol, 2.0 to 2.5 ng of 5-oxo-ETE was formed at both concentrations of added hematin, identical to the results reported in Fig. 6. After boiling the cytosol and adding hematin (0.5 and 5 μM), the catalytic activity of size exclusion fractions eluting between 7 and 8 min (Fig. 6B) were also not increased (data not shown).

Additional experiments were also carried out with treatment of the cytosol preparation with the serine protease trypsin (2.5 mg/ml final concentration). Measurement of the catalytic activity of the crude cytosol after trypsin treatment revealed no loss of activity (Table II). However, size exclusion separation of the trypsinized cytosol resulted in substantial loss of catalytic activity in the components eluting between 7 and 8 min (Fig. 6C).

**DISCUSSION**

The murine peritoneal macrophage has been known for sometime to be an active cell in eicosanoid biosynthesis. It expresses cyclooxygenase, both COX-1 and COX-2 (29), as well as 5-lipoxygenase, which can metabolize arachidonic acid to 5(S)-HpETE and leukotrienes. As found in the studies reported here, the macrophage is also capable of efficient conversion of 5-HpETE into 5-oxo-ETE, another biologically active eicosanoid widely thought to be a product only of the 5-lipoxygenase pathway. Within the macrophage 5-oxo-ETE is conjugated with glutathione to afford the adduct FOG7, which is chemotactic for the eosinophil as well as neutrophil, but does not elevate intracellular calcium in the latter cell type (30). The formation of FOG7 has now been shown to be catalyzed by LTC₄ synthase (31), which is also expressed in the murine peritoneal macrophage. Thus, a host of lipid mediators can result from the oxidation of arachidonic acid within the peritoneal macrophage. However, the biosynthesis of 5-oxo-ETE from 5-HpETE shown in this report is not critically dependent upon the stereochemistry at carbon-5, in that the racemic 5(R, S)-HpETE was fully capable of being converted to 5-oxo-ETE. Because the chiral center in 5-oxo-ETE is lost in this conversion to the α² carbonyl carbon of 5-oxo-ETE, it is impossible to ascertain whether or not an enzymatically derived, chiral hydroperoxide or free radical-derived hydroperoxide (racemic) is the precursor of this conjugated diene eicosanoid. Nonetheless, this pathway describes an efficient way to utilize free radical-derived HpETE.

5-Oxo-ETE biosynthesis has been extensively studied in the human polymorphonuclear leukocyte, where it is specifically derived from 5(S)-HETE by a NADPH-dependent eicosanoid dehydrogenase (7). In the neutrophil, 5-oxo-ETE is produced only after stimulation with phorbol ester that activates NADPH oxidase with consequent elevation of NADP⁺. The microsomal NADPH-dependent dehydrogenase does not convert 5(R)-HETE into 5-oxo-ETE (7). In the peritoneal macrophage, there is no evidence to suggest that this NADPH-dependent eicosanoid dehydrogenase is expressed even though NADPH oxidase is present (32). 5-HpETE was found to be a precursor of 5-oxo-ETE when either 5(S)- or 5(R, S)-HpETE were incubated with the macrophage. Furthermore, the addition of various cofactors as well as other lipid hydroperoxides did not enhance production of 5-oxo-ETE from 5-HpETE. The only effect observed by cofactors was a reduction in the formation of 5-oxo-ETE when NADPH was added to cytosol preparations. This undoubtedly was because of an increased conversion of 5-HpETE to 5-HETE by peroxidases dependent on NADPH (33). When macrophages were incubated with a mixture of stable isotope-labeled 5-HETE and unlabeled 5-HpETE, the resulting 5-oxo-ETE was unlabeled, clearly supporting the hypothesis that 5-HETE was not a precursor in 5-oxo-ETE biosynthesis in the macrophage, but rather 5-HpETE.

A further difference in the biosynthetic pathway of 5-oxo-ETE in the neutrophil versus the macrophage was the primary location of the factors responsible for the synthesis of 5-oxo-ETE. In the peritoneal macrophage the cytosolic fraction retains the major biosynthetic activity, whereas in the neutrophil the microsomal fraction has been described as the locus of 5-hydroxyeicosanoid dehydrogenase. Furthermore, the molecular weight of the macrophage catalytic factor appeared to be ~55,000. The catalytic activity of the cytosolic fraction was observed to be only slightly diminished by boiling when the crude cytosol was used for testing and unaffected by trypsin pretreatment. However, much different results were obtained when attempting to partially purify the component in cytosol responsible for this biochemical conversion in that a substantial loss of activity in the 50–60-kDa region was observed. One possible explanation for the failure to detect the active component after size exclusion chromatography would be formation of hematin or a similar heme prosthetic group released from a heme-containing protein during boiling and trypsin treatment, but this low molecular weight porphyrin was tightly bound by the size exclusion column packing material. Separate experiments clearly showed that hematin did not traverse the HPLC column and became irreversibly absorbed. However, the extensive loss of the high molecular weight factor (Fig. 6, B and C) that catalyzed conversion of 5-HpETE to 5-oxo-ETE was consistent with the presence of a protein catalyzing this conversion process in macrophage cytosol.

