IDENTIFICATION AND BIOLOGICAL ACTIVITY OF NOVEL ω-OXIDIZED METABOLITES OF LEUKOTRIENE B_4 FROM HUMAN LEUKOCYTES

GÖRAN HANSSON, Jan Åke LINDGREN, Sven-Erik DAHLÉN+, Per HEDQVIST+ and Bengt SAMUELSSON
Department of Physiological Chemistry and +*Department of Physiology, Karolinska Institutet, S-104 01 Stockholm, Sweden*

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1. Introduction

The leukotrienes constitute a new group of biologically active compounds derived from polyunsaturated fatty acids [1]. Thus, arachidonic acid can be oxygenated by a lipoxygenase to 5*S*-hydroperoxy-eicosatetraenoic acid [2], which is further converted to an unstable epoxide, 5,6-oxido-7,9,11,14-eicosatetraenoic acid (leukotriene A_4, LTA_4) [3,4]. This intermediate is transformed enzymatically by addition of glutathione into a 'slow-reacting substance of anaphylaxis' (SRS-A), LTC_4 [5,6]. The biological activity of most SRS-A preparations is due to LTC_4 and the two metabolites LTD_4 and LTE_4 [7–9].

LTA_4 can also be hydrolyzed enzymatically to 5*S*,12*R*-dihydroxy-6-*cis*,8,10-*trans*,14-*cis*-eicosatetraenoic acid (LTB_4) [10–12] or non-enzymatically to isomeric 5,12- and 5,6-dihydroxy-eicosatetraenoic acids [13]. We have reported the formation of a novel dihydroxy-acid, 5*S*,12*S*-dihydroxy-6-*trans*,8-*cis*,10-*trans*,14-*cis*-eicosatetraenoic acid (5*S*,12*S*-DHETE), in preparations of human leukocytes [14]. This dihydroxy-acid was not formed via an epoxide intermediate, but by a double oxygenation of arachidonic acid. In addition, an ω-hydroxylated metabolite, 5*S*,12*S*,20-trihydroxy-6-*trans*,8-*cis*,10-*trans*,14-*cis*-eicosatetraenoic acid (5*S*,12*S*,20-THETE), was identified.

This report describes the formation of an ω-hydroxylated metabolite of LTB_4, 5*S*,12*R*,20-trihydroxy-6-*cis*,8,10-*trans*,14-*cis*-eicosatetraenoic acid (20-OH-LTB_4) in human leukocyte preparations and further conversion of this trihydroxy acid to a dicarboxylic acid (20-COOH-LTB_4). In addition, the biological activity of LTB_4, 20-OH-LTB_4 and 20-COOH-LTB_4, on guinea pig lung strips is reported.

2. Materials and methods

2.1. *Cell preparation and incubations*

Human leukocyte suspensions (60×10^6 white cells/ml phosphate-buffered saline containing 0.87 mM $CaCl_2$ (pH 7.4)) were prepared as in [15]. The suspensions (100 or 200 ml) were warmed to 37°C and incubated for 10 min with 5 μM ionophore A23187 (Calbiochem-Behring, La Jolla CA) and 150 μM arachidonic acid (Nu-Check Prep., Elysian MN). The incubations were stopped by addition of 3 vols. ethanol. Incubations under $^{18}O_2$ atmosphere were carried out as in [14].

2.2. *Purification and analytical methods*

Purification of the products was performed as in [14]. This implies ether extraction, silicic acid chromatography, reverse phase (RP-) and straight phase (SP-) high-performance liquid chromatography (HPLC). Gas chromatography–mass spectrometry (GC/MS) of trimethylsilyl ether methyl esters of hydrogenated and non-hydrogenated compounds and oxidative ozonolysis using (–)-menthoxycarbonyl derivatives were done as in [14].

2.3. *Bioassay*

Guinea pig subpleural parenchymal strips (length ~25 mm, cross-sectional area ~7.5–10 mm^2) were suspended in 5 ml organ baths filled with Tyrode's solution. Contractions were recorded isometrically at a resting tension of 2.5 mN and expressed as % of maximal response induced by 100 μM histamine and 40 mM KCl. Before bioassay methyl esters of the leukotrienes tested were hydrolyzed (using 0.1 M LiOH in tetrahydrofurane; 23°C, 18 h) and purified on RP-HPLC.

