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Hypothesis Paper

PROPERTIES OF ENZYMES IN HEPATOCYTES THAT CONVERT 5-HPETE OR LTA_4 INTO LTB_4

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Abstract—Rat hepatocyte homogenates convert 5-hydroperoxyeicosatetraenoic acid (5-HPETE) into biologically active leukotriene B_4 (LTB_4) as well as less active all-trans-LTB_4 (i.e., 6-trans-LTB_4 and 6-trans-12-epi-LTB_4). Here, we present a hypothesis of the reaction mechanism and the minimal structural requirements of the active enzyme based on the following experimental evidence: The ED_{50} of the inhibitors 5,8,11,14-eicosatetraynoic acid (ETYA) and 5,6-dehydro-eicosatetraenoic acid was approximately 100-fold higher than for 5-lipoxygenase. Propanethiol and O_2 were strong inhibitors of LTB_4 formation, whereas butylated hydroxytoluene, nordihydroguaiaretic acid, metyrapone, Desferal and CO had no effect. Cytochrome c, catalase, hematin, and a Fe^{3+}/Fe^{2+} couple, but not iron-free protoporphyrin IX, catalyzed the formation of only all-trans-LTB_4. LTB_4 formation in hepatocyte homogenates was heat- and trypsin-sensitive whereas all-trans-LTB_4 formation was not. We propose that a ferric heme iron forms a ferryl-hydroxo complex upon homolytic scission of the oxygen-oxygen bond in 5-HPETE and the resulting 5,6-trans-epoxide radical is oxidized by the ferryl-hydroxo complex to yield LTA_4. A mechanism for hydrolysis of LTA_4 is described that results in formation of LTB_4 (<1% yield) rather than all-trans-LTB_4.

Keywords—Hydroperoxides, Hepatocytes, Rat liver, 5-HPETE, Leukotriene A_4, Leukotriene B_4, Free radicals

INTRODUCTION

Leukotrienes are a group of endogenous mediators active in hypersensitivity reactions and inflammation. In specialized cells such as polymorphonuclear leukocytes, mast cells, and neutrophils, a 5-lipoxygenase converts arachidonic acid into 5-hydroperoxyeicosatetraenoic acid (5-HPETE), the direct precursor of LTA_4.[1-3] Hydrolysis of LTA_4 by LTA_4-hydrolases[4] leads to the formation of LTB_4, which, at nanomolar concentrations, exerts a variety of distinct biological effects, among them chemotaxis of neutrophils and release of lysosomal enzymes from human polymorphonuclear leukocytes. However, an increasing body of evidence shows that the liver is also involved in biosynthesis[5-10] and degradation[11-12] of eicosanoids. The possible importance of an alternate pathway for LTB_4 production within hepatocytes is increased by the demonstrations that free radical metabolites of drugs can produce 5-HPETE from arachidonic acid[13] and that peroxidized fatty acids can be selectively liberated from phospholipids by phospholipase A_2.[14]

In previous studies we have shown that 5-HPETE and LTA_4 can be converted to biologically active LTB_4 by rat hepatocyte homogenates and human and rat liver microsomal preparations.[6,9,15] In the latter studies the structural and functional identity of LTB_4 was confirmed with mass spectrometry,[16] HPLC retention time, UV absorption, radioimmunoassay, a neutrophil LTB_4 receptor binding assay, and an assay measuring transient increases of neutrophil cytosolic calcium in response to challenges with LTB_4. In addition, the formation of peptido-leukotrienes and LTB_4 from LTA_4 has been measured in cytosolic fractions obtained from whole livers of both guinea pig and rat.[7,8] Although no lipoxygenase activity has been demonstrated in hepatocytes, primary cultures of human and rat hepatocytes, when challenged with ethanol, were found to release a chemotactic factor with chromatographic and radioimmunoassay binding properties similar to LTB_4,[17] substantiating a possible alternative pathway in hepatocytes for generation of leukotrienes.

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The studies presented here indicate that hepatocyte-mediated transformation of 5-HPETE into LTB_4 is not due to the action of the 5-lipoxygenase or LTA_4-hydrolase[1,4] enzymes involved in leukotriene biosynthesis in peripheral cells.[2,3] This paper presents a hypothesis of some of the properties and reaction mechanisms of the enzyme found in hepatocytes that converts 5-HPETE into the biologically fully active LTB_4 as well as the less active all-trans-LTB_4.†

