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Endogenously generated 5-hydroperoxyeicosatetraenoic acid is the preferred substrate for human leukocyte leukotriene A_4 synthase activity

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A single protein from human leukocytes possesses both 5-lipoxygenase and leukotriene A_4 (LTA_4) synthase activities. It has been reported that LTA_4 production is more efficient when the enzyme utilizes arachidonic acid, than when 5-HPETE is exogenously supplied as substrate. In the present study, human leukocyte homogenate 100000 × *g* supernatant was incubated with 100 μM octadeuterated arachidonic acid and exogenous 5-HPETE (0–80 μM), and the isotopic composition of LTA_4 hydrolysis products was determined by gas chromatography-mass spectrometry. Even though 100 μM deuterated arachidonic acid results in 20–30 μM deuterated 5-HPETE, 80 μM exogenous 5-HPETE in the incubation could reduce the amount of deuterated LTA_4 by only approx. 20%. The present study would thus indicate that the arachidonic acid moiety is preferentially converted to LTA_4 in a concerted reaction without dissociation of a 5-HPETE intermediate.

5-Lipoxygenase; Leukotriene A_4 synthase; Leukocyte; Arachidonic acid; Reaction mechanism; (Human)

1. INTRODUCTION

Leukotrienes constitute a group of biologically active derivatives of arachidonic acid believed to play a role in the mediation of immediate hypersensitivity reactions and inflammation (review, cf. [1]). The biosynthesis of leukotrienes is initiated by the lipoxygenation of arachidonic acid to form 5*S*-hydroperoxy-6-*trans*-8,11,14-*cis*-eicosatetraenoic acid (5-HPETE). 5-HPETE may be further converted into 5,6-oxido-7,9-*trans*-11,14-*cis*-eicosatetraenoic acid (LTA_4) which is an unstable key intermediate in the formation of different leukotrienes [1]. Thus, the initial steps in the formation of leukotrienes (i.e. 5-lipoxygenase and LTA_4 synthase activities) have become the subject of intensive research.

Shimizu and co-workers [2] first showed that an enzyme from potatoes that possesses 5- and 8-lipoxygenase activities could catalyze the synthesis of LTA_4 from either arachidonic acid or 5-HPETE. Later, it was discovered that a single protein from human leukocytes possesses 5-lipoxygenase and LTA_4 synthase activities, and that the synthesis of LTA_4 from 5-HPETE is controlled by the same complex multicomponent system that regulates the 5-lipoxygenase reaction [3]. At the same time, it was found that LTA_4 production was more efficient when the enzyme utilized arachidonic acid than when 5-HPETE was exogenously supplied as substrate [3]. Therefore, our aim was to investigate whether arachidonic acid is subjected to both 5-lipoxygenase and LTA_4 syn-

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Abbreviations: 5-HPETE, 5-hydroperoxyeicosatetraenoic acid; LT, leukotriene; 15-HPETE, 15-hydroperoxyeicosatetraenoic acid; HPLC, high-performance liquid chromatography; GC-MS, gas chromatography-mass spectrometry

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thase activities by the same enzyme molecule in a concerted reaction without dissociation from the enzyme.

2. EXPERIMENTAL

2.1. *Materials*

5,6,8,9,11,12,14,15-Octadeutero-arachidonic acid was a generous gift from Dr Ernst H. Oliw. Its purity was >97% as judged by thin-layer chromatography. In GC-MS, the methyl ester of deuterated arachidonic acid eluted with a *C* value of 19.6, and the mass spectrum showed a molecular ion at *m/e* 326 corresponding to methyloctadeutero-arachidonic acid. 5-HPETE was prepared from the incubation of arachidonic acid (Nu Check Prep) with a partially purified preparation of 5-lipoxygenase from potatoes [4]. The identity of 5-HPETE was verified by GC-MS after reduction with $SnCl_2$, treatment with ethereal diazomethane, and conversion into the trimethylsilyl ester. 15-HPETE was obtained by incubation of arachidonic acid with soybean lipoxygenase (Sigma) as described [5].

2.2. *Leukocyte homogenates*

Human leukocyte homogenate 100 000 × *g* supernatants were prepared as described [6].

2.3. *Incubation conditions*

Assay samples contained 800 μl of 0.5 M Tris-HCl/10 mM ATP/15 mM $CaCl_2$/4 mM dithiothreitol (pH 8.8) and 2.8 ml of the 100 000 × *g* supernatant. In addition, assay samples contained 400 μl phosphatidylcholine vesicles (640 μg) in phosphate buffer [7]/2 mM EDTA/1 mM dithiothreitol (pH 7.1) to bring the total volume to 4 ml. Samples were warmed to 37°C and the reaction was initiated by the addition of 20 μl deuterated arachidonic acid (400 nmol), different amounts of 5-HPETE (0, 80, 160 and 320 nmol, respectively) and 80 nmol 15-HPETE as a stimulatory factor [8]. After incubation for 10 min, the samples were placed on ice and 8 ml cold methanol and the internal standard (PGB_1) were added.

2.4. *Extraction and purification*

The assay samples were diluted with water to give a final solution of 1:9 methanol/water. After acidification to pH 3, LTA_4 transformation products were extracted on Sep-Pak C_{18} cartridges. Absorbed products were purified by consecutive washing with water and hexane and were finally eluted with methyl formate [9]. Methyl formate fractions were evaporated under argon, and the samples were injected onto a reversed-phase HPLC column (Nucleosil C_{18}). LTA_4-derived hydrolysis products were collected separately for the GC-MS procedure.