3. Results

Human leukocytes were incubated with ionophore A23187 and arachidonic acid. Products were purified by ether extraction, silicic acid chromatography and RP-HPLC. The RP-HPLC chromatogram was similar to those obtained in this system [11,14], with the main dihydroxylated products 5*S*,12*S*-DHETE and LTB_4 cochromatographing in one peak eluted after 40 min. In addition a major polar peak with a retention time of 12 min was found (cf [14]). This material was converted to methyl esters with diazomethane and subjected to SP-HPLC (fig.1). We have recently identified one of the two major products, compound C, as 5*S*,12*S*,20-trihydroxy-6-*trans*,8-*cis*,10-*trans*,14-*cis*-eicosatetraenoic acid (5*S*,12*S*,20-THETE) [14]. The other main product, compound D, showed a UV-spectrum (absorption maxima at 260, 270 and 281 nm) almost identical to that of LTB_4

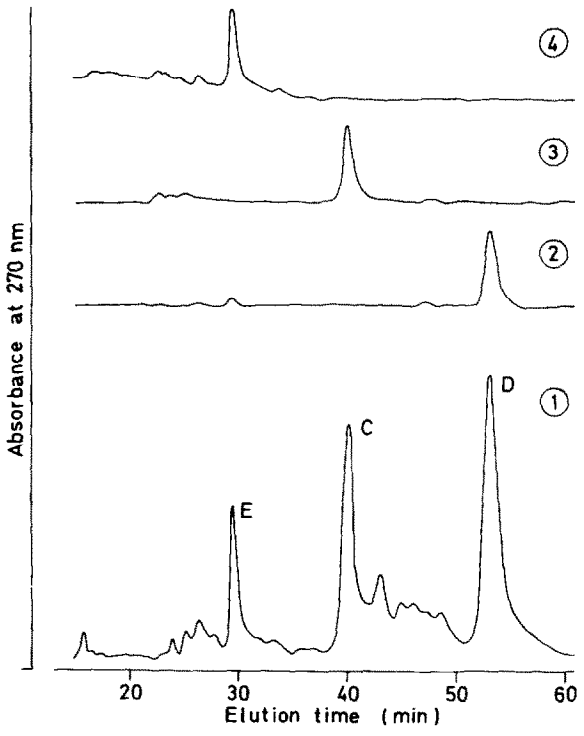

Fig.1. Curve ① shows a SP-HPLC chromatogram of the methyl esters of the polar products isolated from human leukocyte suspensions after incubation with ionophore A23187 and arachidonic acid. Curves ② – ④ show corresponding chromatogram obtained after incubation of human leukocyte preparations with LTB_4, 5*S*,12*S*-DHETE and 20-OH-LTB_4, respectively.

but shifted bathochromically 2 nm compared to that of compound C. Gas chromatography of the trimethylsilyl ether methyl ester of compound D showed a peak with *C*-value 26.7 (1% SE-30). In the mass spectrum (fig.2) prominent ions were found at *m/e*: 582 (M), 567 (M-15), 551 (M-31), 492 (M-90), 383 (M-199, loss of $^{\cdot}CH_2{-}CH{=}CH{-}(CH_2)_4{-}CH_2OSiMe_3$), 327, 317, 293 (M-(199 + 90)), 267, 229, 217 (probably $Me_3SiO{-}CH{=}CH{-}CH{=}O^{+}SiMe_3$ from a rearrangement), 203 ($Me_3SiO^{+}{=}CH{-}(CH_2)_3{-}COOCH_3$ and M-(199 + 90 + 90)), 191 (probably $Me_3SiOCH{=}O^{+}SiMe_3$ from a rearrangement) and 129 (base peak). This indicates that compound D is a tetraunsaturated C-20 acid with 3 hydroxyl groups located at C-5, C-12 and the third at a position beyond C-13. The high relative intensity of ions at *m/e* 129, 217, 267, 293 and 383 (cf. [10,14]) indicate that the first part of the molecule (C1–C-12) is structurally related to LTB_4 [10]. The increase in C-value for the trimethylsilyl ether methyl ester of compound D (C-26.7) compared to the corresponding derivative of LTB_4 (C-23.6 [10]) was 3.1 C, suggesting that the additional hydroxyl group is located at the C-20 position [16,17]. The proposed structure, 5,12,20-trihydroxy-6,8,10,14-eicosatetraenoic acid, was further supported by the mass spectrum of the trimethylsilyl ether methyl ester of hydrogenated compound D (*C*-value 27.3), which was in agreement with the mass spectrum of the same derivative of hydrogenated 5*S*,12*S*,20-THETE [14].