METHODS

Preparation of rat hepatocytes and hepatocyte homogenates

Single cell suspensions of hepatocytes were prepared from fed adolescent male Fischer 344 rats by in situ perfusion of collagenase as described.[18] Separation of hepatocytes from nonparenchymal liver cells was performed according to Smedsrod and Pertoft.[19] Briefly, cell suspensions were filtered through nylon mesh and then centrifuged for 4 min at 50 × g, yielding a pellet enriched in hepatocytes and a supernatant fraction enriched in nonparenchymal cells. The pellet was then washed another four times in Earle's buffer (GIBCO), pH 7.4; it was resuspended, layered on top of a Percoll cushion (25 ml) with a density of 1.070 g/ml, and centrifuged at 130 × g for 10 min. These hepatocytes were washed three times in isotonic Hank's buffer (GIBCO) and homogenized in a buffer containing 10 mM potassium phosphate, pH 8.5, 20% (v/v) glycerol, and 50 μg/ml L-α-dilauroylphosphatidylcholine. They were disrupted by four strokes in a Potter Elvehjem homogenizer at 4°C followed by sonication for 10 min in a bath type sonifier in an ice-water bath. The homogenates were stored in aliquots at −20°C and thawed only once immediately before the experiment.

Incubation with 5-HPETE and HPLC-analysis of product formation

Aliquots of hepatocyte preparations were brought to 400 μl final volume in homogenization buffer and gently bubbled for 15 min with argon in a sealed Reacti-vial at room temperature. 5-HPETE in ethanol, also kept under argon, was added with a gas-tight syringe through the septum of the Reacti-vial in a maximum volume of 20 μl. In inhibition studies, inhibitors were added simultaneously from stock solutions which were also kept under argon. The incubations were performed at room temperature under argon for 15 min. Incubations were stopped by addition of 30 μl of 1 N formic acid, followed by 800 μl of methanol/water, (1:1, v/v), containing 25 ng/800 μl of prostaglandin B_1 as an internal standard. Samples were extracted with 5 ml dichloromethane, washed twice with 1 ml of water, taken to dryness under nitrogen, resuspended in 100 μl eluent and injected into the HPLC. Separation of products was achieved by HPLC on a NUCLEOSIL 5 column (C18, 0.46 × 25 cm, 5 μm, Machery–Nagel, Duren, FRG, operated with a guard column) in a methanol/water/acetic acid (72:28:0.02, v/v) solvent mixture, adjusted to pH of 5.7 with triethylamine, at a flow rate of 1.0 ml/min. Relative peak heights were used to quantify the internal standard prostaglandin B_1 and LTB_4 in individual chromatograms. The amounts of LTB_4 generated were determined by external standardization with synthetic LTB_4. Material eluting with the same retention time as synthetic LTB_4 was collected and rechromatographed in the same eluent system prior to further structural and biological characterization.

Molecular modeling

The computer molecular modeling program XICAMM (XIRIS Corporation, Middletown, NY), which uses a classical molecular mechanics algorithm, was used to search for pairs of low energy conformations of LTA_4 and LTB_4 in which the C5 to C8 segments of the fatty acid chain could be overlaid. These conformers were sought by making suitable rotations about the 4–5, 6–7, and 8–9 single bonds of LTA_4, modeling these conformers into a low energy conformation with XICAMM, then testing whether it was possible to make suitable rotations about the 4–5, 5–6, and 7–8 single bonds of LTB_4 that, when also modeled into a low energy conformation, could be overlaid with that of the LTA_4 conformer.

Other methods

The following techniques were used throughout our studies to ascertain the structural and functional identity of the hepatocyte homogenate-derived LTB_4. Mass spectra of LTB_4 were obtained by direct chemical ionization mass spectrometry (DCIMS) as previously described.[16] Samples were applied to a polyimide-coated fused silica fiber that was inserted directly into the ionization plasma of the chemical ionization source of a modified Hewlett Packard 5985A quadrupole mass spectrometer. The equilibrium binding of [^{3}H]LTB_4 to human neutrophils in the absence and presence of non-radioactive preparations of LTB_4, generated upon in-

†In this paper, the term all-trans-LTB_4 is used to designate the products 6-trans-LTB_4 and 6-trans-12-epi-LTB_4, which are detected in equal amounts and were referred to as peaks I and II, respectively, in chromatograms or organic extracts of 5-HPETE incubations with rat hepatocyte homogenates.[15]

cubation of 5-HPETE in hepatocyte homogenates, was assessed as described.[15] The capacity of synthetic and hepatocyte homogenate-derived preparations of LTB_4 to induce a transient increase in the cytosolic calcium concentration of human neutrophils were compared as described in recent reports.[15] [^{3}H]LTB_4 radioimmunoassays were performed according to the manufacturer's guidelines. The crossreactivity (at 50% B/Bo replacement) of the LTB_4-antiserum employed was 3.3% towards the diastereoisomers of 5,12-dihydroxy-6,8,10-trans-14-cis-eicosatetraenoic acid, 1.6% towards the diastereoisomers of 5,6-diHETE and less than 0.03% towards LTC_4 and LTC_4. Protein concentrations were estimated by the Bio–Rad assay procedure using bovine serum albumin as a standard.

