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Lipoxins: Novel series of biologically active compounds formed from arachidonic acid in human leukocytes

(lipoxygenase interaction products/conjugated tetraenes/human neutrophils)

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ABSTRACT **Trihydroxytetraenes, a novel series of oxygenated derivatives formed from arachidonic acid in human leukocytes, were recently isolated [Serhan, C. N., Hamberg, M. & Samuelsson, B. (1984) *Biochem. Biophys. Res. Commun.* 118, 943–949]. The structure of the major compound was established—i.e., 5,6,15*L*-trihydroxy-7,9,11,13-icosatetraenoic acid. The present study reports the structure of a second member of the trihydroxytetraene series of compounds—i.e., 5*D*,14,15*L*-trihydroxy-6,8,10,12-icosatetraenoic acid. When added to human neutrophils, 5,6,15*L*-trihydroxy-7,9,11,13-icosatetraenoic acid stimulated superoxide anion generation and degranulation at submicromolar concentrations without provoking a substantial aggregation response. With respect to superoxide anion generation, 5,6,15*L*-trihydroxy-7,9,11,13-icosatetraenoic acid proved to be as potent as leukotriene B_4. In contrast, the compound was approximately 2 orders of magnitude less potent than either leukotriene B_4 or fMet-Leu-Phe at provoking degranulation. The results indicate that interaction(s) between the 5- and 15-lipoxygenase pathways of human leukocytes leads to formation of a new series of oxygenated derivatives of arachidonic acid that may be involved in regulating specific cellular responses. The trivial names lipoxin A (5,6,15*L*-trihydroxy-7,9,11,13-icosatetraenoic acid) and lipoxin B (5*D*,14,15*L*-trihydroxy-6,8,10,12-icosatetraenoic acid) are proposed for the new compounds.**

Nonesterified arachidonic acid may be subject to oxygenation by either the cyclooxygenase or lipoxygenase pathways (1). Three major lipoxygenase pathways have been described in mammalian tissues (1–3). These include the 5-, 12-, and 15-lipoxygenases, which stereospecifically insert molecular oxygen into unconjugated double-bond systems via mechanisms involving hydrogen abstraction (4). Each of these enzymes transforms arachidonic acid into a number of biologically active derivatives. For example, via the 5-lipoxygenase pathway, arachidonic acid is converted to (5*S*)-5-hydroperoxy-6,8,11,14-icosatetraenoic acid (5-HPETE), which may be further transformed into leukotrienes (1). Leukotrienes, particularly leukotrienes B_4, C_4, and D_4, contain a conjugated triene structure as the distinguishing feature and serve as mediators in both immediate hypersensitivity reactions and in inflammation (for review, see ref. 5).

Recently we described the isolation of a previously unreported series of oxygenated derivatives of arachidonic acid containing conjugated tetraene structures (trihydroxytetraenes) and reported the structure of the major compound of this series (6). In this paper, we report the biological activities of the major compound (in human neutrophils) and the structure of the next major compound. Since the trihydroxytetraenes appear to arise from the interaction(s) of multiple distinct lipoxygenase pathways, we propose the name lipoxins for the series.

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MATERIALS AND METHODS

Cytochalasin B (Cyto B), cytochrome *c*, superoxide dismutase, and *N*-*tert*-butoxycarbonyl-L-alanine-*p*-nitrophenyl ester (*N*-*t*-Boc-L-Ala-*p*NP) were from Sigma. Arachidonic acid was from Nu-Chek Prep (Elysian, MN), and soybean lipoxygenase (EC 1.13.11.12) type I was from Sigma. (15*S*)-15-Hydroperoxy-5,8,11,13-icosatetraenoic acid (15-HPETE) was prepared by incubation of arachidonic acid with soybean lipoxygenase (7). A23187 was from Calbiochem–Behring.

HPLC equipment was from Waters Associates (pump 6000A, injector U6K) and Laboratory Data Control (Riviera Beach, FL) (UV detector, LDC-III). HPLC grade solvents were used in all studies.

