# Lipoxin Biosynthesis by Trout Macrophages Involves the Formation of Epoxide Intermediates†

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ABSTRACT: Rainbow trout macrophages incubated with calcium ionophore A23187 or zymosan synthesize a range of lipoxygenase products, including lipoxins from endogenous arachidonic and eicosapentaenoic acids. The profile of products formed was consistent with the presence of 5- and 12-lipoxygenase activity in intact cells, whereas freeze–thaw disruption of macrophages revealed a further 15-lipoxygenase activity. To examine the mechanism of lipoxin biosynthesis in these cells, macrophages from the hemopoietic head kidney were incubated with potential intermediates and substrates, including 5-hydroxyeicosatetraenoic acid (5-HETE), 5-hydroperoxyeicosatetraenoic acid (5-HPETE), 15-HETE, 15-HPETE, 5,15-dihydroperoxyeicosatetraenoic acid (5,15-diHPETE), 5,15-dihydroxyeicosatetraenoic acid (5,15-diHETE), and $LTA_4$. Only 5-HPETE caused an increase in $LXA_4$ formation, while incubation with 15-HETE resulted in the appearance of $LXB_4$, a product not formed from endogenous substrates. Alcohol trapping experiments were conducted to evaluate the formation of epoxide-containing intermediates during lipoxin biosynthesis. Both 12-*O*-methoxy and 6-*O*-methoxy derivatives of $LTA_{4/5}$ were formed, together with three groups of tetraene-containing trapping products, one of which co-chromatographed with the methanol trapping products generated from a synthetic 5(6)-epoxy tetraene. The time course of the appearance of tetraene and triene trapping products was similar. Preliminary results are also consistent with the presence of epoxide hydrolase activity in trout macrophages that converted the 5(6)-epoxy tetraene to $LXA_4$. The results of this series of experiments suggest that lipoxin biosynthesis in trout macrophages involves the cooperation of 5- and 12-lipoxygenases to yield an epoxy tetraene-containing intermediate, or its equivalent, that is specifically converted to $LXA_4$. Furthermore, there is no evidence for the direct conversion of $LTA_4$ to lipoxins, suggesting the existence of a novel situation in trout macrophages with two distinct pathways for the generation of lipoxins and leukotrienes.

Lipoxins are a recent addition to the group of fatty acid derivatives termed eicosanoids. These trihydroxy derivatives of arachidonic acid (AA[1]) and eicosapentaenoic acid (EPA) contain a conjugated tetraene structure, giving them a characteristic UV absorbance profile with a $\lambda_{max}$ at *ca.* 300 nm. Two main forms of lipoxin have been identified: one designated lipoxin $A_4$ ($LXA_4$, 5(*S*),6(*R*),15(*S*)-trihydroxy-7,9,13-*trans*,11-*cis*-eicosatetraenoic acid) and the other lipoxin $B_4$ ($LXB_4$, 5(*S*),14(*R*),15(*S*)-trihydroxy-6,10,12-*trans*,8-*cis*-eicosatetraenoic acid) [reviewed in Samuelsson et al. (1987); Dahlén & Serhan, 1991]. The equivalent trihydroxy tetraenes of EPA (i.e., $LXA_5$, 5(*S*),6(*R*),15(*S*)-trihydroxy-7,9,13-*trans*,-11,17-*cis*-eicosapentaenoic acid, and $LXB_5$, 5(*S*), 14(*R*), 15-(*S*)-trihydroxy-6,10,12-*trans*,8,17-*cis*-eicosapentaenoic acid) have also been reported (Wong et al., 1985). Both $LXA_4$ and $LXB_4$ are biologically active compounds with a range of potentially important activities distinct from those of other lipoxygenase products, such as leukotriene $B_4$ ($LTB_4$, 5(*S*),-12(*R*)-8,10-*trans*,6,14-*cis*-eicosatetraenoic acid) [e.g., Dahlén and Serhan (1991)].

Several routes of lipoxin biosynthesis have been described, each operating in different cell types and tissues (Dahlén & Serhan, 1991). The first involves the cooperation of 5- and 15-lipoxygenases (or transcellular metabolism of 15-HETE) to yield an unstable 5(6)-epoxy tetraene (15(*S*)-hydroxy-5(*S*),6(*S*)-epoxy-7,9,13-*trans*,11-*cis*-eicosatetraenoic acid) intermediate (Puustinen et al., 1986), which is converted either enzymatically to form $LXA_4$, $LXB_4$, or 7-*cis*,11-*trans*-$LXA_4$ or nonenzymatically to form 6(*S*)-$LXA_4$, 14(*S*)-8-*trans*-$LXB_4$, 14(*S*)-$LXB_4$, and 8-*trans*-$LXB_4$ (Serhan et al., 1986a,b). The second, more recently described pathway, involves transcellular cooperation between human platelets and neutrophils. In this biosynthetic route, leukotriene $A_4$ ($LTA_4$, (5*S*-*trans*)-5,6-oxido-7,9-*trans*,11,14-*cis*-eicosatetraenoic acid), an unstable epoxide-containing product of 5-lipoxygenase formed by neutrophils, is converted to lipoxins by the action of platelet-derived 12-lipoxygenase (Fiore & Serhan, 1990; Serhan &

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[1] Abbreviations: AA, arachidonic acid; Di-HETrE, dihydroxyeicosatrienoic acid; EBM, Eagle's basal medium; EPA, eicosapentaenoic acid; EpETrE, epoxyeicosatrienoic acid; HBSS, Hank's balanced salt solution; HETE, hydroxyeicosatetraenoic acid; diHETE, dihydroxyeicosatetraenoic acid; HPETE, hydroperoxyeicosatetraenoic acid; diHPETE, dihydroperoxyeicosatetraenoic acid; LT, leukotriene; LX, lipoxin; $PGB_2$, prostaglandin $B_2$; RP-HPLC, reverse-phase high-performance liquid chromatography; a 4/5 subscript denotes products from arachidonic acid and eicosapentaenoic acid, respectively.

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Sheppard, 1990; Edenius et al., 1991; Sheppard et al., 1992). Importantly, studies have shown that neutrophils primed by recombinant human granulocyte/macrophage colony stimulating factor in the presence of thrombin-stimulated platelets synthesize substantial amounts of lipoxins entirely from endogenous substrates (Fiore & Serhan, 1990). Furthermore, COS-M6 cells transfected with 12-lipoxygenase cDNA incubated with $LTA_4$ generate lipoxins (Sheppard et al., 1992), a finding that provides direct evidence for the participation of 12-lipoxygenase in lipoxin biosynthesis. Recently, two additional biosynthetic routes for lipoxin have been reported. One involves the conversion of 14,15-$LTA_4$ to $LXA_4$ and $LXB_4$ by permeabilized platelets (Romano & Serhan, 1992), while 5(*S*),6(*R*)- and 5(*S*),6(*S*)-dihydroxy-7,9-*trans*,11,14-*cis*-eicosatetraenoic acids (5,6-diHETEs) are also converted by platelets to $LXA_4$ and 6(*S*)-$LXA_4$ (Tornhamre et al., 1992). Thus, different routes of lipoxin biosynthesis can be evoked that are dependent upon the cell type(s) of origin.

