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Formation of a novel 20-hydroxylated metabolite of lipoxin A_4 by human neutrophil microsomes

Hideki Sumimoto[a], Ryuichi Isobe[b], Yoichi Mizukami[a] and Shigeki Minakami[a]

[a]*Department of Biochemistry, Kyushu University School of Medicine, Fukuoka 812, Japan* and [b]*Faculty of Pharmaceutical Science, Kyushu University, Fukuoka 812, Japan*

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Lipoxin A_4 (LXA_4) is a biologically active compound produced from arachidonic acid via interactions of lipoxygenases. Incubation of LXA_4 either with human neutrophils or with the neutrophil microsomes leads to formation of a polar compound on a reverse-phase high-performance liquid chromatography. We have identified the metabolite as 20-hydroxy-LXA_4, a novel metabolite of arachidonic acid, on the basis of ultraviolet spectrometry and gas chromatography-mass spectrometry. The LXA_4 ω-hydroxylation requires both molecular oxygen and NADPH, and is inhibited by carbon monoxide, by antibodies raised against NADPH-cytochrome P-450 reductase, or competitively by leukotriene B_4 (LTB_4) and LTB_5, substrates of LTB_4 ω-hydroxylase. These findings indicate that the formation of 20-hydroxy-LXA_4 is catalyzed by a neutrophil cytochrome P-450, the LTB_4 ω-hydroxylase.

Lipoxin A_4; ω-Hydroxylation; Cytochrome P-450; Human neutrophil

1. INTRODUCTION

Unesterified arachidonic acid, released upon cell stimulation, is converted via lipoxygenase pathways to biologically potent products, leukotrienes and lipoxins [1–3]. The leukotrienes, produced through the 5-lipoxygenase pathway, participate in host defense reactions and play a role in immediate hypersensitivity and inflammation [1]. Interactions of 5-, 12- and 15-lipoxygenases lead to the formation of the lipoxins, a novel class of biologically active eicosanoids with a conjugated tetraene as a common structural feature [1–3]. Among them, lipoxin A_4 (LXA_4) is one of the most potent compound and possesses a diverse array of biological activities such as vasodilation [1,3], bronchoconstriction [1,3], inhibition of natural killer cell cytotoxicity [1,3], activation of isolated protein kinase C [4] and enhancement of the growth of myeloid progenitor cells [5]. In addition, recent studies indicate that LXA_4 displays actions counterregulatory to the proinflammatory effects of the leukotrienes. LXA_4 inhibits leukotriene B_4 (LTB_4)-induced inflammation in the hamster cheek pouch [6] and prior exposure of neutrophils to the lipoxin blocks the chemotactic response to LTB_4 [7,8]. Both the cellular and in vivo actions of leukotriene D_4 in renal microcirculation are antagonized by LXA_4 [9]. These activities suggest that LXA_4 may be associated with human diseases. The lipoxin is indeed detected in bronchoalveolar lavage fluids obtained from patients with pulmonary diseases [10].

Rapid conversion to inactive compounds should be expected for such potent molecules as a mechanism to terminate their actions. There have been considerable studies on the metabolism of the leukotrienes. Human neutrophils rapidly metabolize LTB_4 via ω-oxidation to 20-hydroxy-LTB_4 (20-OH-LTB_4) and subsequently to 20-carboxy-LTB_4 [11–14], reactions which are catalyzed by a microsomal LTB_4 ω-hydroxylase, a cytochrome P-450 (P-450) with a high affinity for LTB_4 (to be designated P-$450_{LTB\omega}$) [15–20]. LTB_4 is also ω-oxidized by rat hepatocytes [21–23], in which 20-carboxy-LTB_4 formed is further metabolized via β-oxidation along the ω-terminus [24]. ω-Hydroxylation followed by oxidation to the dicarboxylic acid and β-oxidation from the ω-end has also been recognized as the major pathway for the degradation of the cysteinyl leukotrienes [25–29].