Cell Preparation and Incubation Conditions. Human leukocytes obtained from peripheral blood were prepared as described (8). These preparations represent a mixed population of leukocytes (neutrophils, basophils, eosinophils, etc.) in which the neutrophil contribution represents >90% as determined by Giemsa staining and light microscopy. Cells were washed and suspended in a buffered salt solution (138 mM NaCl/2.7 mM KCl/8.1 mM Na_2PO_4/1.5 mM KH_2PO_4/1.0 mM $MgCl_2$/0.6 mM $CaCl_2$, pH 7.45) at 100×10^6 cells per ml.

Leukocytes (100–500 ml of 100×10^6 cells per ml) were warmed to 37°C in a water bath with slow continuous stirring for 5 min. 15-HPETE (100 μM) and the divalent cation ionophore A23187 (5 μM) were added simultaneously in ethanol [final concentration, <1% (vol/vol)] and the incubations were continued for an additional 30 min. Incubations were stopped by addition of 2 vol of methanol.

Extractions and Purification of Compounds I and II. Procedures for ether extraction and silicic acid chromatography were as described (3). The ethyl acetate fraction from silicic acid chromatography was evaporated, dissolved in methanol, treated with diazomethane, and then subjected to TLC. This TLC step was essential since nonenzymatic products of 15-HPETE interfered with both the structural analysis and bioassay of the compounds of interest. Thus, methyl 11,12,15-trihydroxy-5,8,13-[1-^{14}C]icosatrienoate and methyl 11,14,15-trihydroxy-5,8,12-[1-^{14}C]icosatrienoate were prepared (9) and added to the material eluted in the ethyl acetate fractions. TLC was carried out with plates coated with Silica gel G and with ethyl acetate/2,2,4-trimethylpentane, 5:1 (vol/vol), as solvent. A Berthold Dünnschichtsscanner II was used for localization of labeled material on TLC plates. The zone containing methyl esters exhibiting tetraene UV spectra (i.e., λ_{max} of 301; see ref. 6) but not methyl 11,14,15-trihydroxy-5,8,12-[1-^{14}C]icosatrienoate or methyl 11,12,15-

Abbreviations: 15-HPETE, (15*S*)-15-hydroperoxy-5,8,11,13-icosatetraenoic acid; RP-HPLC, reversed-phase high-pressure liquid chromatography; MS, mass spectrometry; Cyto B, cytochalasin B; *N*-t-Boc-L-Ala-pNP, *N*-*tert*-butoxycarbonyl-L-alanine-*p*-nitrophenyl ester; Me_3Si, trimethylsilyl.

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trihydroxy-5,8,13-[1-^{14}C]icosatrienoate were scraped off, and the material was recovered from the silica gel by elution with methanol. The samples were extracted with ether, dried under N_2, and injected into a reversed-phase HPLC (RP-HPLC) column. The column (500 × 10 mm, Polygosil C_{18}) was eluted with methanol/water, 70:30 (vol/vol), at 3.0 ml/min. A UV detector set at 301 nm recorded the absorption of the eluate. Fractions showing a tetraene UV spectrum (Fig. 2) were collected separately and rechromatographed in the same HPLC system before structural or biological studies.

Analytical Procedures. (−)-Menthoxycarbonyl derivatives of methyl α-hydroxyheptanoate and of dimethyl α-hydroxyadipate were prepared as described (1, 10). (−)-Menthoxycarbonyl derivatives of methyl esters of trihydroxytetraenes were prepared and purified as described (11).

Oxidative ozonolysis was performed by bubbling an excess of O_3 into a solution of the purified (−)-menthoxycarbonyl derivative (5–10 μg) in 0.5 ml of chloroform at −20°C. After 10 min at room temperature, the solvent was evaporated under a stream of argon, and the residue was treated with 0.25 ml of acetic acid and 0.05 ml of 30% hydrogen peroxide at 50°C for 18 hr. The mixture was taken to dryness, treated with diazomethane, and analyzed by GLC.

Catalytic hydrogenation was carried out by stirring the unsaturated compound (10–20 μg) with 2 mg of Adams catalyst in 2 ml of methanol for 15 min under H_2.

GLC was carried out with an F & M biomedical gas chromatograph model 402. The stationary phases used were 1% SE-30 on Gas-Chrom Q (derivatives of trihydroxytetraenes) and 5% QF-1 on GasChrom Q (ozonolysis products).