Lipoxin biosynthesis is not only confined to mammalian leukocytes or platelets. For example, macrophages from a number of species of bony fish, including rainbow trout, Atlantic salmon, and carp, generate lipoxins following exposure to either calcium ionophore A23187 or zymosan (Pettitt et al., 1989a, 1991; Rowley, 1991). The profile of lipoxin generation in these fish also differs. For example, in trout and salmon, $LXA_4$ is the main product (Pettitt et al., 1989a), while in carp both $LXA_4$ and $LXB_4$ are formed (Rowley, 1991). Lipoxins appear to be important pro-inflammatory molecules in rainbow trout (Sharp et al., 1992), suggesting a key role for such compounds in this species. Fish macrophages are an ideal model system for studying the mechanism of lipoxin biosynthesis, as these cells synthesize appreciable amounts of these compounds from endogenous sources of both AA and EPA (Pettitt et al., 1991). The present study was undertaken to investigate the pathways involved in lipoxin biosynthesis in rainbow trout (*Oncorhynchus mykiss*) head kidney leukocytes. These studies show that trout macrophages generate epoxide-containing intermediates that are converted to predominantly $LXA_4$.

## EXPERIMENTAL PROCEDURES

*Materials.* Calcium ionophore A23187, prostaglandin (PG) $B_2$, streptomycin, penicillin, fatty acid-free bovine serum albumin, AA, EPA, and Percoll were purchased from Sigma Chemical Co. Ltd. (Poole, U.K.). Synthetic lipoxins, including $LXA_4$, $LXB_4$, 11-*trans*-$LXA_4$, and 6(*S*)-$LXA_4$, and all other lipoxygenase products [5(*S*)-hydroxyeicosatetraenoic acid (5-HETE), 5-hydroperoxyeicosatetraenoic acid (5-HPETE), 5,15-DiHETE, 15-HETE, 15-HPETE, 12-HETE, 8,15-diHETE, 6-*trans*,12-*epi*-$LTB_4$, 6-*trans*-$LTB_4$, $LTB_4$, $LTA_4$ methyl ester, and $LTA_5$ methyl ester] were obtained from Cascade Biochem Ltd. (Reading, U.K.). Similarly, the cytochrome P-450 products, 5,6-DiHETre [5(*R*/*S*),6(*R*/*S*)-8,11,14-*cis*-dihydroxyeicosatrienoic acid) and 5,6-EpETrE (8,-11,14-*cis*,*rac*-*cis*-5,6-epoxyeicosatrienoic acid] were also obtained from Cascade Biochem Ltd. The 5(6)-epoxy tetraene methyl ester was a kind gift of Professor K. Nicolaou (Department of Chemistry, Research Institute of Scripps Clinic, La Jolla, CA), and the free acid was prepared by LiOH saponification in tetrahydrofuran (Nicolaou et al., 1991). Hank's balanced salt solution with $Ca^{2+}$ and $Mg^{2+}$ (HBSS) and Eagle's basal medium were from Gibco (Paisley, U.K.). All solvents were of HPLC grade and/or redistilled in glass.

*Fish.* Rainbow trout, *Oncorhynchus mykiss*, were obtained from Whitesprings Fisheries (Pontardulais, U.K.) and maintained as described previously (Pettitt et al., 1989b). Additional fish were obtained from the Plymouth Rock Trout Co. (Plymouth, MA).

*Preparation of Macrophages and Macrophage Lysates.* Macrophages were isolated from the hemopoietic head kidney of rainbow trout and placed in short-term culture as described previously (Pettitt et al., 1991). Briefly, the head kidney was removed, passed through a fine mesh sieve into cold (4 °C) Eagle's basal medium (EBM) containing a 0.01% (final concentration) penicillin/streptomycin mix (1:1). After centrifugation, leukocytes were separated from erythrocytes on 54% Percoll continuous gradients, washed twice in $Ca^{2+}$/$Mg^{2+}$-free HBSS, and resuspended in EBM. Aliquots (*ca.* $1 \times 10^7$ macrophages in 5 mL of EBM) were plated out into 25-$cm^3$ flasks and left to adhere for 1–2 h at 18 °C. Subsequently, nonadherent cells (mainly lymphocytes) were removed by vigorous washing with EBM. Cells (>95% macrophages) were routinely incubated in EBM for 24–48 h prior to use. Macrophage lysates were prepared by rapid freezing and thawing of macrophages in 25-$cm^3$ flasks in HBSS with a dry ice/acetone bath. The process was repeated twice and the lysate centrifuged (20000*g*, 30 min, 4 °C). Resulting supernatants were stored at −70 °C as small beads prepared by dropping supernatant from a Pasteur pipet into liquid nitrogen.

*RP-HPLC Analysis.* Lipoxygenase products were routinely separated by RP-HPLC using an Ultrasphere $C_{18}$ column (5 μM packing, 4.6 × 250 mm, Beckman RIIC, High Wycombe, U.K.). Briefly, lipoxygenase products were eluted with a solvent gradient from 100% water/methanol/acetonitrile/acetic acid (45:30:25:0.05, pH 5.7) to 100% methanol in 40 min with a flow rate of 0.6 mL/min. A Waters 991 diode array detector was used and adjusted to collect spectral data between 210 and 340 nm with a resolution of 1.3 nm. Identification of products was by cochromatography with authentic standards, by on-line spectral analysis, and by reference to products synthesized by rainbow trout macrophages ($LXA_5$, $LTB_5$, and 11-*trans*-$LXA_5$) previously verified by gas chromatography–electron impact mass spectrometry (Pettitt et al., 1989b, 1991). Quantification of the products separated by RP-HPLC was by reference to the internal standard ($PGB_2$) using known molar extinction coefficients of HETEs, DiHETEs, and lipoxins.

*Time Course Experiments.* Macrophages (*ca.* $1 \times 10^7$ per flask) were incubated for 1–20 min with 5 μM calcium ionophore A23187 (optimum concentration for eicosanoid generation) at 18 °C. At the end of these time periods, the supernatants were rapidly decanted into 2 vol of cold methanol, the cells were gently rinsed twice with HBSS at 18 °C, and 5 mL of ice-cold methanol was added to each flask. After the flasks were left on ice for 30 min, cells were removed with a plastic cell scraper, and both supernatants and cell suspensions were centrifuged (1500*g*, 10 min, 4 °C) prior to the addition of the $PGB_2$ (100 ng) internal standard. The samples were Sep-Pak extracted (Pettitt et al., 1989b) and separated by RP-HPLC.