The formation of various conjugated dieneone eicosanoids has been studied extensively, particularly as products of the reaction of hydroperoxides with iron-containing metalloproteins and heme derivatives. Dix and Marnett (23) found that 13-hydroperoxy-9-cis-11-trans-octadecadienoic acid was converted into 13-keto-9,11-octadecadienoic acid as a major product in the presence of hematin, the oxidized form of heme. Other epoxyhydroxy and trihydroxy fatty acid metabolites were also formed by this reaction, which was suggested to proceed through formation of an alkoxyl radical lipid hydroperoxide in a one-electron reduction and the concomitant formation of a ferryl-hydroxyl complex of hematin. Whereas the stereochemistry of this initial reduction step was not investigated in these studies, the hypothesis that the initial reduction involves the hydroperoxy group rather than attack at the hydroperoxy carbon atom
suggests that this reaction would be insensitive to the chirality of the hydroperoxide. Hematin was also found to effectively convert 10-hydroperoxyoctadec-8-enoic acid into 10-oxo-octadec-8-enoic acid in almost 80% yield. The formation of this latter \( \alpha,\beta \)-unsaturated ketone was thought to be a result of a one-electron oxidation of the alkoxide radical by \( \text{Fe}^{3+} \rightarrow \text{O} \), which is the oxidized heme formed during the initial reduction of the hydroperoxide to the alkoxyl radical (34). The conversion of lipid hydroperoxides and in particular 13-hydroperoxy-9,11-octadecadienoic acid into the corresponding conjugated diene, 13-keto-9,11-octadecadienoic acid, was also found to be catalyzed by hemoglobin in a rather efficient manner (24). Whereas the mechanism responsible for formation of this diene was not investigated, its formation would be consistent with an intermediate alkoxyl radical by a one-electron reduction reaction, followed by a one-electron oxidation to the corresponding ketone after removal of a hydrogen atom from the chiral center. While the conversion of a hydroperoxide to a keto compound, namely 5-oxo-ETE, by the mechanisms suggested above can be efficiently oxidized at carbon-5 of arachidonate by radic.

The facile formation of 5-oxo-ETE from 5-HpETE in the macrophage, but not the neutrophil, the metabolism of 5-oxo-ETE to FOG\(_7\), and the lack of a stereochemical requirement for lipoxigenase-derived 5(S)-HpETE as precursor, suggest a unique pathway to signal free radical-based lipid peroxidation in the macrophage. We have previously found that specific phospholipid molecular species, namely plasmalogen phospholipids containing arachidonate esterified at the sn-2 position, can be efficiently oxidized at carbon-5 of arachidonate by radical reactions while still in the ordered membrane bilayer (38). Thus, initiation of peroxidation at lipid membranes could result in an elevated production of racemic 5-HpETE esterified to the phospholipid backbone. Subsequent action of phospholipase \( \text{A}_2 \) would release racemic 5-HpETE. Both enantiomers of this hydroperoxide could then be converted into a single product, namely 5-oxo-ETE, by the mechanisms suggested above (Fig. 7). Whereas 5-oxo-ETE is known to exert potent biological activities, it is highly lipophilic and likely would not leave the biosynthetic cell because of membrane association and affinity to fatty acid-binding proteins. However, it is known that the macrophage can efficiently convert 5-oxo-ETE into FOG\(_7\), which is substantially less lipophilic and is readily released from cells. Furthermore, FOG\(_7\) retains considerable biological activity, being a potent chemotactic factor for eosinophils and neutrophils (31). Thus, it is possible that an amplification of cellular events mediated by the 5-oxo-ETE/FOG\(_7\) pathway could result from free radical oxidation of arachidonic acid. The conversion of 5-HpETE to 5-oxo-ETE/FOG\(_7\) could also serve as a unique signal following exposure to reactive oxygen species. Whereas it is unknown at the present time whether one or several iron-containing metalloproteins are involved in the process of reduction and oxidation of 5-HpETE into 5-oxo-ETE, it is clear that this alternative pathway of 5-oxo-ETE biosynthesis can operate in relevant cells involved in the inflammatory response.

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