Although increasing interest has been paid to the biological activities of LXA_4, little is presently known about the metabolic fate of this potent compound. Boucher et al. [30] have shown, in the only report on this subject, that LXA_4 is converted to a more polar metabolite by incubating with human intact leukocytes or with rat liver microsomes, and the compound is charac-

Correspondence address: H. Sumimoto, Department of Biochemistry, Kyushu University School of Medicine, 3-1-1 Maidashi, Higashi-ku, Fukuoka 812, Japan. Fax: (81) (92) 632-2373.

Abbreviations: LXA_4, lipoxin A_4 or (5*S*,6*R*,15*S*)-5,6,15-trihydroxy-7,9,11,13-*E*,*E*,*Z*,*E*-eicosatetraenoic acid; 20-OH-LXA_4, 20-hydroxy-LXA_4 or (5*S*,6*R*,15*S*)-5,6,15,20-tetrahydroxy-7,9,11,13-*E*,*E*,*Z*,*E*-eicosatetraenoic acid; LXB_4, lipoxin B_4; LTB_4, leukotriene B_4; 20-OH-LTB_4, 20-hydroxy-LTB_4; PGA_1, prostaglandins A_1; P-450, cytochrome P-450; P-$450_{LTB\omega}$, cytochrome P-$450_{LTB\omega}$ or human neutrophil LTB_4 ω-hydroxylase.

terized as retaining the conjugated tetraene structure indicated by ultraviolet (UV) spectrometry but the precise structural features are not elucidated.

In the present study we isolated and identified a novel metabolite of arachidonic acid, 20-OH-LXA_4, formed by incubating LXA_4 with human neutrophils or with the neutrophil microsomes. The formation of 20-OH-LXA_4 seems to be catalyzed by the LTB_4 ω-hydroxylase ($P\text{-}450_{LTB\omega}$).

2. EXPERIMENTAL

2.1. *Materials*

Human neutrophils were isolated from the blood of healthy volunteers by dextran sedimentation, hypotonic lysis and the Conray-Ficoll method, and the neutrophil microsomes and cytosols were prepared by differential centrifugation technique as previously described [17].

LXA_4 was purchased from Cascade Biochem., UK; LTB_4 from Cayman Chemical Co., Ann Arbor, USA; lauric acid from Sigma Chemical Co., St. Louis, USA; palmitic acid from Wako Pure Chemical Industry, Osaka, Japan; stearic acid from Nacalai Tesque Inc., Kyoto, Japan; NADPH from Oriental Yeast Co., Tokyo, Japan. All other reagents were of the highest purity commercially available. Antibodies raised against NADPH-cytochrome P-450 reductase in rat liver microsomes were prepared as previously described [17].

2.2. *Measurement of LXA_4 ω-hydroxylase activity*

Human neutrophil microsomes suspended in 100 μl of 100 mM potassium phosphate, pH 7.5, were incubated for the indicated time at 37°C with LXA_4 in the presence of 25 μM NADPH. The reaction mixture was terminated by addition of 100 μl of methanol. After centrifugation, 20 μl of the supernatant was diluted with 80 μl of acetonitrile/water/phosphoric acid (15:85:0.00425, v/v) and the sample (100 μl) was subjected to reverse-phase high-performance liquid chromatography (RP-HPLC). This was carried out using a Shimadzu LC-6A gradient system and a Chemcosorb 5-ODS-H column (5 μm, 4.6 × 150 mm; Chemco Scientific Co., Osaka) with continuous monitoring for ultraviolet absorbance at 301 nm. A flow rate was maintained at 1.0 ml/min. Elution was performed isocratically with acetonitrile/water/phosphoric acid (15:85:0.00425, v/v) for the first 5 min, followed by a linear increase in acetonitrile to 100% for the next 35 min. Typical elution times for LXA_4 and 20-OH-LXA_4 were 23.0 and 16.8 min, respectively. Integrated peak areas were used for quantitative analyses of LXA_4 and its metabolite.

