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New series of lipoxins isolated from human eosinophils

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Granulocytes from human eosinophilic donors were incubated with arachidonic acid or 15-hydroxyeicosatetraenoic acid (15-HETE) and stimulated with the ionophore A23187. The eicosanoids were extracted with reversed-phase cartridges and subjected to RP-HPLC analysis. When extracts from eosinophil-enriched populations were analysed and compared with extracts from human neutrophils, three additional peaks were detected which coeluted with 15-hydroxy-Δ^{13}-trans-15H derivatives of leukotriene C_4, D_4 and E_4 in different HPLC systems. The recorded absorbance spectra of the eluted compounds and the standards were identical and showed a maximum at 307 nm which is characteristic for a conjugated tetraene system with a bathochromic shift by the sulfur moiety in α-position to the tetraene system. The compound which coeluted with the 15-hydroxy-LTC_4 standard was treated with γ-glutamyltransferase and converted to the corresponding leukotriene D_4 derivative. The results indicate that interaction between the 5- and 15-lipoxygenase pathways leads to the formation of a new series of arachidonic acid metabolites in human eosinophils. Since the biosynthetic route is similar to that of lipoxin A_4 and lipoxin B_4, we suggest the trivial names lipoxin C_4, D_4 and E_4.

Lipoxin; Leukotriene; Lipoxygenase; Eosinophil; Inflammation

1. INTRODUCTION

Recently, a new series of arachidonic acid metabolites was reported which is produced by the interaction of the 5- and the 15-lipoxygenase pathway. The compounds contain a conjugated tetraene system and have been termed the lipoxins [1–5]. The lipoxins are biologically active compounds. LXA_4 leads to superoxide anion generation and degranulation in human neutrophils [2], promotes chemotaxis [6], contracts lung parenchymal strips [4] and activates protein kinase C in vitro [7]. Both LXA_4 and LXB_4 inhibit human killer cell activity [8,9].

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Abbreviations: HETE, hydroxyeicosatetraenoic acid; HPETE, hydroperoxyeicosatetraenoic acid; LXA_4, lipoxin A_4; LXB_4, lipoxin B_4; LX, lipoxin; PBS, phosphate-buffered saline; 15-hydroxy-LTC_4, (15*S*)-hydroxy-Δ^{13}-*trans*-15H-leukotriene C_4; LT, leukotriene

(15*S*)-hydroxy-5,6-epoxy-7,9,13-*trans*-11-*cis*-eicosatetraenoic acid was suggested to be the common intermediate in the biosynthesis of lipoxin A_4 and B_4 in human granulocytes [3,4] and LXA_4 was found in extracts from human eosinophils stimulated with the ionophore A23187 and arachidonic acid [10]. Since it has been shown that GSH S-transferase is present in human eosinophils, which is responsible for the formation of leukotriene C_4 and its metabolites in these cells [11,12], it was of interest whether human eosinophils can release amino acid-containing lipoxins. This would give further evidence that (15*S*)-hydroxy-5,6-epoxyeicosatetraenoic acid is the common intermediate in the lipoxin biosynthesis. The formed lipoxins would represent a new class of arachidonic acid metabolites isolated from biological sources. In this paper we describe the extraction, isolation and identification of amino acid-containing lipoxins from human eosinophils. 15-Hydroxylated derivatives of leukotriene C_4, D_4 and E_4 were used as HPLC standards and prepared according to the method of Örning and Hammarström [13].

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2. EXPERIMENTAL

2.1. *Materials*

The lipoxin standards LXA_4, 6*S*-LXA_4, 11-*trans*-LXA_4, 6*S*-11-*trans*-LXA_4, LXB_4, 14*S*-LXB_4, 8-*trans*-LXB_4 and 14*S*-8-*trans*-LXB_4 were generous gifts from Dr J. Rokach (Merck Frosst, Quebec, Canada), leukotriene C_4, D_4 and E_4 and other lipoxygenase metabolites were obtained from Paesel GmbH (Frankfurt, FRG). Arachidonic acid, soybean lipoxygenase, γ-glutamyltransferase, GSH peroxidase, trifluoroacetic acid and ionophore A23187 (free acid) were purchased from Sigma (St. Louis, MO, USA). All solvents used were of analytical grade. PM16 (Serva) supplemented with human albumin (0.1%), glucose (0.1%) and Ca^{2+} (1 mM served as incubation buffer. 15-HETE was either obtained from Paesel GmbH or prepared according to the method of Hamberg and Samuelsson [14].