To investigate the mechanism of formation of compound D, leukocyte preparations were incubated with ionophore A23187 and arachidonic acid under an atmosphere of $^{18}O_2$. The mass spectrum of the trimethylsilyl ether methyl ester of compound D formed in these experiments contained several ions, which were shifted 4 units to higher *m/e* values (*m/e* 586 (M), 571, 555 and 496), while other ions (*m/e* 385, 295, 269, 219 and 205) were shifted 2 units to higher *m/e* values. These data show that compound D carried two atoms of ^{18}O, located at C-5 and C-20. This was also confirmed by GC/MS of the trimethylsilyl ether methyl ester of hydrogenated compound D from the same experiments. The mass spectrum contained ions, which were shifted 4 units (*m/e* 579, 563 and 493), or 2 units (*m/e* 305, 205 and 105) to higher *m/e* values. Thus, the hydroxyl groups at C-5 and C-20 were derived from molecular oxygen, while the hydroxyl group at C-12 was derived from water (cf. [3,14]).

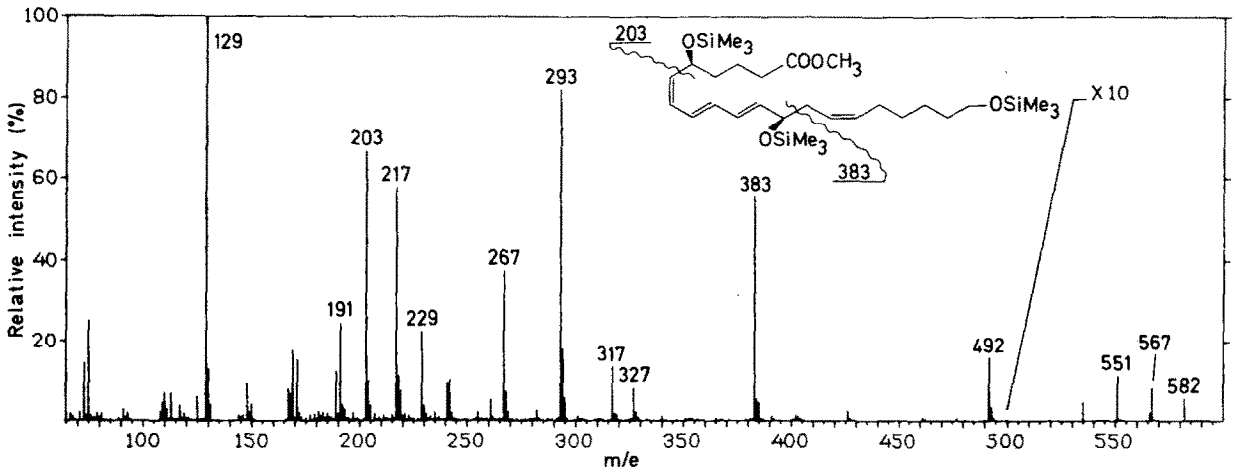

Fig.2. Mass spectrum of the trimethylsilyl ether methyl ester of compound D.

To establish the position of the double bonds and perform steric analysis of the alcohol groups, the (−)-menthoxycarbonyl methyl ester of compound D was subjected to oxidative ozonolysis. After treatment with diazomethane the fragments were analyzed by gas chromatography using a 3% OV-210 column. The products were shown to cochromatograph with the reference compounds (−)-menthoxycarbonyl dimethyl 2*S*-hydroxy adipate and (−)-menthoxycarbonyl dimethyl *R*-malate. These results demonstrate that the structure of compound D is 5*S*,12*R*,20-trihydroxy-6,8,10,14-eicosatetraenoic acid.

To study the pathway of formation of this trihydroxy acid and 5*S*,12*S*,20-THETE (compound C) LTB_4 or 5*S*,12*S*,DHETE (10 μM) were incubated with human leukocyte suspensions (37°C, 10 min). After initial purification, RP-HPLC revealed polar peaks (retention time 12 min), which were converted to methyl esters and subjected to SP-HPLC (see fig.1). As expected, LTB_4 gave rise to one single peak, corresponding to compound D, while 5*S*,12*S*-DHETE was converted to a compound with identical retention time as compound C. GC/MS analysis of the material confirmed the identity with 5*S*,12*R*,20-trihydroxy-6,8,10,14-eicosatetraenoic acid (20-OH-LTB_4) and 5*S*,12*S*,20-THETE, respectively.

