Lipoxin A_4 Metabolism by Differentiated HL-60 Cells and Human Monocytes: Conversion to Novel 15-Oxo and Dihydro Products†

Charles N. Serhan,* Stefano Fiore, Damian A. Brezinski, and Sharon Lynch

Hematology-Oncology Division, Department of Medicine, Brigham & Women's Hospital and Harvard Medical School, Boston, Massachusetts 02115

Received March 3, 1993; Revised Manuscript Received April 30, 1993

ABSTRACT: Lipoxins are tetraene-containing eicosanoids that possess biological activity in several organ systems. To determine their route of further metabolism, [11,12-^{3}H]lipoxin A_4 was prepared and incubated with human neutrophils, promyelocytic leukemia (HL-60) cells, and adherent monocytes. Intact neutrophils and undifferentiated HL-60 cells did not significantly metabolize [11,12-^{3}H]LXA_4, while HL-60 cells differentiated with PMA to monocyte/macrophage lineage rapidly (<15 s) transformed this eicosanoid. The major radiolabeled LXA_4-derived metabolites were characterized by physical methods and were shown to be 15-oxo-LXA_4, 13,14-dihydro-15-oxo-LXA_4, and 13,14-dihydro-LXA_4. Substrate competition with cell-free supernatants from differentiated HL-60 cells suggests that lipoxins compete for 15-hydroxyprostaglandin dehydrogenase activity or an equivalent enzyme system. In addition, adherent monocytes exposed to [11,12-^{3}H]LXA_4 rapidly metabolized (>60% within 30 s) the label to its oxo and dihydro derivatives. These results indicate that, unlike leukotrienes, LXA_4 is subject to dehydrogenation and reduction of its conjugated tetraene to form triene-containing products. Moreover, they suggest that monocytes participate in lipoxin metabolism in their local milieu.

Activation of lipoxygenases (LOs) is held to be an important event in wounding, in senescence, and during the inflammatory response in plants (Rouet-Mayer et al., 1992), whereas in mammalian tissues the products of these enzymes are known to be potent mediators in inflammation (Samuelsson et al., 1987). The lipoxin series is a more recent addition to the family of bioactive eicosanoids. They are unique in that they are tetraene-containing compounds derived via the interactions of individual lipoxygenases (Serhan et al., 1984) and possess selective activities [reviewed in Serhan (1991)]. Lipoxins are generated in microgram amounts by macrophages from rainbow trout (Pettitt et al., 1991), suggesting that they are phylogenetically ancient structures. Their generation by human cells is in the nanogram range and appears to require transcellular metabolism involving at least two cell types (Serhan, 1991). Platelets, for example, generate lipoxins by the action of 12-LO from leukocyte-derived leukotriene A_4 (Tornhamre et al., 1992; Romano & Serhan, 1992), a pathway that is deficient in myeloproliferative disorders (Stenke et al., 1991a). Although platelets can be a rich source of lipoxins, these cells do not enzymatically transform LXA_4[1] (Brezinski & Serhan, 1991) or LXB_4 (Romano & Serhan, 1992). Eosinophils and human airway epithelial cells also produce lipoxins by transcellular routes, but in this case formation involves 15-LO (Edenius et al., 1990). Thus, two major routes of lipoxin formation have been identified, one initiated by 5-LO and the other by 15-LO and subsequent reactions [reviewed in Serhan (1991)].

LXA_4 and LXB_4 are the two main lipoxins. They each possess potent actions in both in vitro and in vivo models that have proven to be distinct from those of other mediators. Despite their stereoselective impact, in some tissues micromolar amounts are required to elicit responses, while in others, subnanomolar levels trigger actions. For example, nanomolar levels elicit prompt vasodilation (Busija et al., 1989; Katoh et al., 1992). In the subnanomolar range (10^{-10} M) they both stimulate GM-CSF-induced myeloid colony formation (Stenke et al., 1991b), and at 10 nM they enhance activity of protein kinase C (PKC) in nuclei of erythroleukemia cells (Beckman et al., 1992). Counterregulatory roles have also been demonstrated; prior exposure to LXA_4 (nanomolar levels) blocks renal vasoconstrictor actions of LTD_4 (Katoh et al., 1992), inhibits chemotaxis of PMN (Lee et al., 1991), and blocks LTB_4-induced inflammation in the hamster cheek pouch (Raud et al., 1991). These findings suggest that LXA_4 may serve as an endogenous chalone. However, in lung tissue $\gg$100 nM is required to elicit a response in this organ (Wikström et al., 1992; Meini et al., 1992). Administration of LXA_4 in micromolar amounts via inhalation blocks bronchoconstriction in asthmatic patients (Christie et al., 1992). Thus, the wide concentration range (subnanomolar to micromolar) observed for LX may reflect nonspecificity for LX-evoked responses or more likely may represent a combination of (i) the presence of specific receptors, (ii) differential modes of action in signal transduction that are cell type-specific (Fiore et al., 1992),

† These studies were supported in part by National Institutes of Health Grant GM-38765 (C.N.S.). The HP diode array detector was purchased with funds from the Proctor Fund, Harvard Medical School. C.N.S. is under the tenure of an Established Investigator Award from the American Heart Association and is a recipient of the Clifford M. Clarke Science Award from the National Arthritis Foundation. D.A.B. was a Research-Resident of the Brigham & Women's and Beth Israel Hospitals during these studies, and S.F. is a Fellow of the National Arthritis Foundation.

* Address correspondence to this author at the Longwood Medical Research Center, 221 Longwood Ave., Boston, MA 02115 (Fax: 617-739-3324).

[1] Abbreviations: GC/MS, gas chromatography/mass spectroscopy; 15-HETE, 15(*S*)-hydroxy-5(*Z*),8(*Z*),11(*Z*),13(*E*)-eicosatetraenoic acid; HL-60, human acute promyelocytic leukemic cell line; LXA_4, 5(*S*),6-(*R*),15(*S*)-trihydroxy-7(*E*),9(*E*),11(*Z*),13(*E*)-eicosatetraenoic acid (lipoxin A_4); 11-*trans*-LXA_4, 5(*S*),6(*R*),15(*S*)-trihydroxy-7(*E*),9(*E*),11-(*E*),13(*E*)-eicosatetraenoic acid; LXB_4, 5(*S*),14(*R*),15(*S*)-trihydroxy-6(*E*),8(*Z*),10(*E*),12(*E*)-eicosatetraenoic acid (lipoxin B_4); Me_3Si (OTMS), trimethylsilyl; NAD, β-nicotinamide adenine dinucleotide; PBM, peripheral blood monocytes; PBS, Dulbecco's phosphate-buffered saline; PGE_1, (11α,13*E*,15*S*)-11,15-dihydroxy-9-oxoprost-13-enoic acid; 15(*S*)-15m-PGE_1, 15-methyl-PGE_1; PMA, phorbol 12-myristate 13-acetate; PMN, polymorphonuclear leukocytes; RP-HPLC, reverse-phase high-performance liquid chromatography.