2.2. *Methods*

2.2.1. Preparation of lipoxin standards

LTD_4 or LTE_4 (2.5 μg) were incubated with 20 μg soybean lipoxygenase (2500 units) in 1 ml PBS buffer, pH 7.4, at room temperature under vigorous shaking. 100 μg soybean lipoxygenase were used to transform LTC_4. The reaction was followed by UV spectrophotometry with scanning between 250 and 350 nm. When the reaction was complete, either GSH peroxidase (20 μg, 2.8 units) and GSH (1 mM) or 500 μl methanol and sodium borhydride were added to reduce the formed hydroperoxides and the mixture was incubated at room temperature for 15 min. Then, GSH peroxidase reaction was stopped with 500 μl methanol and when sodium borhydride was used, the mixture was acidified to pH 6 with HCl, extracted and analysed as described below.

2.2.2. Cell isolation and incubation

Suspensions of human polymorphonuclear leukocytes were prepared according to the method of Hjorth et al. [15]. The suspensions obtained from the eosinophilic donors contained more than 50% eosinophils. The polymorphonuclear leukocytes (5 ml, 4.0×10^7 cells/ml) were preincubated with 15-HETE (20 μM) for 2 min. The reaction was started with ionophore A23187 (5 μM). After 5 min (unless otherwise mentioned in the text), the reaction was stopped with methanol, the tubes were chilled on ice, diluted with buffer to a methanol content lower than 30%, centrifuged ($800 \times g$, 10 min) and extracted.

2.2.3. Extraction procedure

The lipoxins were extracted according to the method described in [16,17]. The extraction procedure in brief: Baker C-18 disposable columns were conditioned with 2 ml methanol, 2 ml water, 2 ml of 0.1% aqueous EDTA solution and 2 ml water. The samples were then applied to the columns and washed with 3 ml water and 3 ml of 25% methanol. Finally, the lipoxins were eluted with 100% methanol, the extract was evaporated to dryness with a stream of nitrogen and resuspended in a small volume of methanol. 10 μl of this solution were injected into the HPLC apparatus.

2.2.4. Analytical methods

HPLC was carried out on a Waters Radial-Pak cartridge (100 mm × 5 mm i.d.) packed with Novapak C-18 (particle size 4 μm) which was obtained from Waters (Eschborn, FRG). The solvent system was either methanol/water/trifluoroacetic acid or acetonitrile/water/trifluoroacetic acid. A flow rate of 1.2 ml was used and the peaks were monitored with a Waters 481 UV detector set at 380 nm. UV spectra were recorded with a Beckman DU-50 spectrophotometer connected on line with the HPLC equipment.

2.2.5. Conversion of lipoxin C_4 to D_4

The LXC_4 extract was evaporated to dryness, 87.5 μl PBS buffer, pH 7.5, 10 μl γ-glutamyltransferase solution (200 μg/ml, 24 U/mg) and 2.5 μl cysteine solution (final concentration 100 μM) were added and incubated for 30 min at 25°C. Then, 20 μl of this solution were directly analysed by HPLC.