2.3. *Identification of 20-OH-LXA_4*

The 20-OH-LXA_4, formed by human neutrophils or by the neutrophil microsomes, was identified by UV spectrometry and GC-MS. The UV spectrum of the 20-OH-LXA_4 separated by RP-HPLC was recorded in methanol using a Hitachi 340 UV/vis spectrophotometer. The lipid was methylated with ethereal diazomethane and subjected to straight-phase HPLC using Chemcosorb 5-Si (4.6 × 150 mm, Chemco Scientific Co., Osaka). The methyl ester was eluted isocratically at a flow rate of 1.0 ml/min with *n*-hexane/2-propanol (80:20, v/v). Typical elution times for the methyl ester derivatives of LXA_4 and 20-OH-LXA_4 were 4.3 and 15.0 min, respectively. The methyl esters were converted to their trimethylsilyl ether derivatives prior to gas chromatography-mass spectrometry (GC-MS) analyses with hexamethyldisilazane/trimethylchlorosilane. The samples were dissolved in hexane and injected to a gas chromatography/mass spectrometer equipped with a fused silica column (NB-1, equivalent to OV-1, GL Sciences Inc., Tokyo) (30 m × 0.25 mm). The mass spectrometer employed was a JEOL JMS-DX 300 double-focusing model interfaced to a JEOL JMA-3500 data system (Tokyo, Japan). The temperature of the injector and the column were kept at 300 and 280°C, respectively. The equivalent chain lengths (C-values) of the compounds on GC were determined using methyl ester derivatives of long-chain fatty acids (chain length: 16, 18, 20, 22, 24, 26, 28 and 30) as standards. The mass spectra were obtained by electron impact ionization with an ionization energy of 30eV.

3. RESULTS AND DISCUSSION

Incubation of LXA_4 with human neutrophils led to formation of a more polar product (compound I) with an elution time of 16.8 min on an RP-HPLC as detected by UV absorption at 301 nm (data not shown). The compound was also formed coincident with disappearance of LXA_4, when LXA_4 was incubated with the neutrophil microsomes under aerobic conditions in the presence of NADPH (Fig. 1). Similar analyses of the incubated samples, either with the neutrophils or with the microsomes, at different wavelengths (320, 270 and 230 nm) showed essentially the same profiles in RP-HPLC as those at 301 nm with no indication for formation of other metabolites (data not shown), suggesting that LXA_4 is converted solely to compound I under the conditions used in the present study.

To identify this novel metabolite, we performed UV and GC-MS analyses. The UV spectrum of compound I showed a triplet of absorption bands at 288, 301 and 316 nm (Fig. 2a), which is identical to that of LXA_4 (Fig. 2b). This suggests that the conjugated tetraene structure in LXA_4 is retained in the metabolite. Gas chromatographic analysis of the trimethylsilyl ether, methyl ester derivative of compound I showed a peak with a C-value of 26.9 (data not shown). The mass spectrum (Fig. 3A) was characterized by ions at *m/e*: 670 (M^+), 580 (M^+–90, loss of trimethylsilanol), 490 [M^+–(90+90)], 482, 467 [M^+– 203], 377 [467–90], 305 [Me_3SiO^+=CH-CH($OSiMe_3$)-$(CH_2)_3$-COOMe], 261 [Me_3SiO^+=CH-$(CH_2)_4$-CH_2-$OSiMe_3$], 215 [305–90], 203 [base peak, Me_3SiO^+=CH-$(CH_2)_3$- COOMe], 171 [261–90 and 203–CH_3OH], 129 and 113 [203–90]. Several ions were the same as those for the corresponding derivative of LXA_4 at *m/e* 482 [M^+–100; probably rearrangement followed by loss of O=HC-$(CH_2)_4$-CH_3, according to ref. 31], 305, 215, 203, 129 and 113 (Fig. 3A and B), suggesting that the structure of the C-1 through C-15 part of compound I is the same as that of LXA_4. Other remaining ions (*m/e* 670, 580, 490, 467, 377 and 261) were shifted 88 mass units up in comparison with ions in the spectrum of LXA_4, a finding consistent with the presence of an additional hydroxyl group at a position beyond C-16. The difference in C-value between a hydroxylated fatty acid metabolite and the parent compound is often used to determine the site of hydroxylation [11,32–35]. The C-value for the trimethylsilyl ether methyl ester of compound I (26.9) was 3.0 C longer than that for the corresponding derivative of LXA_4 (23.9, data not shown). The difference is consistent with those between 5*S*,12*S*,20-trihydroxytetraenoic acid and 5*S*,12*S*-dihydroxytetraenoic acid (3.0 C) [35], between 20-OH-LTB_4 and LTB_4 (3.1 C) [11], and between 20-