0006-2960/93/0432-6313$04.00/0

and/or (iii) rapid local metabolism that ultimately leads to inactivation of LX as is the case for other eicosanoids.

Other LO-derived products such as leukotrienes are metabolized by ω-oxidation in various tissues followed by β-oxidation (Huwyler et al., 1992). Preliminary findings suggest that LX may also be hydroxylated at either ω-20 or ω-1 by cytochrome P-450 monogenase(s) in human leukocytes, as well as rat and human liver microsomes (Boucher et al., 1991). The present work demonstrates that, unlike leukotrienes, LXA_4 is rapidly metabolized by intact PMA-differentiated HL-60 and adherent monocytes to several new products, the structures of which have been determined.

MATERIALS AND METHODS

HL-60 cells were purchased from American Type Culture Collection (Rockville, MD). Other cell culture reagents and versene (EDTA) were from BioWhittaker (Walkersville, MD). Synthetic 11,12-acetylenic LXA_4 methyl ester and lipoxins were from Cascade Biochemical (Reading, U.K.). 15(*S*)-15m-PGE_1, PGE_1, and 5-HETE were from Cayman Chemical Co. (Ann Arbor, MI). [11,12-^{3}H]LXA_4 was prepared from 11,12-acetylenic LXA_4 using Lindlar catalyst as a custom tritiation (NET-259, lot no. 2793-275, New England Nuclear, Boston, MA). Tritiated products were isolated using RP-HPLC (Fiore et al., 1992; Serhan, 1990). Methoxyamine and NAD were from Sigma Chemical Co. (St. Louis, MO). Manganese dioxide and Adam's reagent were from Aldrich Chemical Co. (Milwaukee, WI).

Human PMN were obtained from healthy volunteers by gradient centrifugation of heparinized fresh venous blood (Böyum, 1986). HL-60 cells were seeded in RPMI supplemented with penicillin (100 units/mL), streptomycin (100 μg/mL), and fetal bovine serum (10%) (Hyclone, Logan, UT) and incubated (37 °C with 5% CO_2 atmosphere) in plastic 250-mL flasks. Individual flasks containing $\sim 5 \times 10^7$ HL-60 cells/mL were incubated in the presence or absence of PMA (10 or 16 nM, 24–27 h), and adherence was monitored for induction of macrophage-like phenotype as described in Collins (1987). Peripheral blood monocytes were obtained (Goldyne et al., 1984) after fresh mononuclear cells were plated onto plastic Petri dishes (15 cm) containing PBS with glucose (1 mg/mL) for 1 h at 37 °C. Nonadherent cells were removed and adherent mononuclear cells were gently resuspended using versene (7 mL/plate) and washed in PBS. PMN (>98%), adherent monocytes (>95%), and HL-60 cells were enumerated by light microscopy and suspended in PBS for incubations, and <2–3% in each case were permeable to trypan blue. For some experiments, cell-free supernatants were prepared from HL-60 cells treated with PMA (10–16 nM) for 24–72 h. After being harvested, the differentiated cells were washed and then subjected to freeze–thaw lysis (repeated 3 times) and ultracentrifugation (100000*g*, 1 h).

Incubations with eicosanoids were stopped with cold methanol containing either PGB_2 or 5-HETE as internal standards (5-HETE was used when 15-oxo-ETE was quantified). Products were extracted using Sep-pak C_{18} and routinely chromatographed as described in Serhan (1990). This HPLC system consisted of an LKB gradient dual pump equipped with an Altex Ultrasphere-ODS (4.6 mm × 25 cm) column, flow rate 1 mL/min, eluted (T_0 –20 min) with methanol/H_2O/acetic acid (65:35:0.01 v/v/v) and MeOH/acetic acid (99.99:0.1 v/v) in a linear gradient (20–45 min) that was used to quantitate the ω-metabolites of LTB_4 (i.e., 20-COOH and 20-OH LTB_4) as well as LXA_4 (as in Figure 1). Recovery of internal standards was 82.2 ± 7.9, mean ± SD (n = 13).

Compounds I–IV (Figure 2) were separated using an Altex Ultrasphere-ODS column (10 mm × 25 cm) eluted at a flow rate of 3.0 mL/min with methanol/H_2O/acetic acid (60:40:0.01 v/v/v). Formation of 15-oxo-ETE by 100000*g* supernatants [cf. Agins et al. (1987), Xun et al. (1991), and Sok et al. (1988)] was quantified after RP-HPLC using an ODS column (4.6 mm × 25 cm) eluted with methanol/H_2O/acetic acid (70:30:0.01 v/v/v) and monitored at 280 nm with a flow rate of 1 mL/min. Monocyte-derived products were also chromatographed using a Hypersil column (5 μm, 4 mm × 300 mm) eluted with methanol/H_2O/acetic acid (60:40:0.01 v/v/v) at a flow rate of 1 mL/min. On-line spectra were recorded using a diode array detector (Hewlett-Packard 1040M series II) equipped with HPLC^{3D} ChemStation software (DOS series). (Spectra were acquired using step = 4 nm, *Bw* = 10 nm, and range = 235–360 nm with a sampling interval of 1.28 s.)

GC/MS was performed with a Hewlett-Packard 5971A mass selective detector quadrupole equipped with a HPG1030A workstation and GC 5890. The column was an HP-Ultra 2 (cross-linked 5% phenyl methyl silicone gum phase; 25 m × 0.2 mm × 0.33 μm), and injections were made in the splitless mode in bis(trimethylsilyl)trifluoroacetamide (BSTFA). The temperature program was initiated at 150 °C and reached 250 °C at 10 min and 325 °C at 20 min. Standard saturated fatty acid methyl esters C16–C26 gave the following retention times (min:s; mean of n = 6): C16, 8:03; C18, 9:77; C20, 12:22; C22, 16:11; C24, 20:72; C26, 23:62; these were used to calculate respective *C* values of LX-derived metabolites as described in Serhan (1990). Diazomethane was prepared, and the methyl ester products were treated with BSTFA (Pierce Chemical Co., Rockford, IL) to obtain Me_3Si derivatives. Methyl ester *O*-methoxime derivatives were prepared as described in Kelly and Abel (1983). Catalytic hydrogenations were performed in methanol (1 mL) with Adam's reagent (Aldrich, Milwaukee, WI) by saturating the platinum(IV) oxide (~1–2 mg) with a stream of bubbling hydrogen (20 min, room temperature). After extraction, materials were treated with diazomethane followed by BSTFA (overnight; room temperature).