3. RESULTS AND DISCUSSION

Human eosinophils were incubated with 15-HETE and stimulated with the ionophore A23187 for 5 min. Fig.1A shows a chromatogram

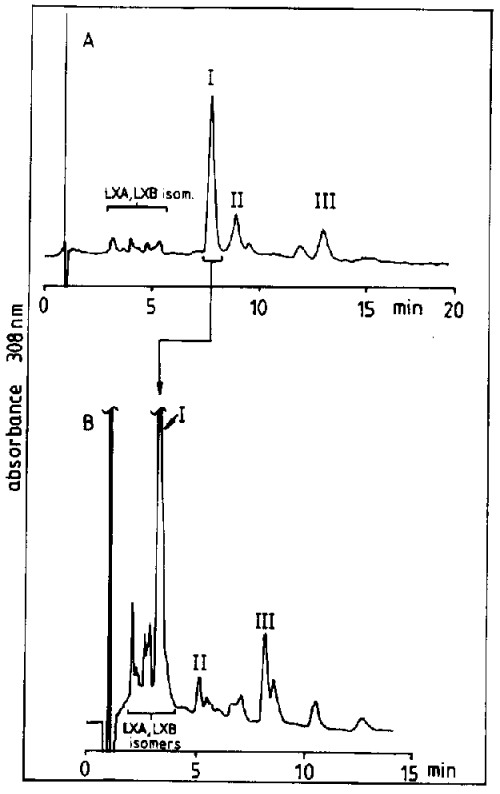

Fig.1. RP-HPLC chromatograms of the lipoxins extracted from human eosinophils after incubation of the cells with ionophore for 5 min. The column was eluted with 66:34:0.008 methanol/water/trifluoroacetic acid (A) and 44:56:0.008 acetonitrile/water/trifluoroacetic acid (B). The wavelength selector of the detector was set at 308 nm.

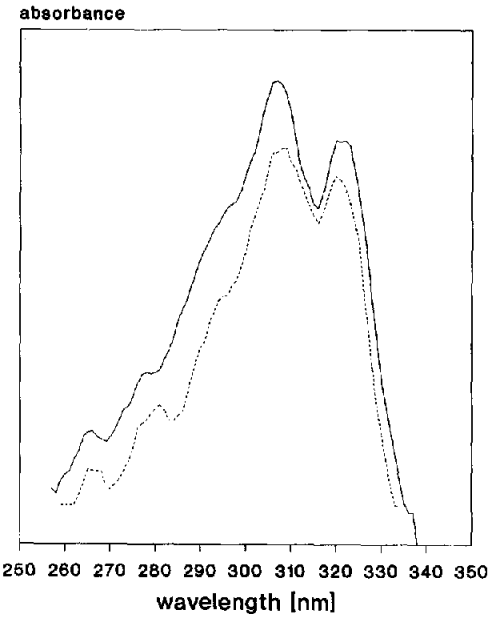

Fig.2. UV-spectrum of peak I (dotted line) and LX-standard (straight line). The spectrum was recorded in 62:38:0.008 methanol/water/trifluoroacetic acid.

obtained after extraction of the lipoxins with reversed-phase cartridges. The chromatogram shows 3 peaks that did not coelute with any HPLC standard of the LXA or LXB series. The retention times of these peaks were strongly affected by the trifluoroacetic acid concentration in the mobile phase, which is typical for arachidonic acid metabolites containing an amino acid residue [18]. The peaks showed UV spectra which were similar to the spectra obtained from LXA_4 and LXB_4, except for a bathochromic shift of 6 nm leading to a maximum at 307 nm with shoulders at 294 and 321 nm (fig.2). Since these spectra are typical for compounds having a conjugated tetraene structure with an allylic thioether substituent [13], there was evidence that these compounds are formed from 15-hydroxy-5,6-epoxyeicosatetraenoic acid by the action of the GSH S-transferase which is present in human eosinophils. This would lead to the (5*S*,15*S*)-dihydroxy-6-glutathionyl-7,9,11,13-eicosatetraenoic acid and the corresponding metabolites of the D- and E-series (see fig.4). Therefore, HPLC standards of these compounds were prepared as described in section 2.2. Peak I coeluted with the (5*S*,15*S*)-dihydroxy-(6*R*)-*S*-glutathionyl-eicosatetraenoic acid standard, while the peaks II and III coeluted with the corresponding 15-hydroxy derivative of LTE_4 and LTD_4, respectively (fig.1A). Both HPLC standards and the peaks I, II and III showed the same UV spectra (fig.2). For peak identification, a second mobile phase consisting of water/acetonitrile/trifluoroacetic acid was used. Under these chromatographic conditions, the amino acid-containing HPLC standards are widely separated from each other (retention times: 15-hydroxy-LTC_4 3.26 min, 15-hydroxy-LTE_4 5.08 min, 15-hydroxy-LTD_4 8.27 min with 44:56:0.008 acetonitrile/water/trifluoroacetic acid and 7.16, 10.96 and 19.69 min with 36:64:0.008, respectively). Fig.1B shows a HPLC chromatogram of the lipoxins extracted from human eosinophils and eluted with 44:56:0.008 acetonitrile/water/trifluoroacetic acid as the mobile phase. Under these chromatographic conditions, the 15-hydroxy-LTC_4 derivative elutes at very short retention times and cochromatographs with an LXA_4 isomer.