*Incubation of Macrophages with Exogenous Substrates.* Macrophages were incubated with either exogenous 5-HETE, 5-HPETE, 15-HETE, 15-HPETE, 5,15-diHETE, 5,15-diHPETE, or $LTA_4$ to determine the potential intermediates and substrates involved in lipoxin biosynthesis. The products formed were separated and quantified by RP-HPLC. Macrophages ($1 \times 10^7$ per flask) were incubated in the presence of the divalent cation ionophore A23187 (5 μM) with either 25 or 50 μM eicosanoid for 10 min at 18 °C. The resulting

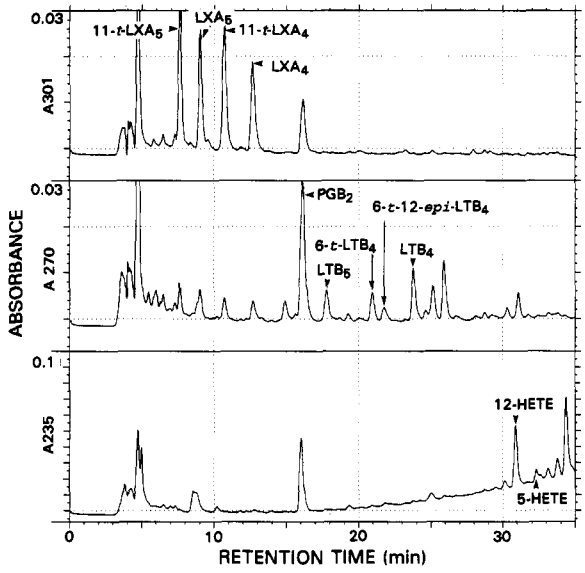


FIGURE 1: Lipoxygenase products formed by intact trout macrophages. Trout macrophages ($1 \times 10^7$ cells) in HBSS were incubated with 5 $\mu$M calcium ionophore for 20 min at 18 °C, and supernatants were Sep-Pak-extracted and separated by RP-HPLC as described in the Experimental Procedures. The amounts of 11-*trans*-$LXA_{4/5}$ were highly variable from one preparation to another.

supernatants were subsequently decanted into cold methanol (2 vol) containing $PGB_2$ (100 ng), Sep-Pak extracted, and subjected to RP-HPLC analysis.

*Trapping Experiments.* To examine the nature and time course of the production of potential unstable intermediates in the biosynthesis of lipoxins, trout macrophages ($5 \times 10^7$ in 2 mL of HBSS) were incubated with 5 $\mu$M calcium ionophore for either 1, 2, 5, 10, or 20 min at 18 °C, with or without exogenous AA or EPA (50–200 $\mu$M). The incubates were immediately decanted into 10 vol of cold acidified (apparent pH 3.0) methanol for 30 min, and the apparent pH was adjusted to pH 7.0. Following centrifugation (1500*g*, 10 min, 4 °C), methanol-containing supernatants were taken to dryness by rotary evaporation, resuspended in water by vortexing in round-bottom flasks, acidified with 0.1 M HCl to pH 3.5, and rapidly loaded onto Sep-Pak $C_{18}$ cartridges. These were rapidly washed with *ca.* 15 mL of distilled water and eluted with hexane followed by methyl formate. The methyl formate fractions were taken to dryness with a stream of $N_2$ and resuspended in methanol prior to separation by RP-HPLC. Trapping products of $LTA_{4/5}$ and the 5(6)-epoxy tetraene (from the 4-series, i.e., AA-derived) were identified by reference to authentic standards prepared in parallel for each trapping experiment.

*Conversion of the 5(6)-Epoxy Tetraene to Lipoxins by Macrophage Lysate Supernatants.* Macrophage lysate supernatant (*ca.* 2 mL) or HBSS was incubated with the 5(6)-epoxy tetraene (7.6 $\mu$M) for 20 min at 20 °C; the reaction was terminated with 2 vol of cold methanol and prepared as for trapping products. Sep-Pak-extracted materials were separated by isocratic RP-HPLC using an Altex Ultrasphere (4.6 × 250 mm i.d.) column and a mobile phase of methanol/water/acetic acid (65:35:0.01, pH 5.7) at a flow rate of 1 mL/min. Detection was at 301 nm with a Waters Model UVM484 detector.

*Conversion of 5,6-EpETrE by Trout Macrophage Lysate Supernatants.* Macrophage lysate supernatant (2 mL), heated (60 °C for 30 min) macrophage lysate (2 mL), or HBSS (2 mL) was incubated at 18 °C for 20 min with 200 $\mu$M 5,6-EpETrE. Following Sep-Pak extraction, samples were separated by isocratic RP-HPLC using a Beckman Ultrasphere (4.6 × 250 mm i.d.) column with a mobile phase of methanol/water/acetic acid (80:20:0.05, pH 5.7) and a flow rate of 0.8 mL/min. Detection was at 210 nm using a Waters 991 diode array detector.

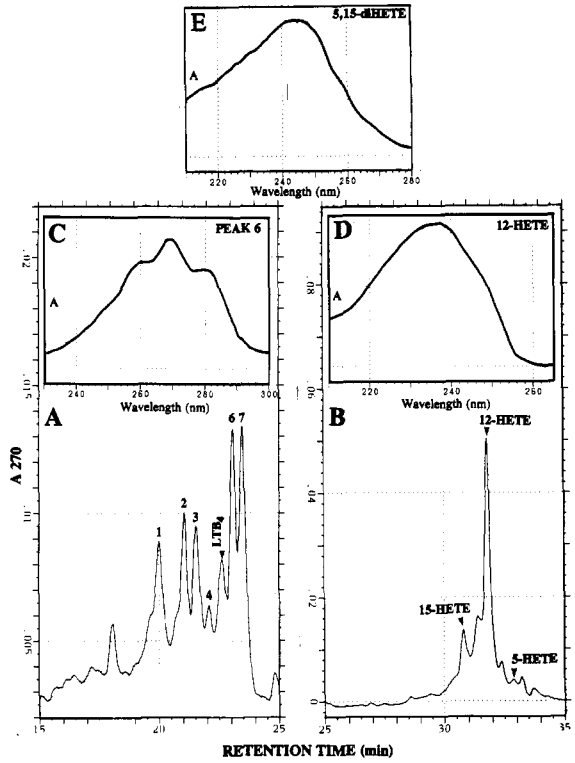


FIGURE 2: Lipoxygenase products obtained from macrophage lysate supernatant. Trout macrophages (*ca.* $2.5 \times 10^8$ total cells) in HBSS were disrupted in an acetone/dry ice bath and centrifuged at 20000*g* for 30 min at 4 °C to yield a lipoxygenase-containing supernatant. Supernatants were incubated with 100 $\mu$M AA for 20 min at 18 °C, Sep-Pak-extracted, and separated by RP-HPLC as described in the Experimental Procedures. Peaks 1–7 (A) all show spectra characteristic of conjugated trienes with a $\lambda_{max}$ of *ca.* 270 nm (C), while the conjugated diene-containing 5-, 12-, and 15-HETE generated had a $\lambda_{max}$ at *ca.* 235 nm (B, D). Peak 2, as well as containing a diHETE with a $\lambda_{max}$ of *ca.* 270 nm, also contained a compound with a conjugated diene with a $\lambda_{max}$ of 243 nm (E) that cochromatographed with authentic 5,15-diHETE. This particular cell-free lysate preparation did not yield lipoxins, although in some other preparations small amounts of $LXA_4$ and 6(*S*)-$LXA_4$ were noted.