RESULTS

Metabolism of LXA_4. Intact neutrophils from peripheral blood of healthy donors did not significantly metabolize exogenous LXA_4 while cells from the same donors rapidly transformed LTB_4 via ω-oxidation. In contrast, PMA-treated HL-60 cells that displayed monocyte/macrophage-like characteristics rapidly transformed LXA_4 (Figure 1). Within the first 60 s of exposure, >70% of LXA_4 was metabolized. In the absence of PMA-induced differentiation, neither intact HL-60 cells (undifferentiated) nor their cell-free supernatants (100000*g*) transform LXA_4 (n = 3).

Differentiated HL-60 cells incubated with LXA_4 converted this eicosanoid to several products. A representative profile of the products obtained from HL-60 cells after harvesting, washing, and incubation with LXA_4 and [11,12-^{3}H]LXA_4 is given in Figure 2. Labeled LXA_4 was transformed to four main products that carried tritium (denoted compounds I–IV), which were collected for further analysis.

Structures of Compounds I–IV. To obtain quantities of these compounds enabling structural studies, their retention times in RP-HPLC were established using the ^{3}H-label elution profile to mark boundaries, and unlabeled samples pooled from several incubations were chromatographed and individually collected from within these regions for GC/MS

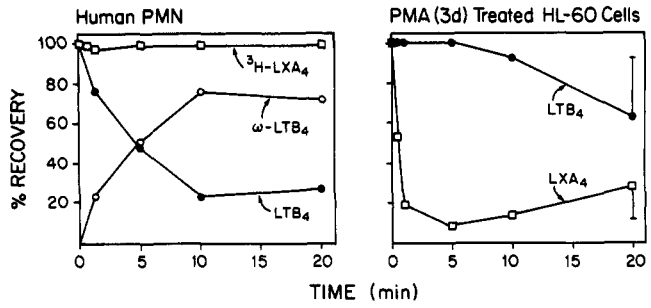

FIGURE 1: Time course of conversion for LTB_4 and LXA_4; comparison between human PMN and HL-60 cells. Left: PMN ($\sim 2 \times 10^7$ cells/mL of PBS) were incubated (37 °C) with 1 μg of either LTB_4 or LXA_4 (with tracer [^{3}H]LXA_4). ω-LTB_4 denotes the sum of 20-OH and 20-COOH LTB_4. Right: HL-60 cells were treated with PMA (15 nM for 72 h), harvested, washed, and incubated (10^6 cells) with either LTB_4 or LXA_4. At indicated intervals, aliquots were removed, placed in cold MeOH (2 volumes), extracted, and chromatographed by RP-HPLC (see Materials and Methods). Results are the mean of two separate time courses with $n = 4 \pm$ SD at $t = 20$ min.

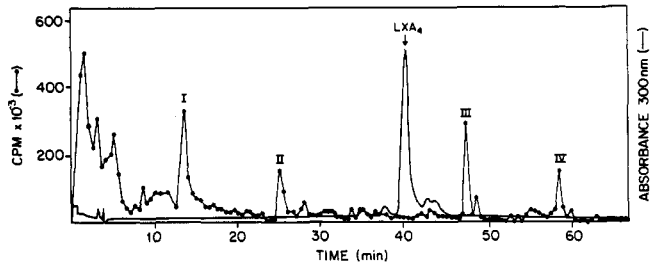

FIGURE 2: RP-HPLC profile of products from HL-60 cells incubated with LXA_4. HL-60 cells were treated (72 h with PMA; 10 nM), washed, harvested, and incubated with LXA_4 ($n = 17$) and [^{3}H]-LXA_4 ($\sim$5000 cpm, $n = 5$). The profile is representative for pooled samples suspended in MeOH/H_2O (50:50 v/v) and injected into an Ultrasphere-ODS (10 mm i.d. × 25 cm) eluted with methanol/water/acetic acid (60:40:0.01 v/v/v) as solvent at a flow rate of 3.0 mL/min. The arrow denotes the LXA_4 standard recorded (—) with the UV detector set at 300 nm.

analysis. Selected ion monitoring of the products obtained after treatment with diazomethane and BSTFA revealed that compounds **I–IV** each displayed prominent ions at m/z 203 [–CH(OSiMe$_3$)(CH$_2$)$_3$COOCH$_3$], indicating that carbons 1–5 of LXA_4 (carboxylic carbon is number 1) were not modified, although each product gave a different retention time than LXA_4 (Figure 2). The methyl ester trimethylsilyl derivative of LXA_4 displays prominent ions in its electron impact spectrum at m/z 203 (base peak) and 173, with its molecular ion at 582 (M^+). Other ions of diagnostic value in this derivative of LXA_4 are observed at m/z 171 (203 – 32), 409 (M – 173), 379 (M – 203), 482 (M – 100), and 492 (M – 90) (Serhan et al., 1984; Serhan, 1990). It is noteworthy that the lipoxins in general are known to give extremely weak molecular ion peaks (Serhan et al., 1984). Nevertheless, compounds labeled **I** and **II** (Figure 2) also possess prominent ions at m/z 173 [Me$_3$SiO$^+$=CH(CH$_2$)$_4$CH$_3$], indicating that the carbon 15–20 fragment of these LXA_4-derived products was intact, while the ion at m/z 173 was not evident in compounds **III** and **IV**. Thus, the conclusion that compounds **I–IV** are metabolites of LXA_4 was based upon their physical properties (HPLC and GC/MS) and the finding that they carry tritium label, as well as the absence of these products in incubations with HL-60 cells not treated with PMA.