RESULTS AND DISCUSSION

In a recent report[15] we have shown that rat hepatocyte homogenates convert 5-HPETE into biologically fully active LTB_4. In typical experiments, a total of $1.5 \pm 0.2\%$ and $0.7 \pm 0.2\%$ (five experiments) of the 5-HPETE consumed was transformed into all-trans-LTB_4 and LTB_4, respectively, after a 30 min incubation of rat hepatocyte homogenates (0.8 mg protein per 400 μl) with 100 μM starting substrate concentration. After the same incubation period, $79 \pm 15\%$ (five experiments) of the initial amount of 5-HPETE could be recovered intact. This capacity to convert 5-HPETE into LTB_4 points to a presumably potent role, at least in vitro, of hepatocytes in leukotriene biosynthesis. Besides transport from other cells, hepatic lipid peroxidation, which occurs to a considerable extent under low but physiological O_2-tensions in the presence of hepatotoxins such as halothane or carbon tetrachloride, may contribute to the availability of precursors (i.e., 5-HPETE[13]) of leukotriene biosynthesis in hepatocytes. Of course, the amount of LTB_4 available is the result of a delicate, O_2-dependent equilibrium between synthesis and metabolism[8,11,12,20] of LTB_4 within hepatocytes. However, it is likely that the sub-nanomolar concentrations of LTB_4 sufficient to evoke intra- or inter-cellular signaling could be achieved locally.

Inhibition of 5-HPETE transforming activity

Here, we have attempted to further characterize the enzyme responsible in hepatocytes for the transformation of 5-HPETE into biologically active LTB_4 by comparing its functional properties with those of 5-lipoxygenase and LTA_4-hydrolase using inhibitors of these enzymes. Rat hepatocyte homogenates were incubated with 5-HPETE and the incubation mixtures were extracted with acidified dichloromethane and analyzed on RP-HPLC. As shown in a previous publication[9] (see Fig. 1 therein), three prominent UV-absorbing peaks (280 nm), labelled peaks I, II, and III, could reproducibly be detected. Peaks I and II co-chromatographed with and exhibited identical retention times as 6-trans-LTB_4 and 6-trans-12-epi-LTB_4, respectively, corresponding to products formed in model systems upon non-enzymatic mild acid hydrolysis of synthetic LTA_4 free acid,[22] whereas peak III co-eluted with synthetic LTB_4 and was thoroughly characterized. The presence of peaks I and II in the chromatograms of extracts of incubations of rat hepatocyte homogenates with 5-HPETE suggested that LTA_4 might be a common intermediate in the conversion process.

We have found that some known inhibitors of arachidonic acid metabolism[23] are able to moderately inhibit the formation of LTB_4 from 5-HPETE by rat hepatocyte homogenates; these results are summarized in Table 1. At concentrations 100-fold higher than those that inhibit lipoxygenases[23], 5,8,11,14-eicosatetraynoic acid (ETYA, 1 mM) and 8,11,14-5,6-dehydro-eicosatetraenoic acid (50 μM) also inhibited LTB_4 formation by 53% and 43%, respectively.

However, they were without effect in hepatocyte homogenates when measured at the ED_{50} for inhibition of 5-lipoxygenase. 5-Lipoxygenase possesses dual enzymatic activities: Not only does it incorporate molecular oxygen into arachidonic acid to yield 5-HPETE, it also transforms 5-HPETE into LTA_4.[21] Therefore, the effect of nordihydroguaiaretic acid (NDGA), an inhibitor considered to be a probe for the active site of 5-lipoxygenase,[24] was measured. NGDA did not have a significant effect on the production of LTB_4. Butylated hydroxytoluene (BHT, 1 mM) had no effect on LTB_4 or all-trans-LTB_4 production. Based on this finding, we suggest that, in the course of the transformation of 5-HPETE to LTB_4 by rat hepatocyte homogenate, no freely diffusible or long-lived radical intermediates are formed. Propanethiol occupies the sixth ligand position of the heme groups in cytochrome P450 and myoglobin and inhibits cytochrome P450-dependent drug metabolism.[25] It also reduces formation of all-trans-LTB_4 by 27% and that of LTB_4 by 49%. However, metyrapone, a classical inhibitor of cytochrome P450 functions, is not an inhibitor of this activity under our experimental conditions. Potassium cyanide (2.5 mM) and CO (1 atm) were used to probe for a role of reduced heme proteins in this reaction. Neither compound significantly inhibited all-trans-LTB_4 formation but they both slightly decreased LTB_4 formation. The iron-chelating agent Desferal (Ciba-Geigy, 1 mM) had no effect on LTB_4 formation from 5-HPETE, indicating that 5-HPETE transformation to LTB_4 in rat hepatocyte homogenates is not primarily