## RESULTS

*Generation of Lipoxygenase Products by Intact Macrophages and Macrophage Lysate Supernatant.* Rainbow trout macrophages incubated with calcium ionophore yielded a range of lipoxygenase products derived from endogenous sources, including lipoxins as previously identified (Pettitt et al., 1989a, 1991; Figure 1). The principal product was 12-HETE (*ca.* 33 ng/$10^6$ macrophages, 20-min incubation with 5 $\mu$M ionophore at 18 °C), with smaller amounts of other products including $LXA_4$ (23 ng/$10^6$ cells), 11-*trans*-$LXA_4$ (26 ng/$10^6$ cells), $LTB_4$ (18 ng/$10^6$ cells), 6-*trans*,12-*epi*-$LTB_4$, 6-*trans*-$LTB_4$, and 5-HETE, as well as the equivalent 5-series products (Figure 1). No 15-HETE was detected with intact cells, and only on rare occasions was 5,15-diHETE observed, and then at low levels (<0.5 ng/$10^6$ cells). Macrophage lysates incubated with AA (25–100 $\mu$M) yielded a profile different from that observed with intact cells (Figure 2). The most notable difference was the appearance of the 15-lipoxygenase products 15-HETE and 5,15-diHETE. A number of diHETEs containing conjugated trienes ($\lambda_{max}$ *ca.* 270 nm), as well as

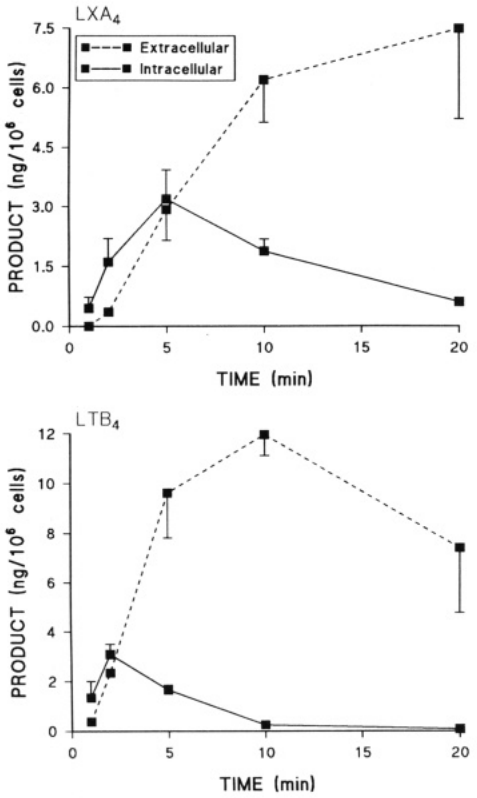


FIGURE 3: Time course of intracellular and extracellular leukotriene $B_4$ and lipoxin $A_4$ generation by trout macrophages. Trout macrophages ($1 \times 10^7$/flask) were incubated with 5 $\mu$M calcium ionophore A23187 for either 1, 2, 5, 10, or 20 min, and the incubations were terminated with excess cold methanol. Following the addition of $PGB_2$ (100 ng), samples were Sep-Pak-extracted and separated by RP-HPLC as described in the Experimental Procedures. Quantification of $LXA_4$ and $LTB_4$ was by reference to the internal standard ($PGB_2$) using $\epsilon_{280}^{PGB_2}$ = 28 000 M cm$^{-1}$, $\epsilon_{301}^{lipoxins}$ = 50 000 M cm$^{-1}$, and $\epsilon_{280}^{leukotrienes}$ = 44 000 M cm$^{-1}$ (mean values ± SE, $n$ = 5 separate experiments).

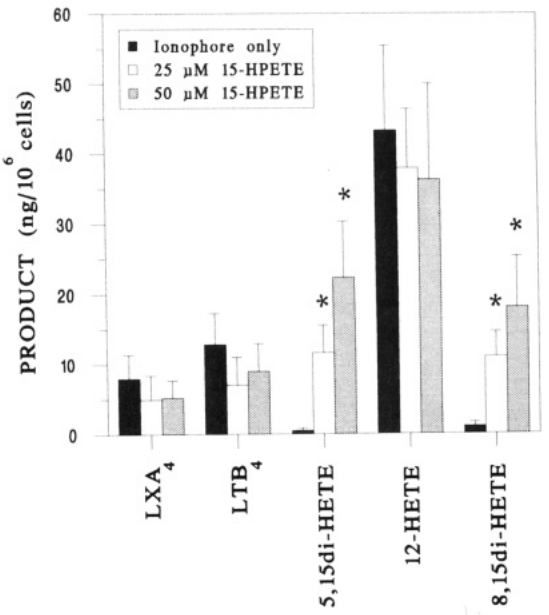


FIGURE 4: Evaluation of exogenous 15-HPETE on lipoxygenase product formation by trout macrophages. Macrophages ($1 \times 10^7$/flask) were incubated with either ionophore alone or ionophore and 25 or 50 $\mu$M 15-HPETE in HBSS for 10 min at 18 °C. Supernatants were subsequently decanted and centrifuged (2000$g$, 5 min, 4 °C) to pellet any cells, and the supernatants were immediately Sep-Pak-extracted and lipoxygenase products separated by RP-HPLC. Products were identified by coelution with authentic standards and by reference to previous GC–mass spectrometry. Quantification of products was as described in the legend for Figure 3 (mean values ± SD, $n$ = 4 separate experiments). * indicates $P < 0.05$ compared with the ionophore only control (Student's $t$-test).

$LTB_4$, were also observed, which were, for the most part, absent in supernatants from intact macrophages challenged with ionophore. In most instances, lipoxins were not observed following the incubation of macrophage lysate supernatants with either AA or EPA. Thus, the macrophage lysates and intact cells displayed 12-lipoxygenase activity as the major activity, and only intact cells consistently generated lipoxins from endogenous fatty acids.

*Time Course for Lipoxin and Leukotriene Generation.* To gain initial insight into the dynamics of the biosynthesis of lipoxins compared with other major lipoxygenase products generated from endogenous sources, such as $LTB_4$, both the intracellular and extracellular appearance of lipoxins and leukotrienes was determined. The time course for the biosynthesis and release of lipoxins and other lipoxygenase products such as $LTB_4$, $LTB_5$, and 12-HETE differed. Within 60 s postchallenge with ionophore, lipoxins and leukotrienes were cell-associated (Figure 3). In the same time period, $LTB_4$ and products other than lipoxins were also present extracellularly. The peak intracellular amount of $LXA_4$ was found at 5 min postchallenge, while for $LTB_4$ and $LTB_5$ the highest cell-associated levels were at 2 min (Figure 3). Similarly, the greatest extracellular level of $LTB_4$ was observed 10 min postchallenge with ionophore, while for $LXA_4$ this occurred at intervals >20 min.