Next, we focused on compounds **III** and **IV** since it appeared that they represent metabolites with structural modifications in the carbon 15–20 fragment of LXA_4. Since ω-oxidation (hydroxylation at carbon 20) was a possibility, ions that could result from the respective 20-OH and 20-COOH forms of LXA_4 after derivatization, namely, m/z 261 and 217 [Me$_3$SiO$^+$=CH(CH$_2$)$_4$CH$_2$OSiMe$_3$ and Me$_3$SiO$^+$=CH(CH$_2$)$_4$-CO$_2$Me], were scanned in the acquired GC/MS data profiles. Neither **III** nor **IV** displayed prominent ions at either m/z 261 or 217, indicating that these products were not likely the result of ω-oxidation. Figure 3, panel A, gives the mass spectrum (C value 24.3) of the Me$_3$Si derivative methyl ester of compound **III**. Prominent ions in its spectrum were observed at m/z 203 [base peak, CH(OSiMe$_3$)(CH$_2$)$_3$COOCH$_3$], 171 (203 – 32; elimination of CH$_3$OH), 215 [(M – 203) – 90; elimination of trimethylsilanol (Me$_3$SiOH)], and 99 [O=C-(CH$_2$)$_4$CH$_3$]. Ions of lower intensity were at m/z 508 (M^+) and 418 (M – 90; loss of Me$_3$SiOH). The presence of these ions suggested that the material that coeluted with ^{3}H-labeled compound **III** (see Figure 2) was the 15-oxo derivative of LXA_4. This is supported by several lines of evidence, namely, the virtual loss of the prominent ion at m/z 173 [Me$_3$-

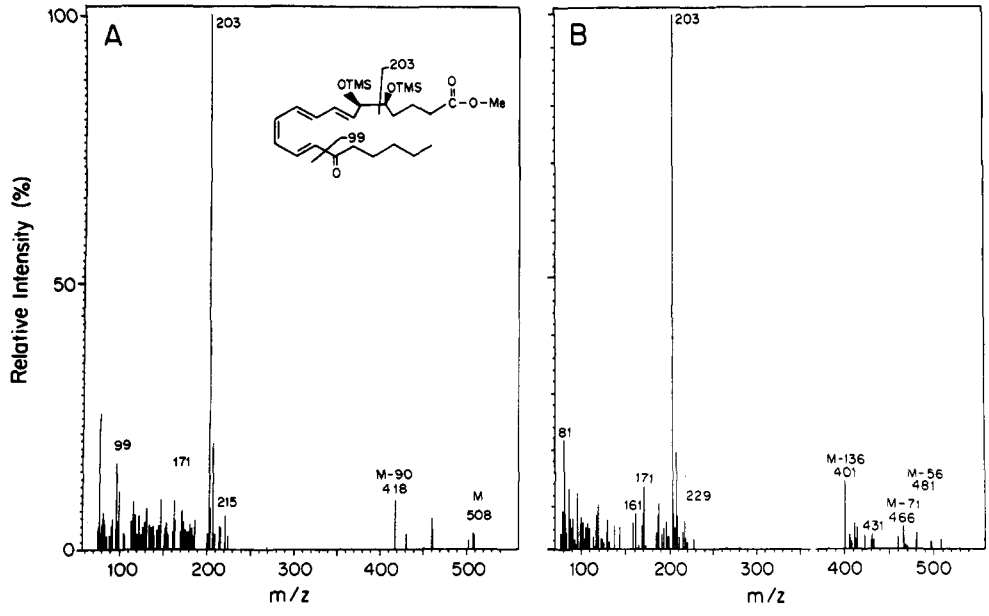

FIGURE 3: (A) Mass spectrum of the Me$_3$Si derivative methyl ester of compound **III**. (B) Mass spectrum of the methyl ester *O*-methoxime Me$_3$Si derivative of **III**. The ions >400 amu were magnified 10 times.

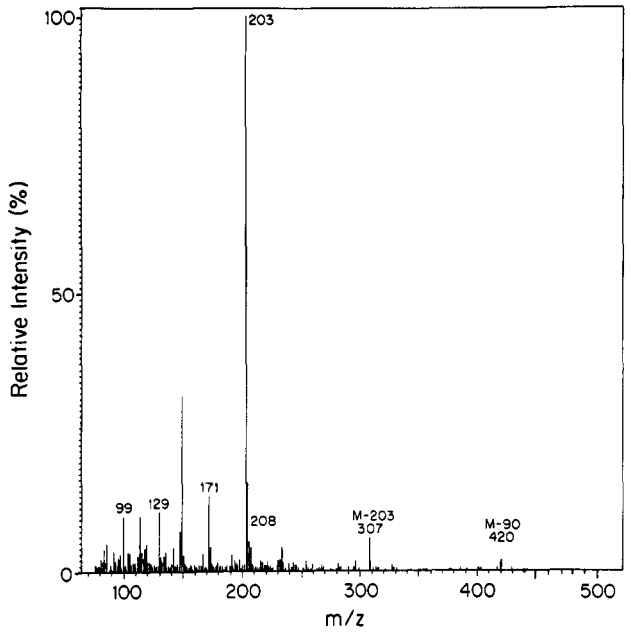

FIGURE 4: Mass spectrum of the Me_3Si derivative methyl ester of compound **IV**.

$SiO^+{=}CH(CH_2)_4CH_3$], the presence of m/z 99 [$O{=}C(CH_2)_4CH_3$], the absence of a tetraene chromophore, and the appearance of a new chromophore at UV λ_{max} 335–340 nm. The tetraenone chromophore was confirmed by treating LXA_4 with MnO_2 in chloroform as used for prostaglandin conversion (Änggård & Samuelsson, 1964). Also, the mass spectrum of the catalytic hydrogenation product gave a C value of 25.1 and prominent ions at m/z 203 (base peak), 99 (66%), 313 (M – 203 or M – $CH(OSiMe_3)(CH_2)_3COOCH_3$; 35%), and 171 (36%) with no prominent ion at m/z 173. Less intense ions were at m/z 516 (M^+) and 426 (M – 90). Thus, the upward shift of 8 amu and fragmentation of this saturated derivative were consistent with the generation of the corresponding 15-oxo derivative.