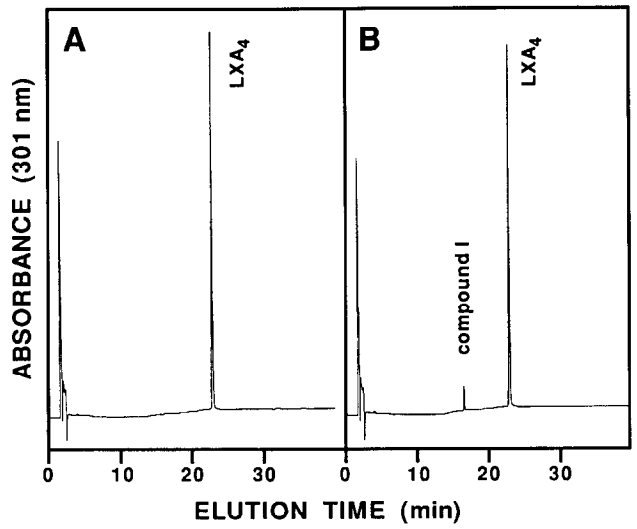

Fig. 1. High-performance liquid chromatograms of LXA and a product (compound I) obtained by incubating human neutrophil microsomes with LXA. LXA (6.0 μM) was incubated for 0 min (A) or for 15 min (B) with 500 μg/ml human neutrophil microsomes in the presence of 25 μM NADPH. The samples were subjected to RP-HPLC as described under Experimental.

hydroxy-prostaglandin B and prostaglandin B (3.1 C) [32–34]. The finding indicates that the additional hydroxyl group is located at the C20 position. Taken together with the UV and GC-MS analyses, compound I was identified as 20-OH-LXA.

Thus, we isolated and identified a novel metabolite of LXA, i.e. 20-OH-LXA, from the incubation of LXA with human neutrophils or with the neutrophil microsomes. In contrast to the present findings, there have been studies reporting that lipoxins including LXA are not transformed via ω-oxidation by intact human neutrophils [36] or in a cell-free system of the neutrophils [16]. Boucher et al., however, have reported that the incubation of LXA with human neutrophils leads to the formation of a more polar metabolite [30], which appears to be 20-OH-LXA. Although the reason for this discrepancy is presently unclear, it could be explained by the difference in analytical procedures including solvents used in HPLC, and by a relatively low rate of the 20-OH-LXA formation compared with that of the LTB ω-hydroxylation (see below). By incubating with human neutrophils or the microsomes, LXA was converted solely to 20-OH-LXA: we could not obtain any evidence for the formation of the 19-hydroxy derivative of LXA using RP-HPLC and GC-MS. This is not surprising because human neutrophils possess only an LTB ω-hydroxylase [11–17], responsible for the LXA ω-hydroxylation as described below, while both ω- and (ω- 1)-hydroxylase activities for LTB exist in rat liver microsomes [21]; both (ω-1)- and (ω-2)-hydroxylase activities in rat polymorphonuclear leukocytes [37].