*Impact of Exogenous Lipoxygenase Products on Lipoxin Biosynthesis.* Of the putative intermediate substrates tested, only 5-HPETE caused an increase in $LXA_4$ formation by ionophore-challenged macrophages (3.4 ± 0.8 ng/10$^6$ cells in the presence of ionophore compared with 5.8 ± 3.1 ng/10$^6$ cells in the presence of ionophore and 50 $\mu$M 5-HPETE; mean values ± SD, $n$ = 4). Incubation of macrophages with A23187 and either 15-HPETE, 15-HETE, 5,15-diHPETE, 5,15-diHETE, or $LTA_4$ (incubated either in the presence or absence of 1% albumin) resulted in only a slight but not statistically significant reduction in the amount of $LXA_4$ generated, compared with cells incubated with ionophore alone. For example, the incubation of macrophages with 15-HPETE caused some reduction in $LXA_4$, $LTB_4$, and 12-HETE and a statistically significant increase in the levels of 5,15-diHETE and 8,15-diHETE (Figure 4). Incubation of macrophages with 15-HETE resulted in the appearance of $LXB_4$ (1.4 ± 0.8 ng/10$^6$ macrophages, mean ± SD, $n$ = 3, following incubation with 25 $\mu$M 15-HETE; Figure 5), a product not formed in macrophages in the absence of this exogenous substrate. Together, these results suggest that 15-HPETE is not the immediate precursor to $LXA_4$ in trout macrophages.

*Trapping Experiments.* Previous studies have used alcohol trapping as a means to identify epoxide-containing intermediates in leukotriene and lipoxin generation [e.g., Borgeat and Samuelsson (1979); Puustinen et al., 1986]. Here, alcohol trapping experiments were conducted to evaluate the formation of epoxide-containing intermediates from macrophage-derived substrates. Typical RP-HPLC chromatograms showing the trapping products obtained from ionophore-stimulated trout macrophages exposed to excess acid alcohol conditions are shown in Figures 6 and 7. Both 12-*O*-methoxy-$LTA_4$ (i.e., 5-hydroxy-12(*R*/*S*)-*O*-methoxy-6,8,10,14-eicosatetraenoic acids) and 6-*O*-methoxy-$LTA_4$ (5-hydroxy-6(*R*/*S*)-*O*-methoxy-6,8,10,14-eicosatetraenoic acids) derivatives were formed from endogenous $LTA_4$, respectively (Figure 6). In addition, the equivalent 5-series trapping products (i.e., derived from

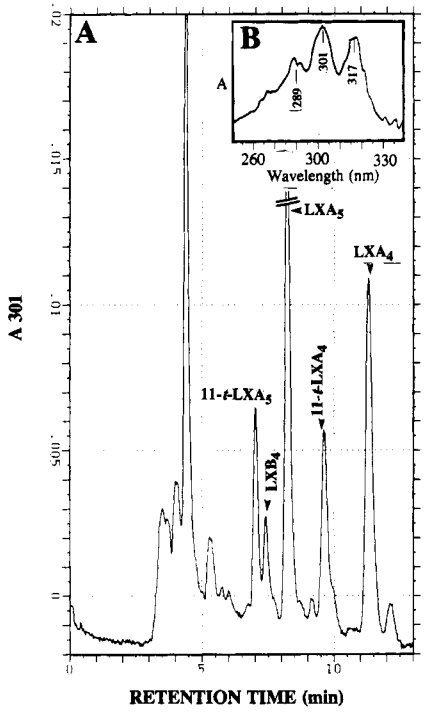


FIGURE 5: Evaluation of 15-HETE as substrate in lipoxin formation by trout macrophages. Macrophages ($1 \times 10^7$/flask) were incubated with 25 $\mu$M 15-HETE and 5 $\mu$M calcium ionophore in HBSS for 10 min at 18 °C. Culture supernatants were Sep-Pak-extracted following the addition of 100 ng of $PGB_2$ and separated by RP-HPLC as described in the Experimental Procedures. Identification of the lipoxins formed was by coelution with authentic standards and on-line UV spectra. A shows a representative chromatogram of lipoxins generated in the presence of 15-HETE. B shows the UV spectrum of the material under the peak identified as $LXB_4$.

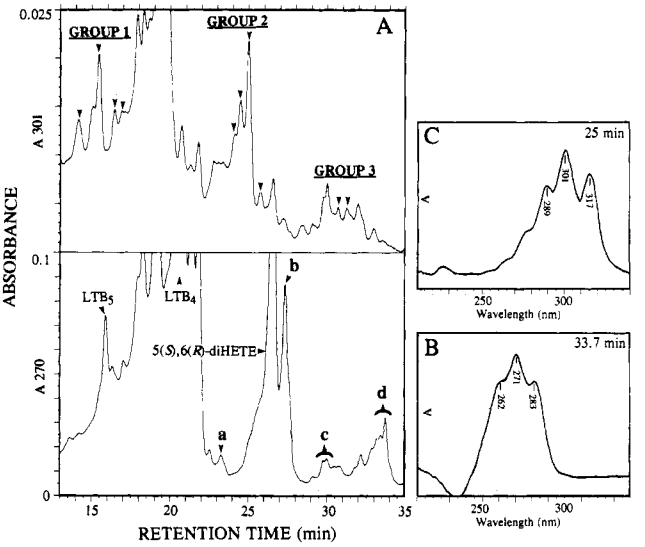


FIGURE 6: Representative RP-HPLC chromatogram and UV profiles of trapping products formed by trout macrophages. Head kidney macrophages ($5 \times 10^7$ cells) in HBSS were incubated with 5 $\mu$M calcium ionophore for 10 min at 18 °C in the presence of exogenous AA (100 $\mu$M). The incubates were rapidly decanted into 10 vol of cold, acidified methanol and left on ice for 30 min. Samples were Sep-Pak-extracted following rotary evaporation and separated by gradient RP-HPLC as detailed in the Experimental Procedures. Three groups (group 1–3) of tetraene-containing trapping products were identified in RP chromatograms (A). Unlabeled arrows indicate peaks in these groups which contain material with UV spectra characterized by a $\lambda_{max}$ at *ca.* 301 nm and shoulders at 289 and 317 nm. The triene-containing trapping products labeled a–d derived from $LTA_4$ and $LTA_5$ were as follows: (a) 5-hydroxy-12(*R*/*S*)-*O*-methoxy-6,8,10,14,17-eicosapentaenoic acids; (b) 5-hydroxy-12(*R*/*S*)-*O*-methoxy-6,8,10,14-eicosatetraenoic acids; (c) 5-hydroxy-6(*R*/*S*)-*O*-methoxy-6,8,10,14,17-eicosapentaenoic acids, and (d) 5-hydroxy-6(*R*/*S*)-*O*-methoxy-6,8,10,14-eicosatetraenoic acids. Identification of trapping products of $LTA_{4/5}$ was by reference to authentic standards treated with acidified methanol and processed simultaneously with macrophage-derived material. B shows the UV scan of the triene-containing material under the peaks with a retention time of *ca.* 33.7 min which coeluted with the 6(*R*/*S*)-*O*-methoxy-$LTA_4$ (5-hydroxy-6-(*R*/*S*)-*O*-methoxy-6,8,10,14-eicosatetraenoic acid) derivatives of $LTA_4$, while C shows the UV scan of the tetraene-containing trapping product in group 2 with a retention time of 25 min.