The fraction (peak I, fig.1A) eluting with the same retention time as the 15-hydroxy-LTC_4 standard under the chromatographic conditions described in fig.1A was pooled and rechromatographed in the acetonitrile/water system. Under these conditions, peak I coeluted with the 15-hydroxy-LTC_4 standard as well (chromatogram not shown). For further confirmation of the GSH moiety, the material was treated with γ-glutamyltransferase. The material was metabolised by this enzyme and a new peak appeared which coeluted with the 15-hydroxy-Δ^{13}-*trans*-15H-LTD_4 standard. Therefore, it can be concluded, that the compound referred to as peak I is (5,15*S*)-dihydroxy-6-glutathionyl-7,9,11,13-eicosatetraenoic acid. Since the stereochemistry of the 15-hydroxylated LTC_4 standard was assigned as (5*S*,15*S*)-dihydroxy-(6*R*)-*S*-glutathionyl-7,9,13-*trans*-11-*cis*-eicosatetraenoic acid [13], the configuration of the coeluting compound (peak I) is probably the same. However, an all *trans* double bond geometry or other configurations at C-5 or C-6 would theoretically be possible but are rather unlikely because of the stereospecificity of the enzymes involved in the biosynthesis of the compounds. Moreover, the capability of the column to separate the *cis* from the corresponding *trans* isomers (e.g. LTC_4 from 11-*trans*-LTC_4, LXA_4 from 11-*trans*-LXA_4, LXB_4

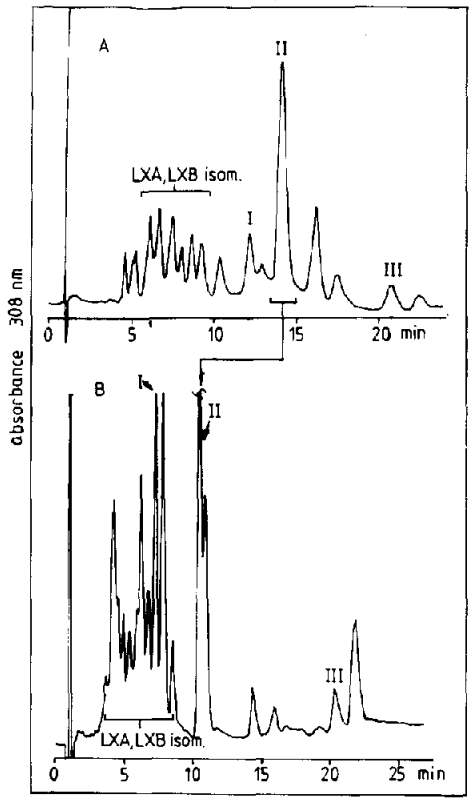

Fig.3. RP-HPLC chromatograms of the lipoxins extracted from eosinophils after incubation of the cells with ionophore for 15 min. The column was eluted with 62:38:0.008 methanol/water/trifluoroacetic acid (A) and 36:64:0.008 acetonitrile/water/trifluoroacetic acid (B).

from 8-*trans*-LXB_4) and the high resolution of the column makes it rather unlikely that the C-5 or C-6 epimers or the all-*trans* isomer coelute exactly with the HPLC standard.