To examine this LXA_4-derived product further, an aliquot of the material eluting beneath the peak labeled **III** was treated with diazomethane followed by methoximation [as described in Bergholte et al. (1987)] and treatment with BSTFA. Its spectrum (Figure 3, panel B), C value of 25.4, showed prominent ions at m/z 203 (base peak), 171 (203 – 32; loss of CH_3OH), and 229 [M – 128 or $CH_3ON{=}C(CH_2)_4CH_3$ – (2 × 90)]. Ions of lower intensity were at m/z 537 (M^+), 466 [M – 71, the α-cleavage ion M – $CH_2(CH_2)_3CH_3$], 481 (M – 56 or M – $CH_2{=}CHCH_2CH_3$, a McLafferty rearrangement ion), 431 [M – 106 (possibly loss of $C_7H_5N^+$)], 401 [M – 136 (elimination of $Me_3SiOH + CH_3 + {\cdot}OCH_3$)], and 460 (M – 77, loss of $NOCH_3$ plus MeOH). Again, an ion at m/z 173 that would have originated from an alcohol-containing C15 fragment [$Me_3SiO^+{=}CH(CH_2)_4CH_3$] was virtually absent in its spectrum. Thus, the ions present are consistent with the methyl ester O-methoxime derivative generated from the 15-oxo-containing derivative of LXA_4. Together, the prominent ions observed with these different derivatives suggest that material eluting beneath the peak labeled **III** was the 15-oxo product of LXA_4 (i.e., 15-oxo-LXA_4).

Figure 4 shows the mass spectrum of the methyl ester Me_3Si derivative (C value 26.0) of compound **IV**. Prominent ions were at m/z 203 [base peak, $CH(OSiMe_3)(CH_2)_3COOCH_3$], 171 (203 – 32; loss of CH_3OH), 99 [$O{=}C(CH_2)_4CH_3$], and 307 [M – 203 or M – $CH(OSiMe_3)(CH_2)_3COOCH_3$]. Ions of lower intensity were at m/z 510 (M^+), 420 (M – 90; loss of trimethylsilanol), and 208 [M – (99 + 203)]. Its UV spectrum recorded on-line showed a triplet of absorbance with maxima at 259, 269, and 280 nm, consistent for a conjugated triene chromophore. The presence of these ions and UV spectrum suggest that **IV** was a dihydro-15-oxo metabolite of LXA_4. This basic structure was supported by the presence of the ion at m/z 99 that is consistent with a keto group at position carbon 15, and the presence of m/z 203 as the base peak revealed that the alcohol groups at carbons 5 and 6 remain intact. In addition, the absence of a trienone chromophore ($\lambda_{cal} \approx 310$ nm) indicates that loss of a double bond was at Δ13–14 position to give the observed triene chromophore. Together, these results indicate that compound **IV** was 13,-14-dihydro-15-oxo-LXA_4.

The methyl ester Me_3SiO derivative of compound **II** (C value 25.4) gave ions at m/z 203 [base peak; $CH(OSiMe_3)(CH_2)_3COOCH_3$], 173 [$Me_3SiO^+{=}CH(CH_2)_4CH_3$], 171 (203 – 32), and 584 (M^+). Its molecular ion was two mass units higher than the LXA_4 derivative. These ions and a triplet band of absorbance recorded on-line gave λ_{max} at 259, 269, and 282 nm, suggesting that compound **II** was a dihydro derivative of LXA_4. The methyl ester Me_3SiO derivative of compound **I** from HL-60 cells gave two products in GC. The major one (C value 25.0) gave ions in its mass spectrum similar to those of LXA_4, but instead its molecular ion was at m/z 586 with ions also present at m/z 555 (M – 31) and 496 (M – 90), indicating that two of the four double bonds were reduced (not shown). However, identical products were not observed with adherent monocytes obtained from peripheral blood (vide infra), and thus the HL-60 cell-derived materials from peak **I** were not further characterized in the present experiments. The structures of **I**–**IV** and results from Figure 1 indicate that LXA_4 is not metabolized by ω-oxidation by intact leukocytes but instead is both dehydrogenated at the carbon 15 alcohol and transformed from a conjugated tetraene to triene structure. Taken together, these observations suggested that LXA_4 may be attacked by NAD-dependent 15-prostaglandin dehydrogenase (15-PGDH), an enzyme known to carry out similar reactions with prostanoids as substrate [Änggård & Samuelsson, 1964; reviewed in Hansen (1976)].

Can LX Compete for 15-PGDH Activity? 15-PGDH activity was recently shown to be induced in HL-60 cells (Xun et al., 1991), and it apparently utilizes 15-HETE as substrate with 92% efficiency compared to PGE_2 (Agins et al., 1987). Indeed, 100000g supernatants prepared from PMA-treated HL-60 cells converted 15-HETE to 15-oxo-ETE, indicating the presence of a dehydrogenase activity after differentiation. LXA_4 competed for catalysis of 15-HETE, giving a $K_i = 8.2 \pm 2.6$ μM (SEM, $n = 6$) calculated from Lineweaver–Burk plots. At equimolar concentrations of LXA_4 and 15-HETE, LXA_4 blocked 15-oxo-ETE formation by ≈50%. The relative conversion for LX compared to PGE_1 by 100000g supernatants (Figure 5) indicated that LXA_4 and 11-*trans*-LXA_4 as well as LXB_4 but not 15-methyl-PGE_1 were converted. Together, these results suggest that LXA_4 > 11-*trans*-LXA_4 > LXB_4 are substrates for 15-PGDH or an equivalent enzyme system.

Conversion of LXA_4 by Monocytes. Since PMA induces differentiation to monocyte/macrophage-like lineage of HL-60 cells (Collins, 1987), we incubated peripheral blood monocytes to determine if they metabolize lipoxin. Lipoxins display potent actions with monocytes (Stenke et al., 1991b), and these cells do not ω-oxidize eicosanoids (Goldyne et al., 1984). When suspensions of both intact monocytes ($n = 5$) and permeabilized cells (freeze–thaw or saponin-treated, $n = 5$), obtained after adherence to plastic, were exposed to LXA_4,

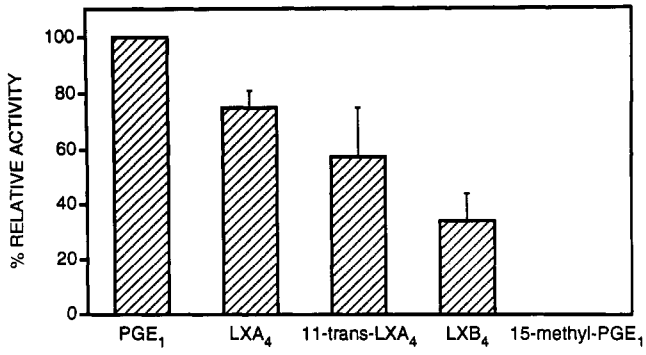

FIGURE 5: Conversion of PGE_1 and lipoxins by differentiated HL-60 cells. PMA-treated (10 ng/mL, 72 h) HL-60 cells were harvested, lysed by freeze–thaw, and taken for ultracentrifugation (100000g, 1 h). Each supernatant (equivalent $\approx 10^7$ cells) was incubated with eicosanoids (20 min, 37 °C). The percent conversion was calculated after HPLC quantitation. Results represent the mean of three experiments ± SE.