eicosapentaenoic acid) were also formed. These macrophage-derived products coeluted with acidified methanol-treated $LTA_4$ and $LTA_5$ standards and possessed conjugated trienes with a $\lambda_{max}$ of *ca.* 270 nm (Figure 6B). A series of tetraene-containing trapping products was also generated from endogenous substrates, which were only detected in the presence of acidified methanol. The first two groups of compounds obtained had retention times between 13 and 17 min (group 1) and between 23 and 26 min (group 2) and were usually the predominant tetraene-containing trapping products (Figure 6A,C). Addition of exogenous AA (50–200 $\mu$M) caused an increase in the group with the shorter retention times (13–17 min, group 1), suggesting that these were derived from this fatty acid. In the presence of exogenous EPA, those trapping products with longer retention times (23–26 min, group 2) were increased. The last group of tetraene-containing trapping products in the chromatographic profile gave retention times ranging between 31 and 32.5 min (Figures 6 and 7) and consisted of two main peaks. These products coeluted with acidic methanol-trapped products derived from the 5(6)-epoxy tetraene, an authentic standard of a 4-series intermediate in lipoxin synthesis (Figure 7). Spectral analysis of the trapping products showed two main peaks and two minor peaks with a $\lambda_{max}$ of *ca.* 301 and shoulders at 289 and 317 nm (Figure 7C). These are consistent with the formation of 5,15-dihydroxy-14(*R*/*S*)-*O*-methoxy-6,8,10,12-*trans*-eicosatetraenoic acid [14(*R*/*S*)-*O*-methoxy derivatives] and 5,15-dihydroxy-6(*R*/*S*)-*O*-methoxy-7,9,13-*trans*,11-*cis*-eicosatetraenoic acid [6(*R*/*S*)-*O*-methoxy derivatives] trapping products of the 5(6)-epoxy tetraene (Puustinen et al., 1986; Luscinskas et al., 1990).

The time courses for the appearance of both tetraene- and triene-containing trapping products were similar, although while the maximum of tetraene trapping products occurred at *ca.* 5 min, the equivalent maximum for the triene trapping products was at *ca.* 10 min (Figure 8). These observations are consistent with the involvement of 5(6)-epoxides in lipoxin formation by rainbow trout macrophages. Therefore, to gain evidence for 5(6)-epoxy tetraene involvement, we incubated this compound with trout macrophage lysate supernatant. In these preliminary experiments, such incubations yielded predominantly $LXA_4$ and the nonenzymatic hydrolysis products of this substrate, 14(*S*)-8-*trans*-$LXB_4$[5(*S*),14(*S*),15(*S*)-trihydroxy-6,8,10,12-*trans*-eicosatetraenoic acid] and 8-*trans*-$LXB_4$[5(*S*),14(*R*),15(*S*)-trihydroxy-6,8,10,12-*trans*-eicosatetraenoic acid; Serhan et al., 1986b], that cochromatograph in this RP-HPLC system (Figure 9). Incubation of the 5(6)-epoxy tetraene in HBSS alone yielded only a nonenzymatic profile of products, with essentially equivalent amounts of 6(*S*)-$LXA_4$ and $LXA_4$ formed as racemic hydrolysis products and predominantly 14(*S*)-8-*trans*-$LXB_4$ and 8-*trans*-$LXB_4$ as the major products observed. To investigate further the apparent epoxide hydrolase activity in trout macrophages, 5,6-EpETrE (200 $\mu$M) was incubated for 20 min at 18 °C with either macrophage lysate supernatant,

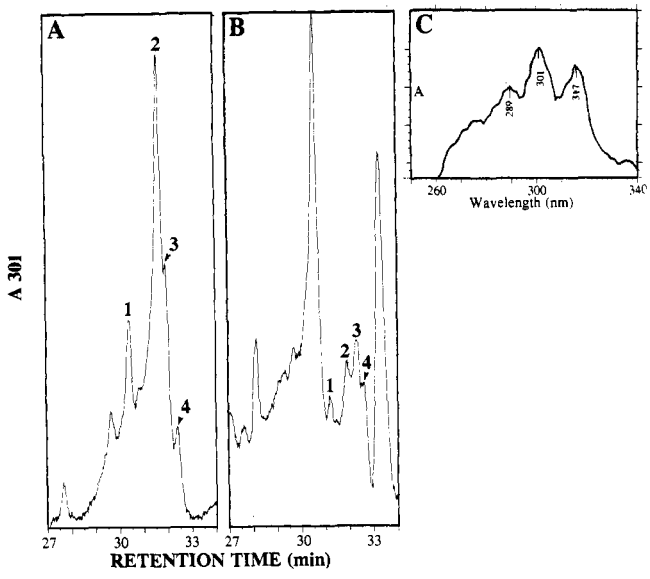


FIGURE 7: Comparison of the acid methanol-trapped 5(6)-epoxy tetraene standard (A) and acid-trapped material derived from ionophore-challenged trout macrophages (B). Authentic 5(6)-epoxy tetraene was treated with acidic methanol, apparent pH 3.0, for 30 min at 4 °C, the pH was adjusted to 7.0, and it was Sep-Pak-extracted and separated by RP-HPLC under conditions identical to the macrophage-derived material as shown in Figures 6 and 7B. (A) Chromatogram showing 5(6)-epoxy tetraene-derived trapping products following treatment with acidified methanol. Material under peaks 1–4 shows a UV spectrum with a $\lambda_{max}$ at *ca.* 301 nm and shoulders at 289 and 317 nm. (B) Chromatogram of trapping products formed by the incubation of macrophages (*ca.* $1 \times 10^8$ cells) for 10 min with 5 $\mu$M calcium ionophore following treatment of cell supernatants with acidified methanol (apparent pH 3.0). Products were extracted and separated as described in the Experimental Procedures. Peaks 1–4 cochromatographed with their corresponding peaks in Figure 6A. (C) UV scan of material under peak 2 in B showing the characteristic absorbance profile of a conjugated tetraene as found in lipoxins, with a $\lambda_{max}$ at *ca.* 301 nm.