In order to get higher quantities of the metabolites of LXC_4, the incubation time was increased to 15 min. Fig.3A shows a chromatogram of the tetraenes, extracted from eosinophils under these conditions. There, only minor amounts of peak I were detected and the compound coeluting with 15-hydroxy-Δ^{13}-*trans*-15H-LTE_4 was the major product. The fraction was collected and rechromatographed in the acetonitrile/water system. In both HPLC systems, peak II coeluted with the 15-hydroxy-LTE_4 standard so that the compound can be assigned as 15-hydroxy-LTE_4, a metabolite which can be formed from 15-hydroxy-LTC_4 by the action of the γ-glutamyltransferase and the dipeptidase in human eosinophils. Fig.3B shows a chromatogram of the tetraenes obtained when acetonitrile/water/trifluoroacetic acid was used as mobile phase.

Peak III coeluted in both HPLC systems with 15-hydroxy-Δ^{13}-*trans*-15H-LTD_4. The amounts of this compound were much less than those of peaks I and II released by the cells after 5 and 15 min. This is in agreement with the metabolism of the amino acid-containing leukotrienes in the cells under our assay conditions. There, only minor amounts of LTD_4 were released which may be due to the rapid metabolism of LTD_4 to LTE_4. 1–3% of the added 15-HETE was converted into lipoxins and in the presence of 20 μM exogenous 15-HETE, peak I (corresponding to 15-hydroxy-LTC_4) was the major arachidonic acid metabolite released from the cells after stimulation with the ionophore. Since human eosinophils possess a 15-lipoxygenase, it was of interest whether arachidonic acid can be transformed to these metabolites by the cells. Therefore, human eosinophils were incubated with either arachidonic acid (50 μM) and ionophore or with ionophore alone.

In incubations with arachidonic acid and ionophore, the same pattern of tetraenes was observed as in the incubations with 15-HETE and ionophore (chromatogram not shown) and no additional peaks were detected, so that a significant formation of 5,15-dihydroxy-14-GSH adducts can be ruled out. These compounds could be formed via the 5-hydroxy-14,15-epoxy-eicosatetraenoic acid, an intermediate which cannot be produced from 15-HETE but from 15-HPETE, which is present in arachidonic acid- and ionophore-stimulated cells. The failure to detect these compounds is in agreement to the finding that incubation with either 15-HETE or 15-HPETE leads to the same LX spectrum in granulocytes [3,4], so that the 15-hydroxy-5,6-epoxy-eicosatetraenoic acid and not the 14,15-epoxide must be regarded as the common precursor of lipoxins in human granulocytes. Moreover, no 14-GSH isomers of leukotrienes were isolated from biological material up to now, which makes it rather unlikely that there is an enzyme in eosinophils which can transform arachidonic acid-derived 14,15-epoxides to 14-GSH adducts. Taken together, these two facts may explain the absence of 14-GSH adducts in our incubations.

The amounts of tetraenes formed in the presence of arachidonic acid were much lower than in the presence of exogenous 15-HETE. This might be due to the lower amount of 15-HETE which is available for the formation of the 15-hydroxy-LTs when the cells were incubated without added 15-HETE. In the incubations with ionophore alone (without 15-HETE and without arachidonic acid), neither 15-HETE nor any tetraenes were detected and the leukotrienes LTC_4, D_4 and E_4 were the predominant lipoxygenase metabolites. The lack of 15-HETE formation under these conditions is in agreement to other reports [12,19] and may be due to the lacking availability of arachidonic acid for the 15-lipoxygenase when the cells are stimulated with the ionophore alone.