In other experiments human leukocyte suspensions were incubated with compound D (10 μM;

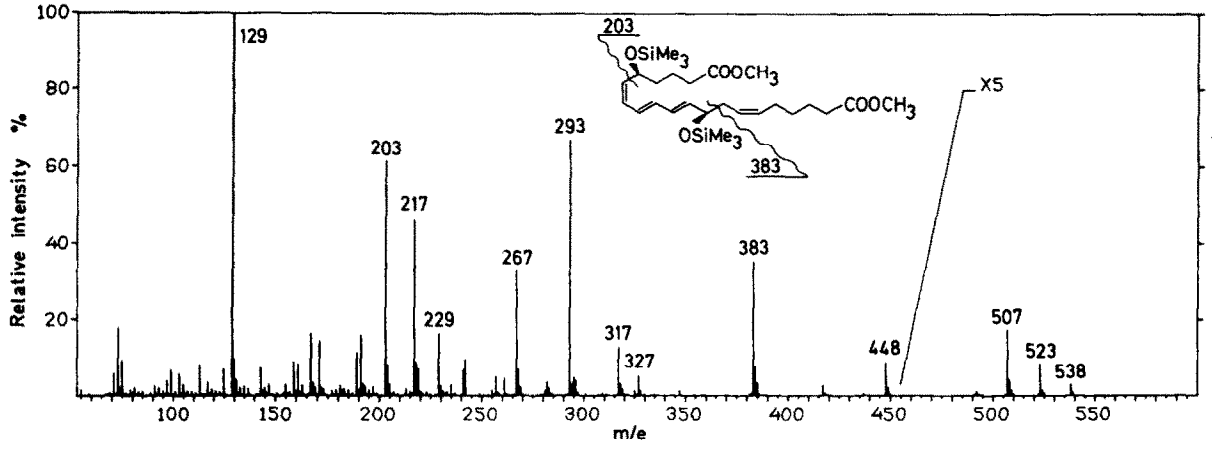

Fig.3. Mass spectrum of the trimethylsilyl ether methyl ester of compound E.

37°C; 30 min). After ether extraction and silicic acid chromatography the products were subjected to anion exchange chromatography using diethylamino-hydroxypropyl Sephadex LH-20 (DEAP-LH-20) [18]. The dicarboxylic fraction from this column was extracted at pH 3 with ethyl acetate, converted to methyl esters and subjected to SP-HPLC. One major peak with the same retention time as compound E appeared (fig.1). This material and compound E were identical as judged by GC/MS of the trimethylsilyl ether methyl ester derivatives showing peaks with *C*-value 26.5. In the identical mass spectra (fig.3) ions of high intensity were present at *m/e*: 538 (M), 523 (M-15), 507 (M-31), 448 (M-90), 383 (M-155, loss of $^{\cdot}CH_2$–CH=CH–$(CH_2)_4$–$COOCH_3$), 293 (M-(155 + 90)), 267, 217, 203 (see above), 129 (base peak), suggesting a tetraunsaturated C-20 dicarboxylic acid with hydroxyl groups at C-5 and C-12. Thus, compound D can be oxidized by human leukocyte preparations to a dioic acid, 5*S*,12*R*-dihydroxy-6,8,10,14-eicosatetraen-1,20-dioic acid (20-COOH-LTB_4).

The biological activity of LTB_4, 20-OH-LTB_4 and 20-COOH-LTB_4 was compared to that of LTC_4 and histamine in the guinea pig lung strip. LTB_4 and its metabolites caused dose-dependent contractions, which were slower in onset and of longer duration than responses evoked by equipotent concentrations of histamine. However, relative to cysteinyl-containing leukotrienes, contractions induced by LTB_4 or its ω-oxidized derivatives were short-lived and returned to resting tension within 15 min. According to dose–response relations assessed non-cumulatively, LTB_4, 20-OH-LTB_4 and 20-COOH-LTB_4 were $\geqslant$10-times more potent than histamine, although 50–100-times less active than LTC_4 (fig.4) [19,20].