GLC/mass spectrometry (MS) was performed with an LKB 9000 S instrument equipped with a column of 1% OV-1 on Chomosorb W. UV spectra were obtained with a Hewlett–Packard model 8450A UV/visible spectrophotometer.

Saponification of Methyl 5,6,15*L*-Trihydroxy-7,9,11,13-Icosatetraenoate. Samples (50–250 μg) obtained from several incubations were dissolved in 500 μl of tetrahydrofurane and placed under N_2 in a −70°C bath with a magnetic stir-bar. LiOH (1 M; 50 μl) was added, and the reaction was continued for 48 hr at 4°C. Samples were extracted and subjected to RP-HPLC with methanol/water/acetic acid, 70:30:0.01 (vol/vol), as the solvent system. The eluates were extracted with ether, and the concentrations of 5,6,15*L*-trihydroxy-7,9,11,13-icosatetraenoic acid, which we call "lipoxin A," were determined by using an absorption coefficient of 50,000 just prior to bioassay.

Preparation of Human Neutrophils for Aggregation, $O_2^{\cdot-}$ Generation, and Elastase Release. Fresh whole blood was obtained from healthy volunteers just prior to bioassay. Neutrophil suspensions were prepared by means of sedimentation with dextran, followed by gradient-centrifugation in Lymphoprep (12). Cells were washed and suspended in Dulbecco's phosphate-buffered saline (pH 7.45). Neutrophil aggregation was studied with a standard Payton aggregometer and recorder (13), and generation of superoxide anion, $O_2^{\cdot-}$, was measured by continuous recording of the reduction of ferricytochrome *c* (14). Release of elastase was determined as described (15, 16) with minor modifications. After the addition of Cyto B (3 min at 37°C), *N-t*-Boc-L-Ala-pNP (10 μM) was added to both reference and sample cuvettes in ethanol (0.1%), and baselines were recorded at 360 nm for 60 sec before addition of test compounds. In each experiment, lipoxin A and LTB_4 were added to cells in ethanol (final concentration, <0.1%). Appropriate solvent controls were added to reference cuvettes.

RESULTS

In order to examine interactions between the 5- and 15-lipoxygenase pathways, 15-HPETE and A23187 were added simultaneously to human leukocytes, and the products formed were characterized. Analysis of the ethyl acetate fractions obtained after silicic acid chromatography revealed strongly absorbing material at 243 nm (5,15-dihydroxyicosatetraenoic acid, called 5,15-DHETE; cf. ref. 17) and 301 nm (trihydroxytetraenes) (Fig. 1). In addition to these UV-absorbing materials, the ethyl acetate fractions contained large amounts of non-UV-absorbing materials derived from 15-HPETE 11,12,15-trihydroxy-5,8,13-icosatrienoic acid, and 11,14,15-trihydroxy-5,8,12-icosatrienoic acid. Since these compounds, which can be formed *inter alia* in the presence of heme proteins (9), interferred with both structural and biological studies, it was necessary to include a TLC purification step (see ref. 6). To obtain samples suitable for structural analysis and biological studies, material present in ethyl acetate fractions was mixed with 11,12,15-trihydroxy-5,8,13-[1-^{14}C]icosatrienoic acid and 11,14,15-trihydroxy-5,8,12-[1-^{14}C]icosatrienoic acid, and the mixtures were treated with diazomethane and subjected to TLC (6). After TLC, 1.02 ± 0.23 μg of tetraene-containing material per 1 × 10^8 human leukocytes was obtained (n = 13).

Isolation of Compounds I and II. After TLC, samples were recovered from the silica gel by elution with methanol, followed by ether extraction. The material was then subject to RP-HPLC (methanol/water, 7:3, vol/vol) with the UV detector set at 301 nm. Two major components showing strong absorption at 301 nm were obtained (Fig. 2). Eluted material in peaks I and II was collected and rechromatographed in the same HPLC system. The UV spectrum of material eluted in peak I (compound I) is shown in Fig. 2*A Inset*. We recently determined the structure of the eluted material in peak II as the methyl ester of lipoxin A (5,6,15*L*-trihydroxy-7,9,11,13-icosatetraenoic acid) (6); its UV spectrum is shown in Fig. 2*B Inset*.