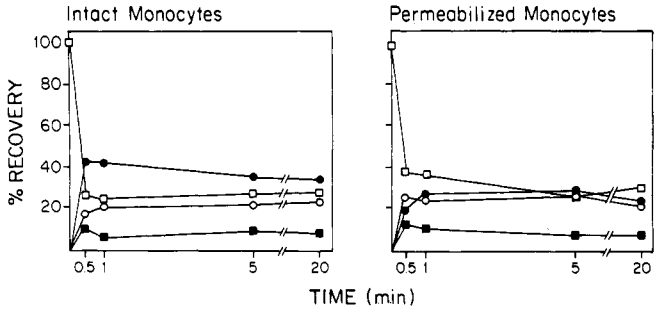

FIGURE 6: Time course of conversion of LXA_4 by human PBM. PBM were isolated by adherence (1 h, 37 °C) and suspended at 125 × 10^6 cells/ 5 mL of PBS before incubation with 500 ng of LXA_4 and 100 000 dpm of [11,12-^{3}H]LXA_4 (37 °C). At indicated intervals, aliquots (1 mL) were removed, placed in 2 volumes of cold MeOH with internal standard, extracted, and chromatographed by RP-HPLC. Results are the mean from cells obtained with two separate donors. Left: Intact cells. Right: Monocytes were permeabilized by rapid freeze–thaw and incubated with 1 mM NAD for 3 min before addition of [11,12-^{3}H]LXA_4 and LXA_4. Symbols: (□) LXA_4; (■) 15-oxo-LXA_4; (○) 13,14-dihydro-15-oxo-LXA_4; (●) 13,14-dihydro-LXA_4.

they converted it to 15-oxo-LXA_4 and conjugated triene-containing products, 13,14-dihydro-LXA_4 and 13,14-dihydro-15-oxo-LXA_4 (see Table I). As with differentiated HL-60 cells, monocytes rapidly converted LXA_4 (>60%) within 30 s (Figure 6). The temporal relationships for formation of these metabolites in both intact and permeabilized monocytes were similar and suggest that the 15-oxo-LXA_4 metabolite is a transient intermediate. Also, in each monocyte suspension incubated with [^{3}H]LXA_4 (d = 33), 13,14-dihydro-15-oxo-LXA_4 and 13,14-dihydro-LXA_4 were the major products carrying radiolabel. It is noteworthy that a product eluting before 13,14-dihydro-LXA_4 at 15.5–17 min was observed that also displayed a triene chromophore and was likely the 11-trans isomer of 13,14-dihydro-LXA_4 that results from cis–trans isomerization encountered during workup. The 11-cis double bond of native LXA_4 is labile and readily isomerizes to all-trans during extraction and isolation (Romano & Serhan, 1992). In a separate group of experiments, we added the cation ionophore A23187 (2.5 μM) along with [^{3}H]LXA_4 and ~0.3 μM LXA_4 (20 min, 37 °C) to isolated intact monocytes to determine whether further cell activation could alter the generation of LXA_4 metabolites. In the presence of A23187, a modest 16.6 ± 5.6% increase (mean ±SD) was registered for 15-oxo-LXA_4, while <6% changes were noted for either 13,14-dihydro-15-oxo-LXA_4 or 13,14-dihydro-LXA_4 (n = 3).

DISCUSSION

Lipoxins have reported actions that range from subnano- to micromolar (Busija et al., 1989; Katoh et al., 1992; Stenke et al., 1991b; Beckman et al., 1992; Lee et al., 1991; Raud et al., 1991; Wikström et al., 1992; Meini et al., 1992; Christie et al., 1992; Fiore et al., 1992). Like other eicosanoids, they may be rapidly inactivated in their local environment by further metabolism. Since lipoxins have potent actions on neutrophils (Lee et al., 1991; Raud et al., 1991; Fiore et al., 1992), induce myeloid stem cell growth (Stenke et al., 1991b), and stimulate the entry of erythroleukemia cells into S phase (Beckman et al., 1992), we evaluated the further metabolism of LXA_4 with human PMN, HL-60 cells, and adherent monocytes in suspensions isolated from peripheral blood. The present results demonstrate that [11,12-^{3}H]LXA_4 is transformed into novel products by an enzyme system that appears to be induced (by PMA) in cultured promyelocytic cells and is present in monocytes. In contrast, neither intact neutrophils nor undifferentiated HL-60 enzymatically transformed [11,12-^{3}H]-LXA_4 (Figures 1 and 2). The major products were identified as 15-oxo-LXA_4, 13,14-dihydro-15-oxo-LXA_4, and 13,14-dihydro-LXA_4. Thus, the metabolism of LXA_4 involves oxidation at C^5 and reduction of the double bond at C13 and C14 to give conjugated triene-containing compounds.

The properties of these leukocyte LX-derived products are summarized in Table I. Generation of the *O*-methoxime from **III** confirmed both the presence and position of the keto group (Figure 3). Also, the presence of a triene chromophore (**IV**) and absence of a trienone auxochrome in its UV spectrum indicated that the double bond between C13 and C14 was the site of reduction to give 13,14-dihydro-15-oxo-LXA_4 (Table I). It is noteworthy that large-scale separations of these metabolites required preparative HPLC to avoid their coelution and appearance of UV peaks with combined tetraene and triene chromophores. The structures of the LXA_4-derived

Table I: Physical Properties of LXA_4 Metabolites from Adherent Human Monocytes and Differentiated HL-60 Cells

	HPLC retention times[a] (min)			GC/MS[c]	
parent compound	A	B	UV $\lambda_{max}^{solvent\ b}$ (nm)	C value	diagnostic ions
LXA_4	40.5	22.0	287, 300, 315	24.1	171, 173, 203 (100%), 582 (M^+)
15-oxo-LXA_4	48.0	30.5	335–340	24.3	99, 171, 203 (100%), 305, 418, 508 (M^+)
13,14-dihydro-15-oxo-LXA_4	58.5	34.8	259, 269, 280	26.0	99, 171, 203 (100%), 307, 420, 510 (M^+)
13,14-dihydro-LXA_4	25.3	20.5	259, 269, 282	25.4	171, 173, 203 (100%), 484, 584 (M^+)