2.5. *Gas chromatography-mass spectrometry*

LTA_4-derived hydrolysis products were collected from HPLC and esterified by treatment with ethereal diazomethane. Trimethylsilyl derivatives were prepared using tris(trimethylsilyl)trifluoroacetamide (25 μl, Sigma) and pyridine (10 μl). The samples were evaporated and dissolved in hexane. GC-MS was performed on a 0.32 mm i.d. × 25 m methyl silicone (SPB-1, Supelco) capillary column on a Finnigan model 4500 mass spectrometer. The oven temperature was 260°C and the energy of the ionization beam was 70 eV. A standard fatty acid methyl ester mixture was used for the conversion of retention times to relative *C* values.

3. RESULTS

When human leukocyte homogenate 100 000 × *g* supernatant was incubated with 100 μM arachidonic acid, 20–30% was converted into 5-HPETE and ~5% into LTA_4 as measured by the

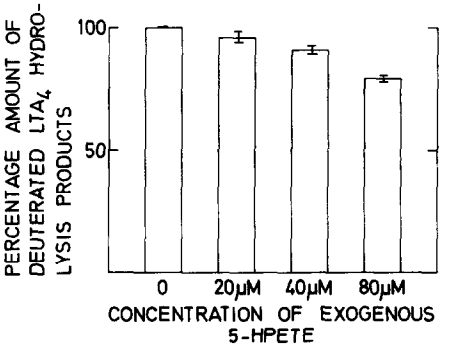

Fig.1. Effect of exogenous 5-HPETE on the formation of deuterated LTA_4 hydrolysis products when human leukocyte homogenate 100 000 × *g* supernatant was incubated with 100 μM octadeuterated arachidonic acid and the indicated concentrations of exogenous 5-HPETE (mean ± SD, $n = 3$).

sum of LTB_4, 6-*trans*-LTB_4 and 12-*epi*-6-*trans*-LTB_4. To investigate whether exogenous 5-HPETE could compete with endogenously generated 5-HPETE for the synthesis of LTA_4, the leukocyte homogenate $100\,000 \times g$ supernatant was incubated with 100 μM octadeuterated arachidonic acid and varying concentrations of 5-HPETE. Thereafter, the isotopic dilution of deuterated LTA_4 hydrolysis products by 5-HPETE was judged by GC-MS.

Gas chromatographic analysis of the trimethylsilyl ether derivatives of methyl esters of 6-*trans*-LTB_4, 12-*epi*-6-*trans*-LTB_4 and LTB_4 showed peaks of equivalent chain lengths of 24.8, 24.8 and 23.6, respectively. The relative *C* values of all these three compounds are in agreement with previously published data [10]. The mass spectra of all three compounds were virtually identical and were in agreement with previous reports [10,11]. For the compounds that were obtained from the incubations with octadeuterated arachidonic acid, the following characteristic ions at high intensity were seen at *m/e* 502 (M), 487 (M − 15), 412 (M − 90, loss of trimethylsilanol), 389 (M − 111, loss of $^{\cdot}CH_2\text{-}CH=CH\text{-}(CH_2)_4\text{-}CH_3$), 299 (M − (111 + 90)), 219 (probably $MeSiO\text{-}CH=CH\text{-}CH=O^+SiMe_3$ from a rearrangement) and 192 (probably $Me_3SiOCH=O^+SiMe$ from a rearrangement). The ion at *m/e* 389 appeared to be the best choice to monitor the isotopic dilution of deuterated LTA_4 hydrolysis products by exogenous 5-HPETE. Therefore, the isotopic dilution of LTA_4 hydrolysis products was calculated on the basis of the relative intensities of ions at *m/e* 389 (derived from octadeuterated arachidonic acid) and at *m/e* 383 (derived from exogenous 5-HPETE).

The percentage amounts of deuterated 6-*trans*-LTB_4, 12-*epi*-6-*trans*-LTB_4 and LTB_4, respectively, of the total amount of these products formed in the incubation of human leukocyte homogenate $100\,000 \times g$ supernatant with octadeuterated arachidonic acid and exogenous 5-HPETE are shown in fig.1.

4. DISCUSSION

In the present study, 100 μM octadeuterated arachidonic acid and 0–80 μM exogenous 5-HPETE were incubated with human leukocyte homogenate $100\,000 \times g$ supernatant and the isotopic composition of LTA_4 hydrolysis products was determined by GC-MS. It is worth noting that 100 μM deuterated arachidonic acid resulted in the formation of 20–30 μM deuterated 5-HPETE and, thus, the highest concentration of exogenous 5-HPETE (80 μM) was ≈3-fold as compared to endogenously produced deuterated 5-HPETE. LTA_4 formed from exogenous 5-HPETE represented, however, only approx. 20% of the total amount of LTA_4 formed during incubations (fig.1).

It has previously been reported that LTA_4 production is more efficient when LTA_4 synthase activity utilizes 5-HPETE generated from arachidonic acid than when 5-HPETE is exogenously supplied as substrate [3]. Thus, taken together with the present data, it appears that endogenously produced 5-HPETE is the preferred substrate for LTA_4 synthase activity. The present study might further indicate that the 5-lipoxygenase/LTA_4 synthase enzyme preferentially utilizes 5-HPETE produced by itself to form LTA_4, rather than using 5-HPETE produced by a different enzyme molecule. It remains to be elucidated how different stimulatory factors, for example Ca^{2+}, affect the ratio of endogenous/exogenous 5-HPETE converted to LTA_4.

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