Structure of Compound I. The UV spectrum of compound I showed a triplet of absorption bands at 287, 301, and 316 nm (solvent, methanol; see Fig. 2*A*). The spectrum was virtually identical to that found for methyl 5,6,15*L*-trihydroxy-7,9,11,13-icosatetraenoate (compound II), thus indicating that compound I possessed a conjugated tetraene structure (Fig. 2*B*).

A sample of compound I was converted into the trimethylsilyl (Me_3Si) derivative and analyzed by GLC/MS. The material was eluted as a relatively broad peak having an equivalent chain length corresponding to $C_{24.0}$–$C_{24.1}$ (1% OV-1). The mass spectrum (Fig. 3) showed prominent ions at m/e 173 [base peak; Me_3SiO^+=CH—$(CH_2)_4$—CH_3] and m/e 203 [Me_3SiO^+=CH—$(CH_2)_3$—$COOCH_3$] as well as weak-

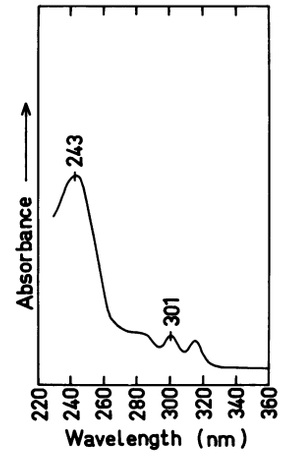

FIG. 1. Representative UV spectrum of samples obtained after partial purification by silicic acid chromatography. Tracings were recorded of free acids in methanol.