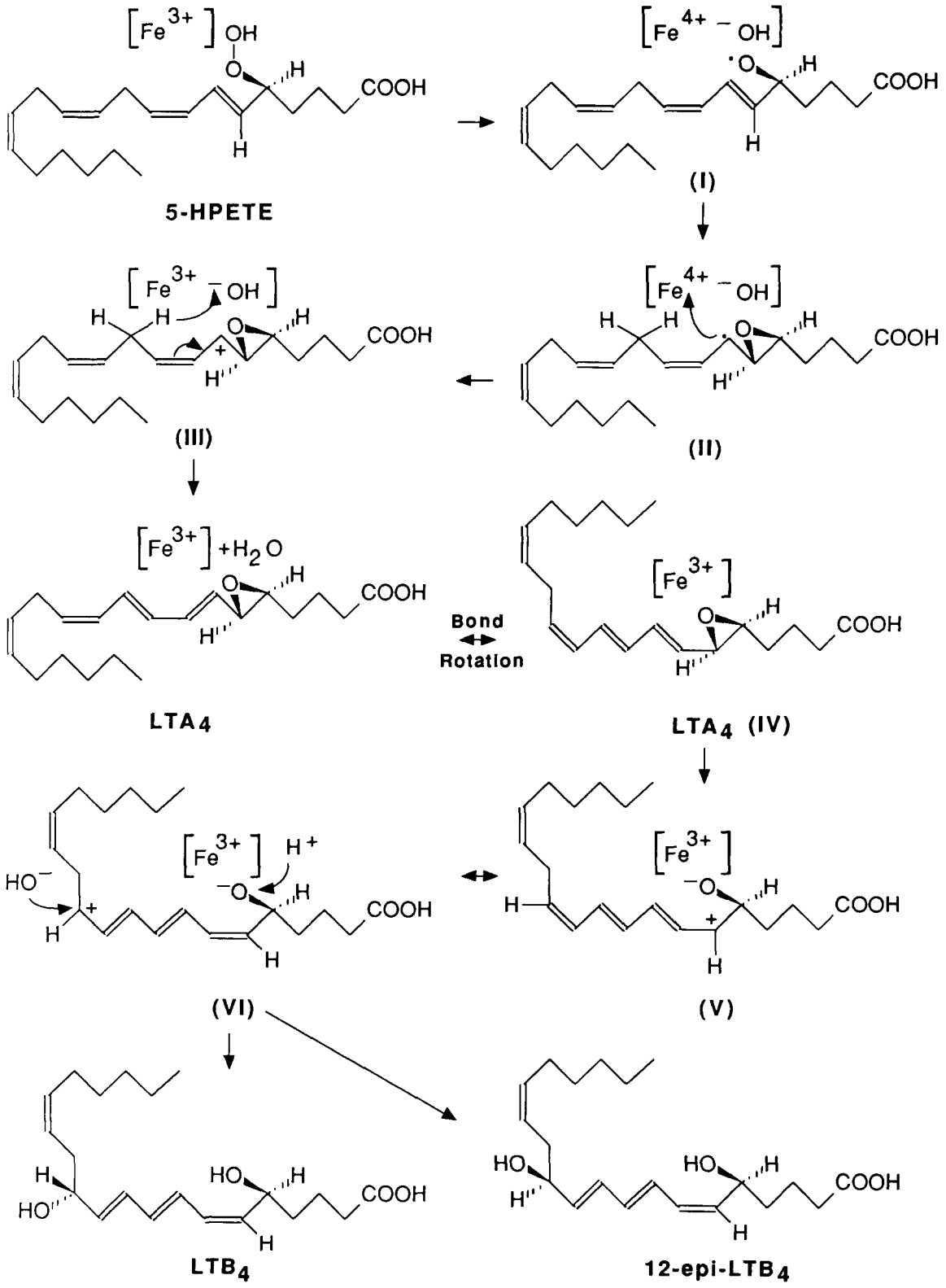

Fig. 1. Hypothetical reaction mechanisms of LTB_4-formation from 5-HPETE. Ferric iron associates with the oxygen-oxygen bond of 5-HPETE to cause homolytic scission and the formation of a ferryl-hydroxo complex and an alkoxyl radical (I); the alkoxyl radical forms a 5,6-trans-epoxide with a carbon-centered radical at C7 (II); the radical electron is oxidized by the ferryl-hydroxo complex to yield a carbonium ion at C7 (III); this carbonium ion loses a proton from the allylic C10 position to yield LTA_4. Hydrolysis of the epoxide in this conformer would yield 6-trans-LTB_4 and 6-trans-12-epi-LTB_4. However, rotation about the single bonds of LTA_4 would yield one of a limited set of conformers in which formation of a 6-cis-double bond would be favored (IV). If hydrolysis of the epoxide is catalyzed by ferric iron while the LTA_4 is in conformer (IV), a carbonium ion would result that could be considered to have a positive charge delocalized between C6 (V) and C12 (VI). Attack of a hydroxide ion at C-12 from below or above the plane of the molecule would yield LTB_4 or 12-epi-LTB_4, respectively.

Table 1. Inhibition of 5-HPETE Conversion to LTB_4

Inhibitor[a]		% Residual Activity: All-trans-LTB_4	% Residual Activity: LTB_4	
BHT	1 mM	102.1 ± 4.8	99.4 ± 2.3	(n = 2)
Potassium cyanide	2.5 mM	87.0 ± 14.0	79.4 ± 10.2[c]	(n = 3)
NDGA	100 μM	90.2 ± 4.8	89.4 ± 1.2	(n = 2)
Metyrapone	1 mM	85.5 ± 8.0	81.5 ± 12.9	(n = 2)
Desferal	1 mM	103.4 ± 1.2	98.1 ± 2.0	(n = 3)
ETYA	1 mM	99.9 ± 18.2	46.9 ± 12.4[cd]	(n = 3)
8,11,14-5,6-dehydroeicosatetraenoic acid	50 μM	88.1 ± 14.9[c]	57.4 ± 7.8[cd]	(n = 3)
Propanethiol	1 mM	73.4 ± 4.1[c]	51.1 ± 10.1[cd]	(n = 3)
O_2[b]	100%	37.5 ± 9.1[c]	33.8 ± 5.8[c]	(n = 5)
CO[b]	100%	84.2 ± 13.0	94.4 ± 4.3[c]	(n = 3)