[a] Retention times were recorded using two separate systems: (A) the column was an Ultrasphere-ODS from Altex (dp 5 μm, 10 mm i.d. × 25 cm) eluted with MeOH/H_2O/acetic acid (60:40:0.01 v/v/v) at 3.0 mL/min flow rate; (B) the column was a Shandon ODS hypersil (5 μm, 4 mm × 300 mm) from Bishoff chromatography eluted with MeOH/H_2O/acetic acid (60:40:0.01 v/v/v) at 1.0 mL/min. [b] Recorded in HPLC solvent on-line with a 4-nm step and 1.28-s sampling acquisition mode using HP 3-D software (in MeOH using a ±1-nm step, 13,14-dihydro-LXA_4 gave λ_{max} at 262, 272, and 283 nm). [c] C values (equivalent chain length) and fragmentation patterns were obtained for the methyl ester of the trimethylsilyl derivative of each parent compound.

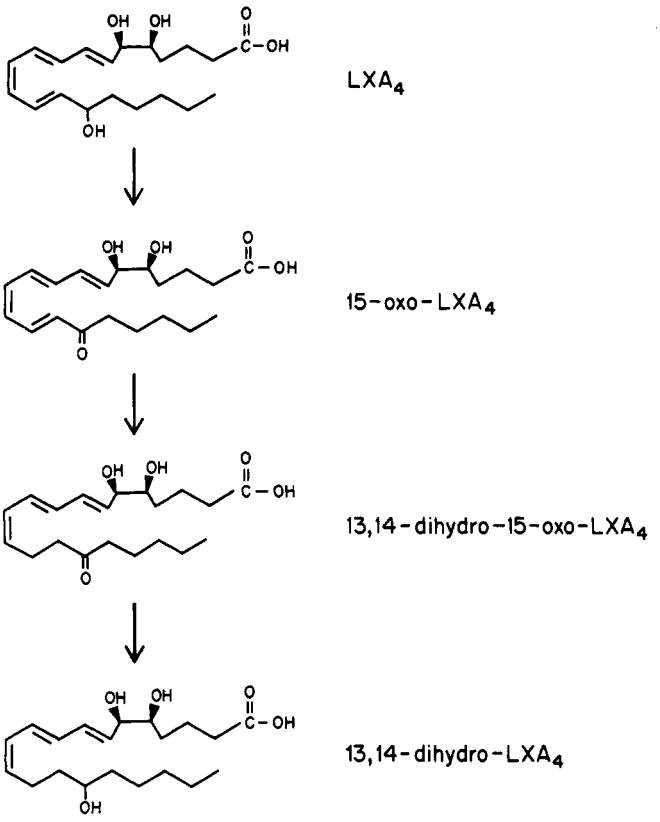

FIGURE 7: Proposed scheme of LXA_4 metabolism by human HL-60 cells and monocytes. The stereochemistry of the alcohol group at carbon 15 in 13,14-dihydro-LXA_4 has not been determined.

products documented (Table I) suggest that these LO products are initially metabolized by human leukocytes in a fashion similar to that proposed for prostanoids [reviewed in Hansen (1976)] rather than ω-oxidiation as used to metabolize LTB_4 [see Huwyler et al. (1992)]. This notion is also supported by results from substrate competition (Figure 5), time–course experiments (Figure 6), and the finding that addition of NAD led to enhancement of 15-oxo-LXA_4 recovery from monocytes. (Nonenzymatic LX degradation was excluded from the present results by adding extraction control incubations, i.e., without cells, in each experimental series.) A proposed scheme for LXA_4 initial further metabolism is given in Figure 7. Although further studies are required to establish the nature of the enzymes involved, it is likely that oxidation at the alcohol (C15) is followed by reduction of the Δ13 double bond by a second enzymatic step as previously demonstrated for prostanoids.

PGDH activity is reported to be in high levels in lung, liver, kidney, placenta, and spleen and is involved in the inactivation of prostanoids (Hansen, 1976). It follows that these organs may also rapidly transform LX. Indeed, many have already been documented as sites of LX action [reviewed in Serhan (1991)]. Apparently, several isoforms of the enzyme are known as well as species differences that give rise to unique substrate specificity. In lung, 15-HETE is the preferred substrate of PGDH (Bergholte et al., 1987). Unlike human placenta, the porcine kidney-derived PGDH catalyzes oxidation of prostaglandins and 12-hydroxyheptadecatrienoic acid (HHT) but not other hydroxy fatty acids (Tai et al., 1985). Human HL-60 cells also catalyze oxidation of 15-HETE (92%), 12-HHT (85%), and 12-HETE (21%) but not 5-HETE when compared to PGE_2 and $PGF_{2\alpha}$ (Agins et al., 1987). Thus, the enzymatic activity from hematopoietic origins is not restricted to attacking prostanoid structures. A K_i value of 8.2 μM for LXA_4 (Results) is consistent with PGDH involvement in the oxidation of lipoxins. The present level of results does not preclude the involvement of similar or equivalent enzyme systems in these reactions. Along these lines, a 5-hydroxyeicosanoid dehydrogenase activity requiring NADP was recently localized in microsomal fractions from human leukocytes (Powell et al., 1992), and a mouse liver microsomal fraction was found to generate 15-oxo-HETE without preference for cofactors (Sok et al., 1988). LXB_4 (which carries alcohol groups at C5, C14, and C15) is also metabolized by PMA-differentiated HL-60 cells (Figure 5). In addition, a 6,7-dihydro-LXB_4 was identified from incubations with 15-HPETE and potato tubers (Ho & Wong, 1989), suggesting that LXB_4 may also be metabolized by similar reactions as observed here for LXA_4 but triggered at carbon 5 of LXB_4.