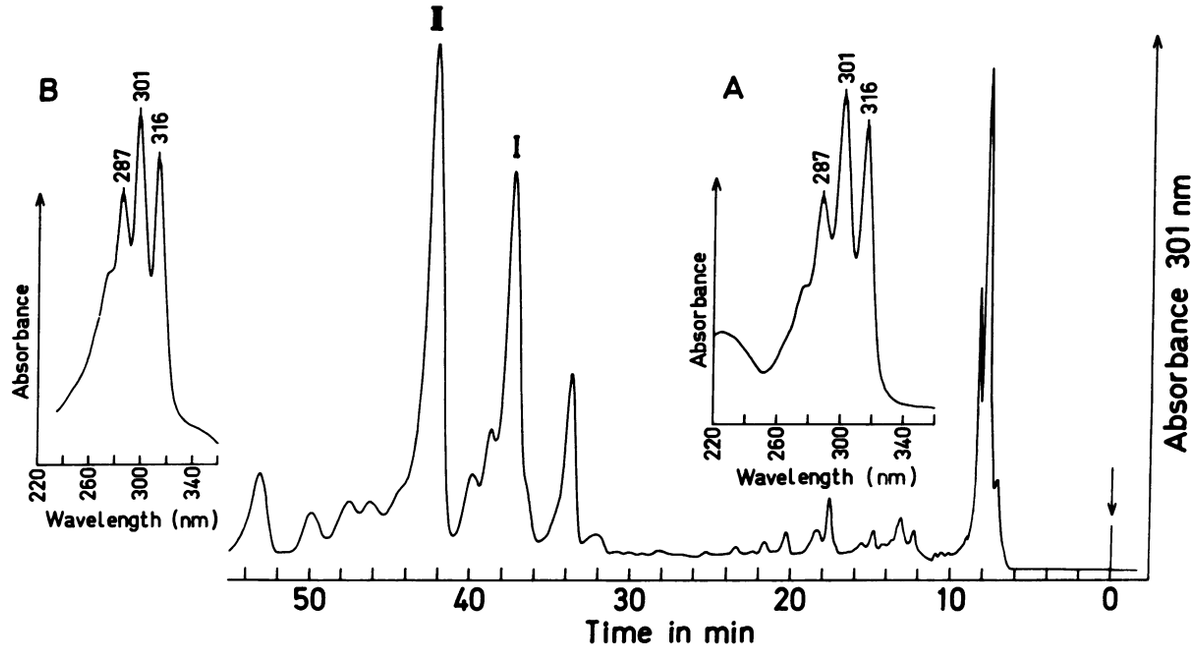

FIG. 2. RP-HPLC chromatogram of products obtained from incubation of human leukocytes with 15-HPETE and ionophore A23187. The incubation (30 min) was terminated by the addition of 2 vol of methanol. After removal of precipitated proteins, acidic ether extraction, and purification by silicic acid column chromatography, samples were treated with ethereal diazomethane and further purified by TLC. Products were eluted from silica gel, extracted, and injected. The UV detector was set at 301 nm, and the column was eluted with methanol/water, 70:30 (vol/vol), at 3.0 ml/min. (*Inset A*) UV spectrum of compound I in methanol. (*Inset B*) UV spectrum of compound II in methanol.

er ions at *m/e* 582 (M), 492 (M − 90; loss of Me_3SiOH), 482 [M − 100; rearrangement followed by loss of $O{=}HC{-}(CH_2)_4{-}CH_3$], 409 (M − 173), 379 (M − 203), 319 [M − (173 + 90)], 301 [M − (101 + 2 × 90); loss of $\cdot CH_2{-}(CH_2)_2{-}COOCH_3$ and 2 Me_3SiOH], 275 [$Me_3SiO^+{=}CH{-}CH(OSiMe_3){-}(CH_2)_4{-}CH_3$], 229 [M − (173 + 2 × 90)], and 171 (203 − 32). The mass spectrum of the Me_3Si derivative of hydrogenated compound I ($C_{25:3}$) showed ions of high intensity of *m/e* 575 (M − 15), 490 [M − 100; rearrangement followed by loss of $O{=}HC{-}(CH_2)_4{-}CH_3$], 417 (M − 173), 399 [M − (101 + 90)], 297 [M − (203 + 90)], 203 (base peak), and 173. A dihydroxy derivative formed by hydrogenolysis also was observed in the gas chromatogram (cf. ref. 6). The mass spectrum of this material was similar to that of the Me_3Si derivative of methyl 14,15-dihydroxyicosanoate (12).

In order to prove the presence of an allylic hydroxyl group at C-5 of compound I and to determine its absolute configuration, the (−)-menthoxycarbonyl derivative of compound I was subjected to oxidative ozonolysis. GLC analysis (column, 5% QF-1) of the ozonolysis product showed the presence of the (−)-menthoxycarbonyl derivative of methyl hy-