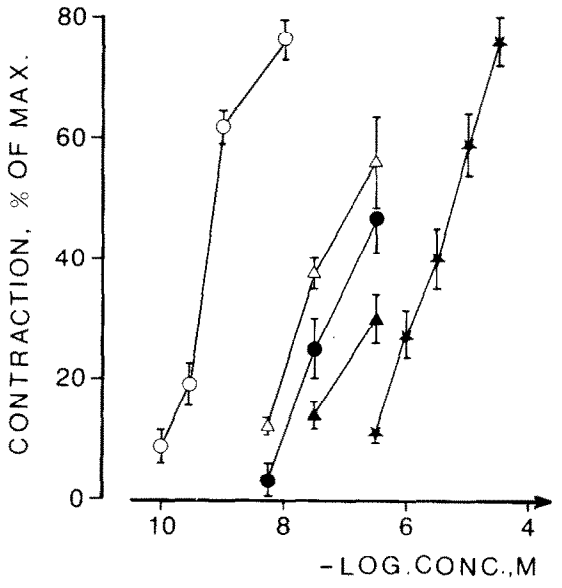

Fig.4. Non-cumulative dose–response relations for LTB_4 (•), 20-OH-LTB_4 (△), 20-COOH-LTB_4 (▲), LTC_4 (○) and histamine (★) in guinea pig lung parenchymal strips. Indicated drug concentrations refer to final concentration in bath fluid surrounding the tissue. Log dose–response relations for leukotrienes were established from mean values of 4–5 obs. in separate tissues at each concentration. Data presented as mean ± SEM, in percent of maximal concentration.

4. Discussion

Here, we describe the identification of a novel ωl-hydroxylated metabolite of LTB_4, 5*S*,12*R*,20-trihydroxy-6,8,10,14-eicosatetraenoic acid (20-OH-LTB_4) isolated from a human leukocyte preparation (fig.5). GC/MS- and UV-absorbance-data of this polar compound indicated that the stereochemistry corresponded to LTB_4 [10,11,14]. Steric analysis showed that the configuration of the alcohol groups at C-5 and C-12 was identical to that of LTB_4 (i.e., 5*S*,12*R*) [10]. As judged by labeling experiments with $^{18}O_2$ the C-5 hydroxyl group was derived from the molecular oxygen, while the C-12 hydroxyl group was formed from water. This indicated the same mechanism of formation as for LTB_4 [3]. Furthermore, LTB_4 could be transformed into compound D by human leukocyte preparations. In the same system 5*S*,12*S*-DHETE, was converted to 5*S*,12*S*,20-THETE.

An ωl position for the additional hydroxyl group in the LTB_4 structure was suggested by gas chromatographic data (increase of *C*-value with 3.1 [16,17]), and by the finding of a prominent ion at *m/e* 103 in the mass spectrum of the trimethylsilyl ether methyl ester of hydrogenated compound D. After ^{18}O-labeling this ion was shifted to *m/e* 105, also showing that the ωl-hydroxyl group was derived from molecular oxygen. Additional support for a terminal hydroxyl group was obtained by the conversion of compound D to a dicarboxylic acid, 5*S*,12*R*-dihydroxy-6,8,10,14-eicosatetraen-1,20-dioic acid (20-COOH-LTB_4), which was also formed in incubations of human leukocytes with ionophore A23187 and arachidonic acid. The same compound has independently been isolated and characterized after stimulation of

human leukocyte suspensions with other agents (Jubiz et al. in preparation).

Since the new leukotrienes were formed by ω-oxidation of LTB_4, they have probably retained the same configuration of the double bonds, i.e., 6-*cis*,8-*trans*, 10-*trans*,14-*cis* [12]. Preliminary results show that 5*S*,12*S*,20-THETE can also be oxidized to a dicarboxylic acid. Omega-oxidation to the dicarboxylic stage is a major pathway for metabolism of other arachidonate derivatives, e.g., prostaglandins [21]. It