It is generally believed that prostanoids are inactivated via PGDH [reviewed in Hansen (1976)]. However, recent results indicate that 13,14–dihydro–PGE_1 retains antiplatelet and vasodilatory properties of PGE_1 (Ney et al., 1991), exemplifying that each metabolite carries distinct bioactions. Along these lines, elimination of LXA_4 double bonds does reduce its airway smooth muscle properties (Dahlén et al., 1989). Several commonly used drugs including aspirin, indomethacin, probenicid, and xylocaine are documented inhibitors of PGDH activity (Hansen, 1976). It is likely that they can also block LX further metabolism, leading to increased levels of lipoxin that can sustain their putative chalone functions. Moreover, it follows that chemical modification of LX at their alcohol groups and/or ω-ends as with PGE and F analogs (Basu et al., 1992) may enhance their bio-half-life and activities by preventing dehydrogenation and subsequent degradation. The biological activities of the LXA_4 metabolites described here are currently being evaluated and will be reported elsewhere. In summary, our results are the first to establish the structures of the major LXA_4 metabolites, indicating that the bio-half-life of lipoxins must be taken into account in evaluating their bioactions.

ACKNOWLEDGMENT

We thank Kelly-Ann Sheppard for expert technical assistance in the early stages of this work and Mary Halm Small for assistance in the preparation of the manuscript.

REFERENCES

Agins, A. P., Zipkin, R. E., & Taffer, I. M. (1987) *Agents Actions 21*, 397–399.

Änggård, E., & Samuelsson, B. (1964) *J. Biol. Chem. 239*, 4097–4102.

Basu, S., Sjöquist, B., Resul, B., & Stjernschantz, J. (1992) *Acta Chem. Scand. 46*, 108–110.

Beckman, B. S., Despinasse, B. P., & Spriggs, L. (1992) *Proc. Soc. Exp. Biol. Med. 201*, 169–173.

Bergholte, J. M., Soberman, R. J., Hayes, R., Murphy, R. C., & Okita, R. T. (1987) *Arch. Biochem. Biophys. 257*, 444–450.

Boucher, J. L., Delaforge, M., & Mansuy, D. (1991) *Biochem. Biophys. Res. Commun. 177*, 134–139.

Böyum, A. (1986) *Scand. J. Clin. Lab. Invest., Suppl. 21* (No. 97), 77–89.

Brezinski, D. A., & Serhan, C. N. (1992) *Biol. Mass Spectrom. 20*, 45–52.

Busija, D. W., Armstead, W., Leffler, C. W., & Mirro, R. (1989) *Am. J. Physiol. 256*, H468–471.

Christie, P. E., Spur, B. W., & Lee, T. H. (1992) *Am. Rev. Respir. Dis. 145*, 1281–1284.
Collins, S. J. (1987) *Blood 70*, 1233–1244.
Dahlén, S.-E., Veale, C. A., Webber, S. E., Marron, B. E., Nicolaou, K. C., & Serhan, C. N. (1989) *Agents Actions 26*, 93–95.
Edenius, C., Kumlin, M., Björk, T., Ängård, A., & Lindgren, J. A. (1990) *FEBS Lett. 272*, 25–28.
Fiore, S., Ryeom, S. W., Weller, P. F., & Serhan, C. N. (1992) *J. Biol. Chem. 267*, 16168–16176.
Goldyne, M. E., Burrish, G. F., Poubelle, P., & Borgeat, P. (1984) *J. Biol. Chem. 259*, 8815–8819.
Hansen, H. S. (1976) *Prostaglandins 12*, 647–679.
Ho, H.-Y., & Wong, P. Y.-K. (1989) *Adv. Prostaglandin, Thromboxane, Leukotriene Res. 19*, 137–140.
Huwyler, J., Jedlitschky, G., Keppler, D., & Gut, J. (1992) *Eur. J. Biochem. 206*, 869–879.
Katoh, T., Takahashi, K., DeBoer, D. K., Serhan, C. N., & Badr, K. F. (1992) *Am. J. Physiol. 263 (Renal Fluid Electrolyte Physiol. 32)*, F436–F442.
Kelly, R. W., & Abel, M. H. (1983) *Biomed. Mass Spectrom. 10*, 276–279.
Lee, T. H., Lympany, P., Crea, A. E. G., & Spur, B. W. (1991) *Biochem. Biophys. Res. Commun. 180*, 1416–1421.
Meini, S., Evangelista, S., Geppetti, P., Szallasi, A., Blumberg, P. M., & Manzini, S. (1992) *Am. Rev. Respir. Dis. 146*, 930–934.
Ney, P., Braun, M., Szymanski, C., Bruch, L., & Schrör, K. (1991) *Eicosanoids 4*, 177–184.
Pettitt, T. R., Rowley, A. F., Barrow, S. E., Mallet, A. I., & Secombes, C. J. (1991) *J. Biol. Chem. 266*, 8720–8726.
Powell, W. S., Gravelle, F., & Gravel, S. (1992) *J. Biol. Chem. 267*, 19233–19241.
Raud, J., Palmertz, U., Dahlén, S.-E., & Hedqvist, P. (1991) *Adv. Exp. Med. Biol. 314*, 185–192.
Romano, M., & Serhan, C. N. (1992) *Biochemistry 31*, 8269–8277.
Rouet-Mayer, M.-A., Bureau, J.-M., & Laurière, C. (1992) *Plant Physiol. 98*, 971–978.
Samuelsson, B., Dahlén, S.-E., Lindgren, J. A., Rouzer, C. A., & Serhan, C. N. (1987) *Science 237*, 1171–1176.
Serhan, C. N. (1990) *Methods Enzymol. 187*, 167–175.
Serhan, C. N. (1991) *J. Bioenerg. Biomembr. 23*, 105–122.
Serhan, C. N., Hamberg, M., & Samuelsson, B. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 5335–5339.
Sok, D.-E., Kang, J. B., & Shin, H. D. (1988) *Biochem. Biophys. Res. Commun. 156*, 524–529.
Stenke, L., Edenius, C., Samuelsson, J., & Lindgren, J. A. (1991a) *Blood 78*, 2989–2995.
Stenke, L., Mansour, M., Edenius, C., Reizenstein, P., & Lindgren, J. A. (1991b) *Biochem. Biophys. Res. Commun. 180*, 255–261.
Tai, H.-H., Liu, Y., Tai, C. L., Yoden, K., & Shen, R.-F. (1985) *Adv. Prostaglandin, Thromboxane, Leukotriene Res. 15*, 159–161.
Tornhamre, S., Gigou, A., Edenius, C., Lellouche, J.-P., & Lindgren, J. A. (1992) *FEBS Lett. 304*, 78–82.
Wikström, E., Westlund, P., Nicolaou, K. C., & Dahlén, S.-E. (1992) *J. Lipid Mediators 5*, 205–217.
Xun, C.-Q., Tian, Z.-G., & Tai, H.-H. (1991) *Biochem. J. 279*, 553–558.