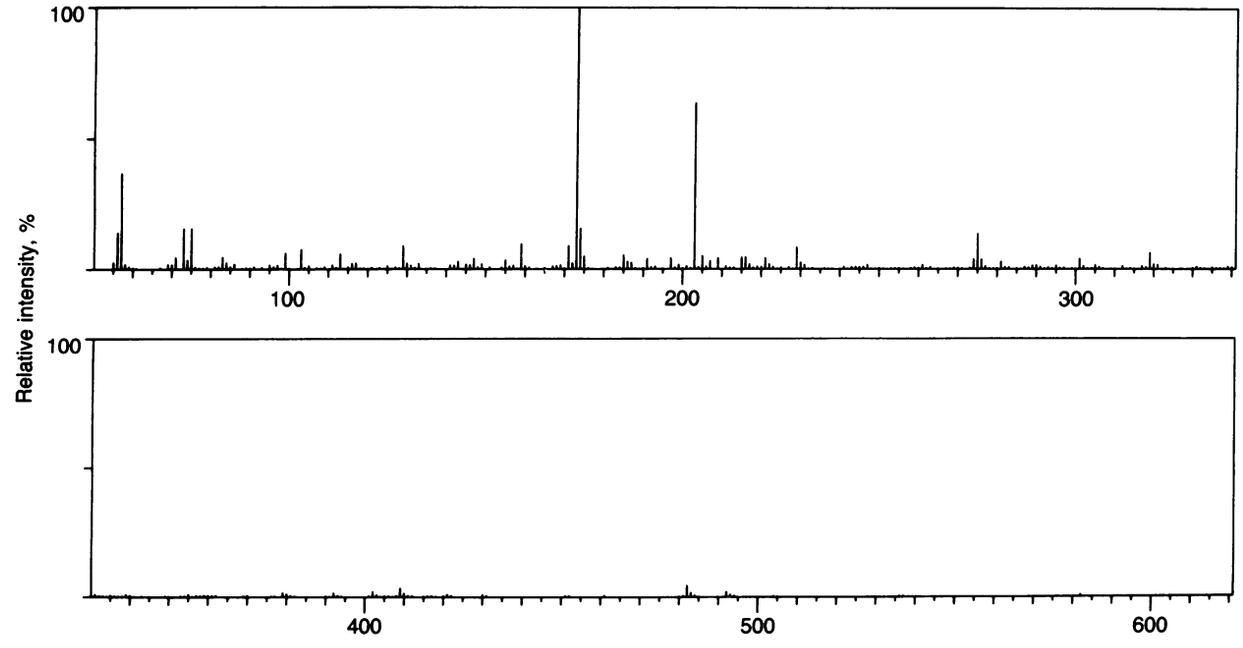

FIG. 3. Mass spectrum of the Me_3Si derivative of compound I.

drogen 2*L*-hydroxyadipate as well as variable amounts (<20%) of 2*L*-hydroxyheptanoic acid. These results showed that compound I mainly was due to a methyl icosatetraenoate containing a hydroxy group at C-5 (D-configuration) and one of its four double bonds at Δ^6. The origin of the (−)-menthoxycarbonyl derivative of 2*L*-hydroxyheptanoic acid (<20%) was probably contaminating methyl 5,6,15*L*-trihydroxy-7,9,11,13-icosatetraenoate (see Fig. 2 and ref. 6).

On the basis of the UV data, MS analyses, and the results of oxidative ozonolysis, the major component present in peak I (Fig. 2) was assigned the structure methyl 5*D*,14,15*L*-trihydroxy-6,8,10,12-icosatetraenoate. Thus, the compound is a positional isomer of methyl 5,6,15*L*-trihydroxy-7,9,11,13-icosatetraenoate isolated previously (6). This is in agreement with their similar behavior on RP-HPLC, compound I being only slightly more polar than compound II (Fig. 2). In addition, it is of interest to note that the UV spectra of both compounds I and II appear to be virtually identical, with λ_{max} of 287, 301, and 316. After saponification and purification of the free acid of compound II, lipoxin A, samples were treated with diazomethane and subjected to RP-HPLC. The reesterified compound comigrated with compound II.

Human Neutrophil Responses to Lipoxin A. To determine whether lipoxin A affected neutrophil responses, we examined $O_2^-\cdot$ generation, elastase release, and aggregation of neutrophils exposed to this compound. Here continuous recording techniques were utilized to examine the kinetics of neutrophil responses upon addition of lipoxin A. Results of these experiments with Cyto B-treated neutrophils are shown in Fig. 4.

Lipoxin A (0.5 μM) induced a rapid burst in the generation of $O_2^-\cdot$ and stimulated the release of lysosomal elastase, while at the same concentration it exerted little to no effect in provoking aggregation (Fig. 4*A*). Representative tracings obtained for aggregation, $O_2^-\cdot$ generation, and elastase release by human neutrophils exposed to 0.5 μM lipoxin A are shown in Fig. 4*A*.

In dose–response studies, neither lipoxin A nor its methyl ester provoked aggregation at 1 μM to 0.1 nM. However, lipoxin A proved to be a potent stimulator of $O_2^-\cdot$ generation (Fig. 4). At concentrations >0.1 μM, lipoxin A provoked $O_2^-\cdot$ generation and, in this respect, proved to be as potent as leukotriene B_4. Under these conditions the synthetic chemotactic peptide fMet-Leu-Phe (0.1 μM) stimulated $O_2^-\cdot$ generation [14.5 ± 5.5 (SD) nmol of cytochrome *c* reduced per 5 min (n = 12)] more than either 0.5 μM LTB_4 (6.5 ± 3.6 nmol of cytochrome *c* reduced per 5 min) or 0.5 μM lipoxin A [(8.1 ± 3.7 nmol of cytochrome *c* reduced per 5 min (n = 12)].