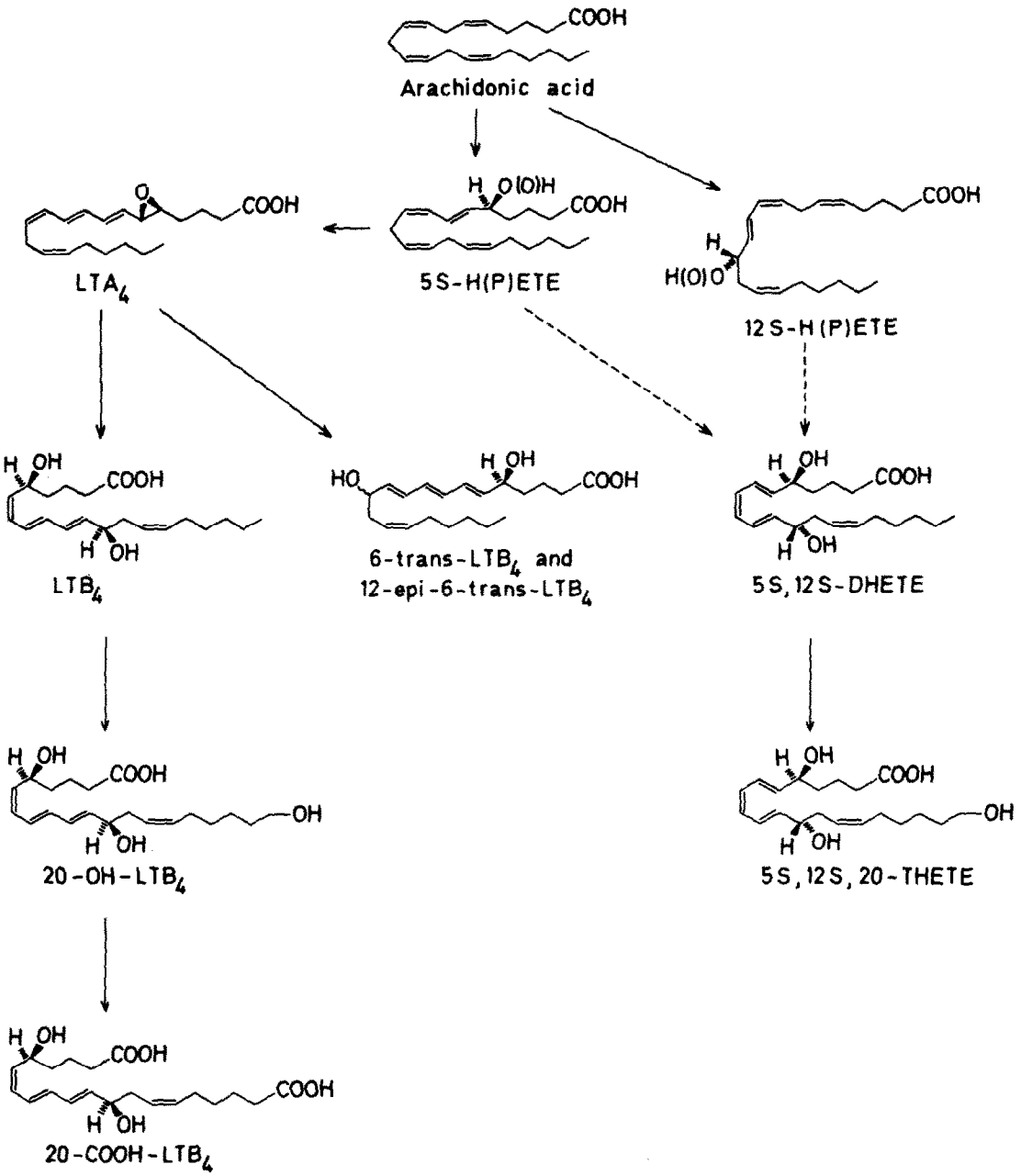

Fig.5. Scheme of transformation of arachidonic acid by preparations of human polymorphonuclear leukocytes. Dashed lines indicate proposed reactions.

is possible therefore, that ω-hydroxylation and further oxidation to dioic acids represent important steps in leukotriene metabolism.

This report shows that 20-OH-LTB_4 and 20-COOH-LTB_4 largely retained the bronchoconstrictor activity of LTB_4. However, although LTB_4 is a potent bronchoconstrictor, it differs from the cysteinyl-containing leukotrienes in being primarily a leukotactic agent and in causing leukocytes to adhere to the endothelium in postcapillary venules [22–24]. 20-OH-LTB_4 and 20-COOH-LTB_4 seem to be ~50-times less potent than LTB_4 in promoting leukocyte adhesion to endothelial cells in the hamster cheek pouch (Björk et al. in preparation). Therefore it seems that ω-oxidation of LTB_4 has variable effects on different biological activities. The reason for this variability needs to be further investigated.

Acknowledgements

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