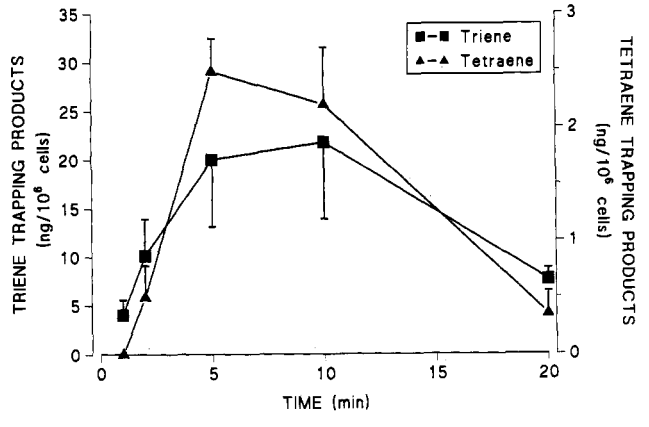


FIGURE 8: Time course for the generation of triene- and tetraene-containing trapping products by stimulated trout macrophages. Macrophages (*ca.* $1 \times 10^7$/flask) in HBSS were challenged with 5 $\mu$M calcium ionophore for 1, 2, 5, 10, or 20 min, and the reactions were terminated by the addition of 10 vol of ice-cold acidified (apparent pH 3.0) methanol. After 30 min at 4 °C, supernatants were dried down by rotary evaporation, Sep-Pak-extracted, and separated by gradient RP-HPLC as detailed in the Experimental Procedures. Data shown are the mean values ± SD ($n = 5$ separate experiments) of the sum of tetraene-containing and triene-containing trapping products.

heat-treated macrophage lysate supernatant, or HBSS alone. As shown in Figure 10, conversion of 5,6-EpETrE to 5,6-diHETrE only occurred in the presence of the macrophage lysate preparation. Finally, incubation of $LTA_4$ with trout macrophage lysate produced $LTB_4$ and nonenzymatic hydrolysis products of $LTA_4$, 6-*trans*-$LTB_4$ and 6-*trans*,12-*epi*-$LTB_4$ (Figure 11), indicating the presence of LTA hydrolase activity in these cells.

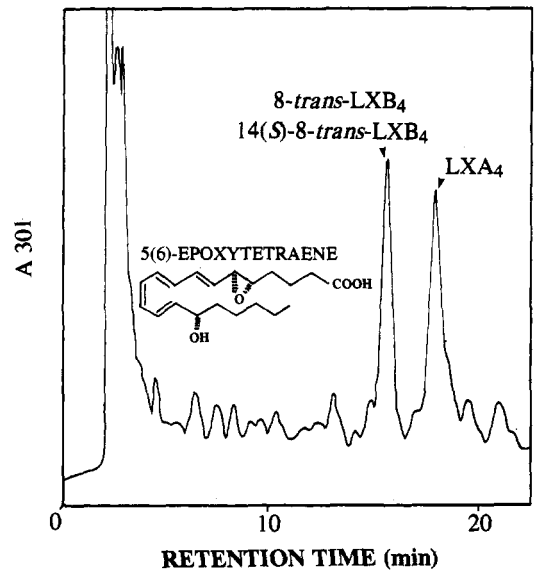


FIGURE 9: RP-HPLC profile obtained from the incubation of the 5(6)-epoxy tetraene with macrophage lysate supernatant. Head kidney macrophages ($5 \times 10^8$ cells) in HBSS were disrupted by repeated freezing and thawing in an acetone/dry ice bath, and the cell debris was removed by centrifugation at 20000*g* for 30 min at 4 °C. Supernatant (*ca.* 2 mL) was incubated for 10 min at 20 °C with 7.6 $\mu$M 5(6)-epoxy tetraene, and the reaction was terminated with 2 vol of cold methanol. Following rotary evaporation, samples were Sep-Pak-extracted and separated by isocratic RP-HPLC eluted with methanol/water/acetic acid (65:35:0.01, pH 5.7) at a flow rate of 1 mL/min. The *all-trans* isomers of $LXB_4$, namely, 14(*S*)-8-*trans*-$LXB_4$ and 8-*trans*-$LXB_4$, coelute. These are the nonenzymatic products of the 5(6)-epoxy tetraene that are not formed by intact trout macrophages. The inset shows the structure of the 5(6)-epoxy tetraene.

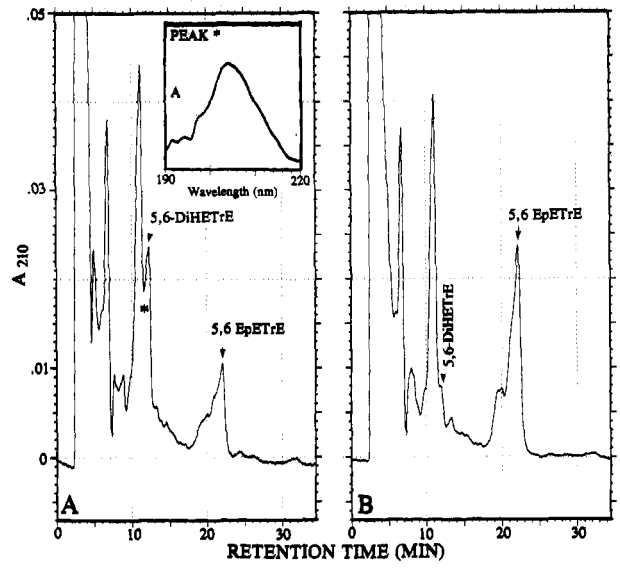


FIGURE 10: RP-HPLC profiles of products formed following the incubation of 5,6-EpETrE with macrophage lysate supernatant (A) or heat-treated macrophage lysate supernatant (B). Note the lack of conversion of 5,6-EpETrE to 5,6-DiHETrE in the heat-treated lysate. The inset shows the UV scan of the product in A that cochromatographs with 5,6-DiHETrE.

## DISCUSSION

Intact trout macrophages generate a range of lipoxygenase products from endogenous fatty acid substrates, indicating

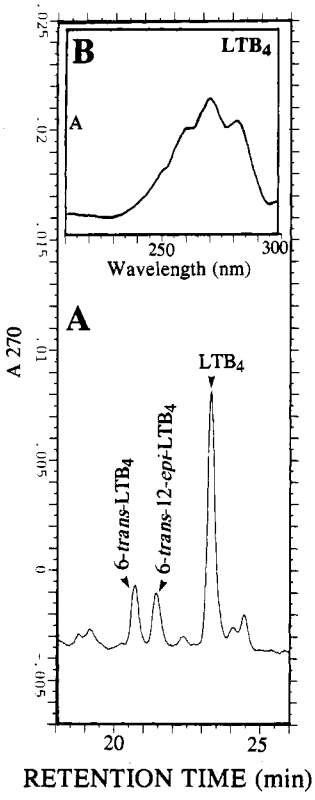


FIGURE 11: RP-HPLC profile of conjugated triene-containing products formed following the incubation of $LTA_4$ with trout macrophage lysate supernatant. $LTA_4$ (50 $\mu$M) was incubated for 10 min at 18 °C with macrophage lysate supernatant prepared as described in the Experimental Procedures and in the legend to Figure 9. Compounds were Sep-Pak-extracted and separated by gradient RP-HPLC using the same conditions employed in the chromatograms in Figures 1, 2, and 5.