Both fMet-Leu-Phe and LTB_4 are potent stimulators of elastase release in human neutrophils (16). When lipoxin A was compared to the effects of these agents, lipoxin A proved to be approximately 2 orders of magnitude less potent than either fMet-Leu-Phe or LTB_4. A group of representative tracings obtained from the same donor are shown in Fig. 4*B*. Here, the response to fMet-Leu-Phe and LTB_4 are shown for purposes of comparison. In each experiment, lipoxin A displayed a longer lag phase (≈30 sec) than either fMet-Leu-Phe or LTB_4 in inducing lysosomal elastase release.

DISCUSSION

In the present paper, we describe the structure of a previously unreported compound isolated from human leukocytes, namely, 5*D*,14,15*L*-trihydroxy-6,8,10,12-icosatetraenoic acid (lipoxin B, parent acid of compound I). The compound has four conjugated double bonds, three hydroxyl groups, and is a positional isomer of 5,6,15*L*-trihydroxy-7,9,11,13-icosatetraenoic acid (lipoxin A, parent acid of compound II), which we have recently reported (6). These compounds appear to be the major products of a novel series of compounds that contain four conjugated double bonds as a distinguishing feature (Figs. 2 and 5). In addition, we report the biological activities of lipoxin A in human neutrophils (Fig. 4).

Samples of the new compounds were isolated from the diethyl ether extracts of incubations of human leukocytes exposed to 15-HPETE and the divalent cation ionophore A23187 as recently described (6). Purification was achieved

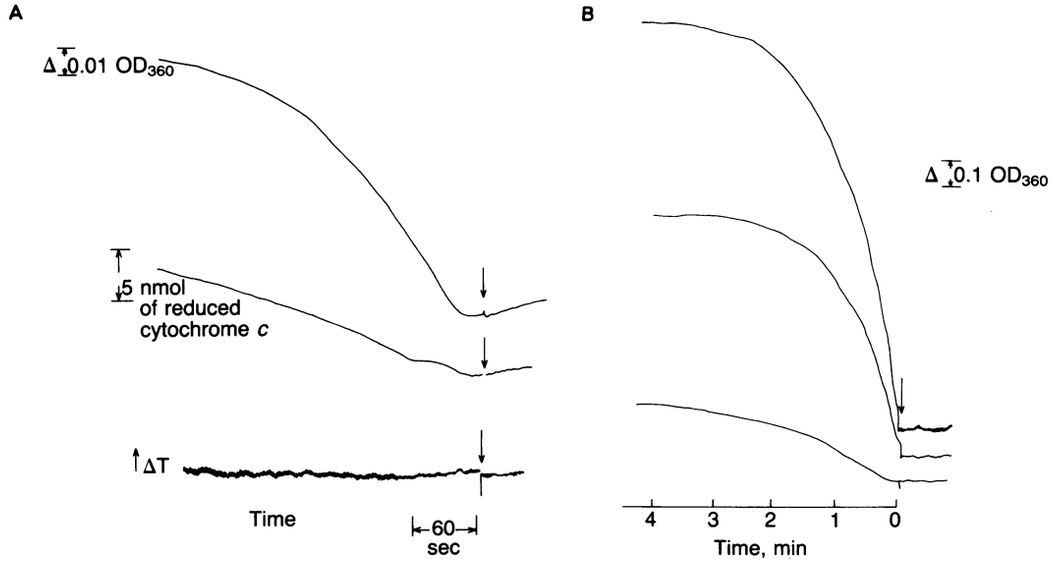

FIG. 4. Human neutrophil aggregation (bottom tracing), $O_2^-\cdot$ generation (middle tracing), and elastase release (top tracing). (*A*) The top tracing is a representative tracing of lysosomal elastase release. Neutrophils (3×10^6 cells per ml) were incubated with Cyto B (5 μg/ml) 3 min before addition of *N*-*t*-Boc-L-Ala-pNP (10 μM), followed by addition of lipoxin A (0.5 μM). The middle tracing is a representative continuous recording of $O_2^-\cdot$ generation. Neutrophils (3×10^6 cells per ml) were incubated with Cyto B (5 μg/ml) 3 min at 37°C. The bottom tracing is a representative aggregation tracing. Neutrophils (3×10^6 cells per 0.1 ml) were incubated with Cyto B (5 μg/ml) 3 min at 37°C before addition of lipoxin A (0.5 μM). Lipoxin A was added to the sample cuvette at the time indicated by the arrow. (*B*) Continuous recording of lysosomal elastase release from human neutrophils. Tracings are representative of those obtained from individual donors: top, fMet-Leu-Phe at 0.5 μM; middle, leukotriene B_4 at 10 nM; bottom, lipoxin A at 0.5 μM.