We further characterized the LXA ω-hydroxylation

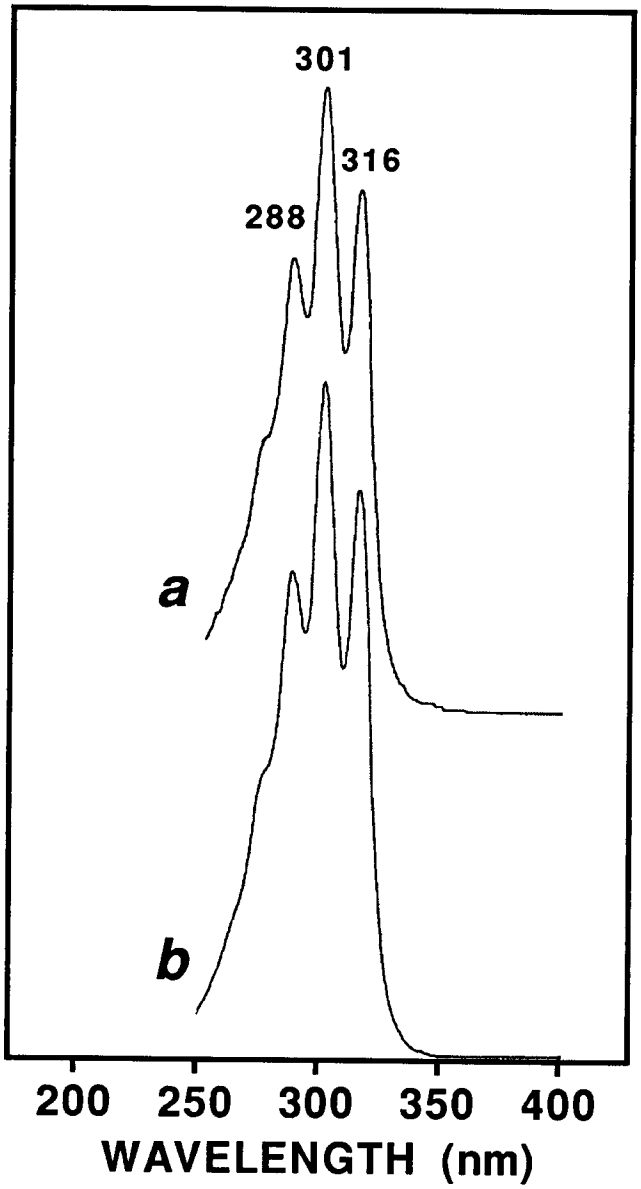

Fig. 2. Ultraviolet spectra of a metabolite of LXA (compound I) (a) and LXA (b).

by human neutrophil microsomes to clarify the enzyme responsible for the reaction. The decrease in LXA corresponded to the formation of 20-OH-LXA at any time studied (data not shown), suggesting that LXA is stoichiometrically converted to 20-OH-LXA. The LXA-hydroxylating activity was proportional to an incubation time of up to 60 min and to the concentration of the microsomal protein below 3.0 mg/ml (data not shown), while the activity was not detected by using human neutrophil cytosols (data not shown). The rate of the 20-OH-LXA formation at 10 μM of LXA was 29 $\pm$ 2 pmol/min/mg of microsomal protein (n = 5), which is approximately 5–10% of the rate for the LTB ω-hydroxylation by human neutrophil microsomes