However, it becomes evident from these results, that the formation of these metabolites is closely related to the presence of 15-HETE and that the presence of this metabolite is responsible for the ratio of LTC_4 and 15-hydroxy-LTC_4. High 15-HETE concentrations lead to the predominant formation of 15-hydroxy-LTC_4, while low 15-HETE concentrations lead to the predominant release of leukotrienes. These results and the fact that no tetraenes were detected in samples containing no 15-HETE but high amounts of LTC_4, D_4 and E_4 (samples of cells stimulated with ionophore alone) clearly demonstrate that these tetraenes are not formed by the attack of the eosinophil 15-lipoxygenase on leukotriene C_4, D_4 and E_4. Therefore, one can conclude that these compounds are produced via the 15-hydroxy-5,6-epoxy-eicosatetraenoic acid like LXA_4 and LXB_4. Since the biosynthetic route is similar to that of LXA_4 and LXB_4, we suggest the trivial names lipoxin C_4, D_4 and E_4. The scheme for the formation of LXC_4, LXD_4 and LXE_4 is given in fig.4. Interaction of the 5- and the 15-lipoxygenase pathway leads to the (15*S*)-hydroxy-5,6-epoxy-7,9,13,-*trans*-11-*cis*-eicosatetraenoic acid which is transformed to lipoxin C_4 by the eosinophil-derived GSH S-transferase. The formed LXC_4 can be further metabolised by the γ-glutamyltransferase and dipeptidase to LXD_4 and LXE_4.

Previous studies have reported that LTC_4 and its metabolites are the predominant 5-lipoxygenase products generated in human eosinophils. In the present study, we have shown that human eosinophils release a novel series of lipoxins in the presence of the 15-lipoxygenase metabolite 15-HETE. Since 15-HETE can be released from eosinophils under certain conditions [12] and from other cells [20,21] and tissues [22], there is strong evidence that these compounds are also produced in eosinophils or by transcellular biosynthesis [23] in vivo. Therefore, it is of interest to elucidate the biological activities of this new series of lipoxins and their physiological and pathophysiological role. Since LXA_4 and LXB_4 show biological activities distinct from leukotriene B_4, this new series of lipoxins may also show other biological effects than the amino acid-containing leukotrienes.

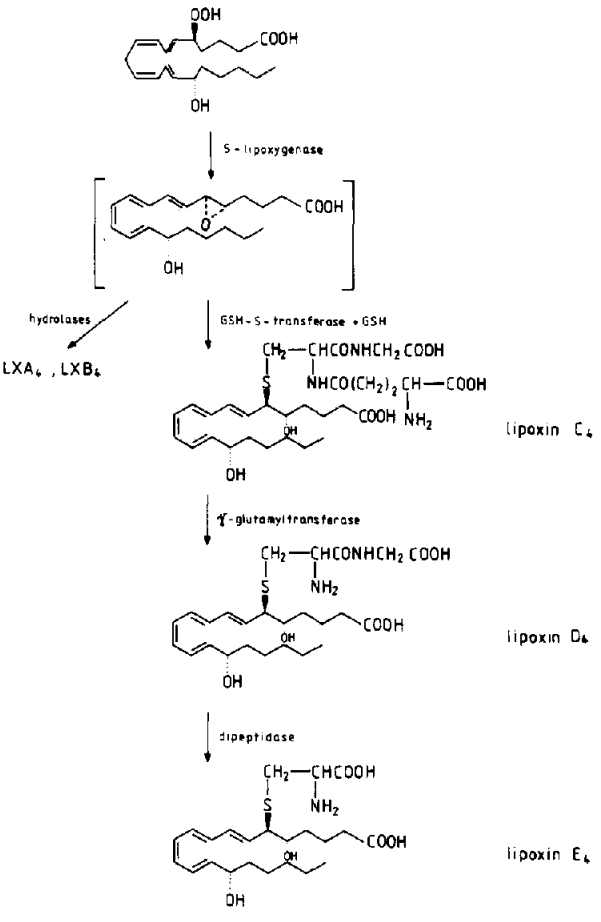

Fig.4. Scheme for the formation of lipoxin C_4, D_4 and E_4 in human eosinophils.

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