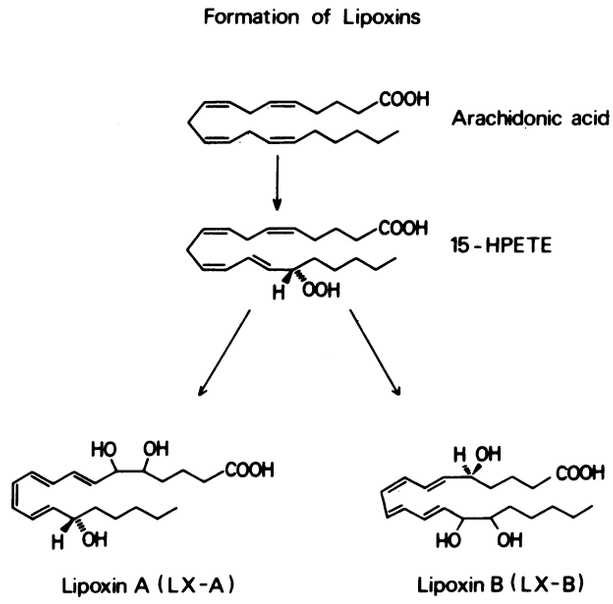

FIG. 5. Scheme of formation of lipoxins A and B. The geometrical configurations of the double bonds have not been determined and thus are tentative assignments.

by silicic acid column chromatography of the free acids, TLC of the methyl esters, and RP-HPLC of the methyl esters (Fig. 2). The presence and positions of three hydroxyl groups as well as four conjugated double bonds of compound I were established by GLC/MS analysis of several derivatives of the compound, including the hydrogenated compound (Fig. 3 and *Results*). Analysis of the fragments obtained after oxidative ozonolysis of the tris[(−)-menthoxycarbonyl] derivative of compound I showed that the hydroxyl group at C-5 had the *D*-configuration and that one of the four double bonds was located at Δ^6. Unlike compound II, which can be isolated with <5% cross-contamination (6), compound I at the present stage appears to be contaminated <20% with an isomer of this compound II (see *Results*). Nevertheless, the ozonolysis data taken together with the UV data indicate that the locations of the double bonds of compound I are at Δ^6, Δ^8, Δ^{10}, and Δ^{12}. Moreover, the finding that the hydroxyl group at C-5 had the *D*-configuration indicates that these compounds arise via reactions catalyzed by 5-lipoxygenase (Fig. 5). Although several biosynthetic routes may lead to the formation of lipoxin A and lipoxin B, it appears likely that they are formed via multiple lipoxygenations.

When added to purified human neutrophils, lipoxin A provoked a rapid and sustained generation of $O_2^{\cdot-}$ and induced degranulation without provoking substantial aggregation (Fig. 4*A*). Here lipoxin A proved to be as potent as LTB_4. However, lipoxin A proved to be ≈2 orders of magnitude less potent than either fMet-Leu-Phe or LTB_4 at inducing degranulation (Fig. 4*B*).

Although LTB_4 may be considered a complete secretagogue in human neutrophils (14), knowledge of its intracellular role(s) is unavailable. Recent studies by Pozzan *et al.* (18) indicate that the chemotactic peptide fMet-Leu-Phe, in addition to stimulating a rise in intracellular Ca^{2+} that may serve as an intracellular signal (19), also generates important "excitatory signal(s)" that function in degranulation and superoxide anion generation. It is possible that arachidonic acid metabolites such as lipoxin A may serve as one of these signals or mimic the action of endogenous compounds in human leukocytes. Nevertheless, our findings suggest that lipoxin A, unlike LTB_4, is a selective secretagogue in human neutrophil.

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