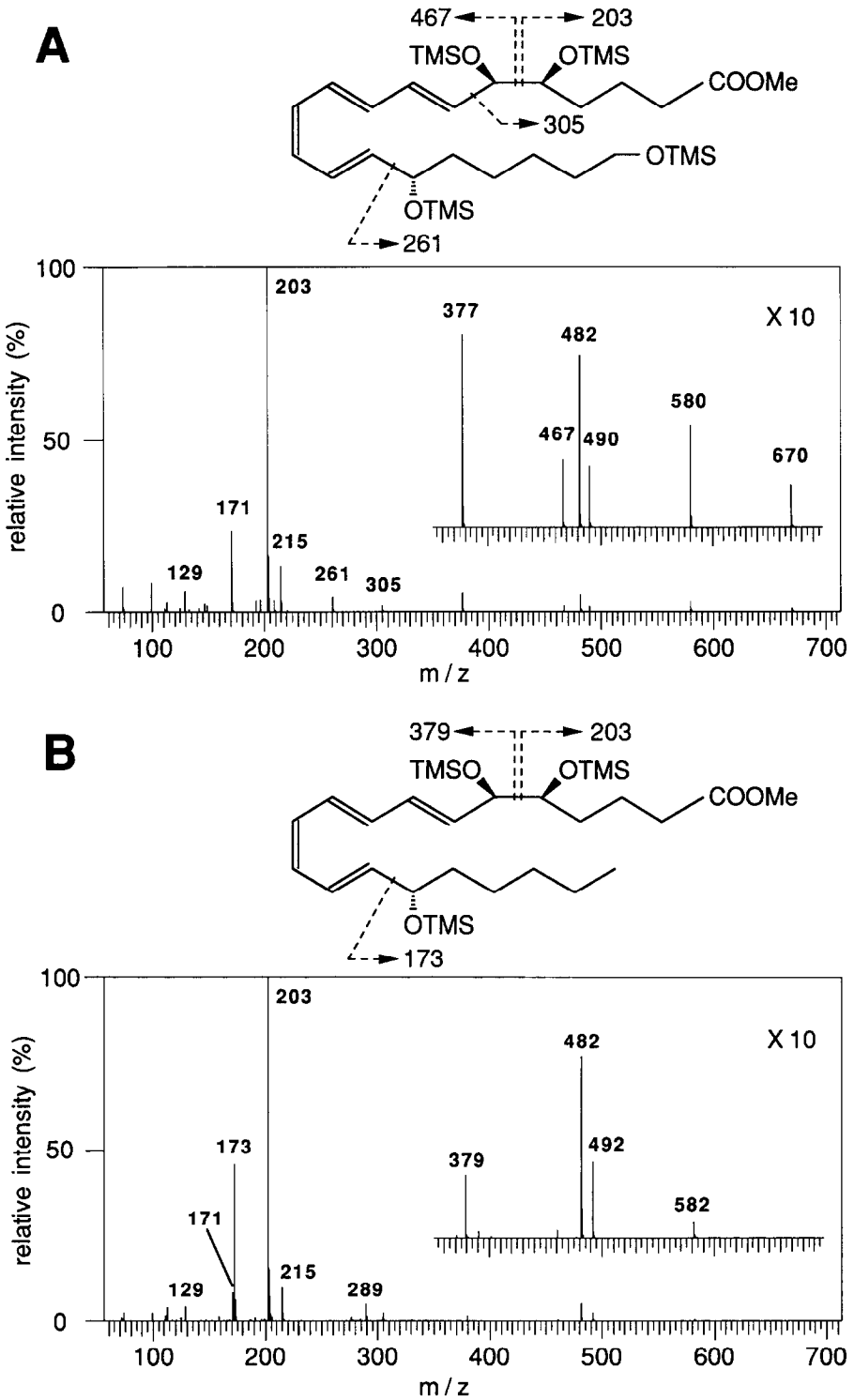

Fig. 3. Mass spectra of the trimethylsilyl ether methyl ester derivatives of a metabolite of LXA_4 (compound I) (A) and LXA_4 (B). Compound I was isolated and derivatized as described under Experimental.