the presence of 5- and 12-lipoxygenase activity. Macrophage cell-free supernatants exhibit a different profile of product generation (for example, HETEs and diHETEs) consistent with the appearance of 15-lipoxygenase activity. Whether this represents a distinct 15-lipoxygenase is uncertain as 12-lipoxygenase can act at the C15 position under certain circumstances (Yokoyama et al., 1986; van der Donk et al., 1992). Studies on the purification of lipoxygenases from the gills of rainbow trout have, however, demonstrated chromatographically distinct 15-lipoxygenase which only "acts" on AA following its separation from the dominant 12-lipoxygenase (German & Berger, 1990; German & Creveling, 1990). A similar situation might exist in trout macrophages, thereby accounting for the apparent lack of 15-HETE generation by intact cells.

Previous studies have reported on several routes by which lipoxin biosynthesis can take place in human tissues with the interaction of either 5- and 12-lipoxygenases or 5- and 15-lipoxygenases, leading to the generation of these compounds [reviewed in Dahlén and Serhan (1991)]. The particular route taken depends on the cell type, the enzymes present, and the initial oxygenation of substrate that commits to the first biosynthetic step. The present study has shown little evidence to support the involvement of 15-lipoxygenase and its products, such as 15-HPETE, in the generation of $LXA_4$ and $LXA_5$ with intact trout macrophages. This is in contrast to cells such as mammalian granulocytes and mononuclear phagocytes, which readily convert 15-HPETE to lipoxins (Serhan et al., 1984; Kim, 1988). Recent reports have also highlighted a pivotal role of granulocyte-derived $LTA_4$ in the transcellular biosynthesis of lipoxins by platelets catalyzed by 12-lipoxygenase (Fiore & Serhan, 1990; Serhan & Sheppard, 1990; Romano & Serhan, 1992; Sheppard et al., 1992; Tornhamre et al., 1992). There is no evidence here to support a similar route in rainbow trout macrophages for a number of reasons. First, macrophages incubated with exogenous $LTA_4$ in the presence or absence of calcium ionophore did not exhibit an increase in the amount of $LXA_4$ formed (if $LTA_4$ was an intermediate, we would expect an increase in its conversion to either the 5(6)-epoxy tetraene or to lipoxins). Second, trapping studies revealed that the time courses for the appearance of LTA and epoxy tetraenes from endogenous fatty acids were similar, which would not be expected if LTA was a direct precursor in the formation of lipoxins via epoxy tetraene(s).

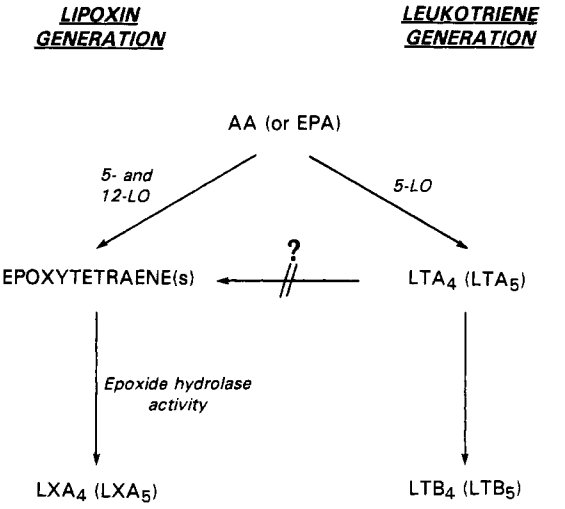


FIGURE 12: Scheme showing the hypothetical parallel pathways of biosynthesis for lipoxins and leukotrienes in trout macrophages. Note the apparent lack of conversion of LTA to the epoxy tetraene intermediate. LO, lipoxygenase.

In view of these results, we propose that two distinct pathways may operate in trout macrophages during the generation of leukotrienes and lipoxins, as shown in Figure 12. In this scheme, one enzyme system independently generates leukotrienes, while another is devoted to lipoxin biosynthesis. In this proposed scheme, the interaction of 5- and 12-lipoxygenases results in the generation of 5(6)-epoxy tetraene intermediates from endogenous AA or EPA, while 5-lipoxygenase alone appears to be devoted to the formation of LTA, which is enzymatically converted to LTB. These steps are consistent with previous studies that demonstrated the generation of epoxy tetraene intermediates by the interaction of 5- and 12-lipoxygenases [e.g. Serhan and Sheppard (1990)] and the formation of $LTA_4$ from AA by 5-lipoxygenase [e.g. Whelan et al. (1990)]. Furthermore, to provide additional evidence that the 5(6)-epoxy tetraene is a potential precursor to lipoxin in trout macrophages, it was added to trout macrophage lysates and found to be converted to predominantly $LXA_4$. These findings not only demonstrate that the 5(6)-epoxy tetraene is an intermediate in lipoxin generation but also suggest the presence of epoxide hydrolase activity in these cells that rapidly converts this to $LXA_4$. The presence of such an enzyme may explain the finding that intact macrophages challenged with ionophore or zymosan fail to generate either $LXB_4$ or the nonenzymatic hydrolysis products of 5(6)-epoxy tetraene [i.e., 14(*S*)-8-*trans*-$LXB_4$, 8-*trans*-$LXB_4$] above detection limits by RP-HPLC. It would be interesting to purify this epoxide hydrolase and compare its properties with these of the metallohydrolase, $LTA_4$ hydrolase,

as both of these enzymes may share a common phylogenetic ancestry.

The products denoted groups 1 and 2 contained conjugated tetraene chromophores and were only observed in methanol trapping experiments. Thus, it is likely that these are also methanol trapping products, although they were not further identified in the present study. Since group 2 products were increased in the presence of exogenous EPA, it is probable that they arise from this precursor. Also, in the presence of exogenous 15-HETE, $LXB_4$ was generated that was not formed by intact cells from endogenous substrate (Figure 5). Its formation from 15-HETE may involve a 14(15)-epoxy tetraene intermediate that could be evoked during cell–cell interactions to generate $LXB_4$. These points remain to be studied further.

In summary, the present and previous results (Pettitt et al., 1991) suggest that trout macrophages possess parallel pathways that can generate lipoxins from this single cell type (Figure 12), while in human tissues the lipoxin pathway may have diverged to involve the cooperation of at least two cell types for their biosynthesis via transcellular events (Serhan & Sheppard, 1990; Fiore & Serhan, 1990; Edenius et al., 1991). There is also no evidence for the involvement of 15-lipoxygenase products such as 15-HPETE in the generation of lipoxins in trout macrophages.

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