[17,18]. When reactions were run at 0°C, in the absence of NADPH or using microsomes pretreated for 10 min at 80°C, no product was formed (Table I). As shown in Table I, a negligible activity to ω-hydroxylate LXA_4 was observed under anaerobic conditions, indicating that the reaction requires molecular oxygen. The hydroxylation was strongly inhibited by carbon monoxide (Table I). The findings of the localization in the microsomes, the requirement of O_2 and NADPH, and the inhibition by CO, suggest that a P-450 is involved in the

reaction. The involvement is further supported by inhibition of the reaction by antibodies raised against NADPH-P-450 reductase (Table I). To confirm that the P-450 is the LTB_4 ω-hydroxylase, we carried out the following experiments. LTB_4, a substrate of $P\text{-}450_{LTB\omega}$, inhibited the LXA_4 ω-hydroxylation by the neutrophil microsomes (Table I). LTB_5, a good substrate but with a lesser affinity than that of LTB_4 [19], also possessed an inhibitory activity but to a lesser extent (Table I). The inhibitions by LTB_4 and by LTB_5 were restored by increasing the concentrations of LXA_4 (data not shown), suggesting that the inhibitors act in a competitive manner. Various fatty acids including lauric acid and PGA_1, all of which are not substrates for $P\text{-}450_{LTB\omega}$ [16,17,19], did not affect the reaction (Table I). The formation of 20-OH-LXA_4 by human neutrophils is, thus, catalyzed by the LTB_4 ω- hydroxylase ($P\text{-}450_{LTB\omega}$), whereas the formation of a polar metabolite by incubating LXA_4 with rat liver microsomes seems to be catalyzed by a P-450 but not by the one(s) responsible for LTB_4 metabolism [30]. Since LXA_4 antagonizes the actions of LTB_4 towards neutrophils [7,8], the competitive inhibition by LTB_4 of the neutrophil-dependent LXA_4 ω-hydroxylation may suggest a regulation in the interaction between these active substances: actions of LXA_4 can be regulated by LTB_4 through the competition at the common metabolizing enzyme.

Since lipoxin B_4 (LXB_4) is also converted to 20-hydroxy-LXB_4 by human neutrophil microsomes (Y. Mizukami and H. Sumimoto, unpublished observation), the conversion to 20-hydroxy metabolites may be important to eliminate lipoxins in the organism. The ω-hydroxylation is an initial step in the formation of the dicarboxylic acid required for subsequent β-oxidation from the ω-end, and this oxidation pathway has now been recognized as the major pathway for the degradation and elimination of the leukotrienes [24,26–29].

Table I

Lipoxin A_4 ω-hydroxylation by human neutrophil microsomes under various conditions

Condition	LXA_4 ω-hydroxylase activity (%)
Control	100
Heat-treated microsomes[a]	0
Without NADPH	0
Anaerobic[b]	2
Anti-NADPH-P-450 reductase IgG (100 μg/ml)	27
Non-immune IgG (100 μg/ml)	103
CO/O_2 (80/20, %)	12
LTB_4 (1 μM)	28
LTB_4 (3 μM)	9
LTB_5 (1 μM)	61
LTB_5 (3 μM)	18
Lauric acid (3 μM)	106
Stearic acid (3 μM)	102
PGA_1 (3 μM)	94
PGE_1 (3 μM)	97

[a] The microsomes were pretreated for 10 min at 80°C.

[b] The microsomes were deoxygenated under a stream of nitrogen followed by incubation with 10 mM glucose, glucose oxidase (50 μg/ml), and catalase (100 μg/ml) for 10 min at 25°C before starting the reaction.

Human neutrophil microsomes (50 μg) suspended in 100 μl of the phosphate-buffered solution, pH 7.5, was incubated for 15 min at 37°C with LXA_4 (6.0 μM) in the presence or absence of NADPH (25 μM) under the indicated conditions. The LXA_4 ω-hydroxylating activity was determined as described under Experimental Procedures. Data represent mean values of two experiments. The activity under aerobic conditions in the absence of the inhibitors (18 nmol/min/mg protein) is set at 100%.

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