Note. Rat hepatocyte homogenates were incubated under an argon atmosphere in presence of 100 μM 5-HPETE for 15 min at room temperature. Extraction of reaction products, separation on HPLC, and quantification of LTB_4 were carried out as described in Methods. The control activity, determined in presence of 0.8 mg protein/400 μl incubation volume and 2 μl vehicle (typically methanol), was 31.0 ± 5.2 ng all-trans-LTB_4 (62.8%) and 11.4 ± 1.8 ng LTB_4 (37.2%) formed per 15 min (n = 8). Inhibitors were added in vehicle from stock solutions which were kept under argon. For each set of inhibitors used, a set of three control incubations was carried out and statistical analysis of inhibition was performed on that particular set of data. Values are mean ± SD and given as percentage of activity remaining in presence of inhibitor. Statistical analysis (two-tailed unpaired t-test) was done with Statview 512+ software on a Macintosh II computer.

[a]Inhibitors and 5-HPETE were added simultaneously, except for 8,11,14-5,6-dehydro-eicosatetraenoic acid, which was added 15 min prior to the substrate.

[b]Argon atmosphere was replaced by 100% o_2 or 100% CO.

[c]Indicates significant difference from control value at the $p < 0.05$ level.

[d]Indicates significant difference from value of all-trans-LTB_4 at the $p < 0.05$ level.

dependent on free iron. Due to the low potency of these inhibitors and the possible complexity of pathways of 5-HPETE decay in hepatocyte homogenates, we have not been able to establish the type of the observed inhibition (i.e., Dixon-plot analysis); however, we were able to recognize a preferential inhibition of LTB_4 formation with respect to all-trans-LTB_4. In fact, except in the presence of O_2 (which inhibited the formation of LTB_4 as well as that of all-trans-LTB_4 from 5-HPETE by 66% and 63%, respectively), the formation of all-trans-LTB_4 proved to be rather insensitive to all the inhibitors employed. In addition, the ratio of 6-trans-LTB_4 to 6-trans-12-epi-LTB_4 was not affected by any of the inhibitors, suggesting that they are formed by a common pathway.

It is noteworthy that oxygen is one of the most potent inhibitors of both LTB_4 and all-trans-LTB_4 formation. A similar strong inhibition by oxygen was observed in the catalysis of LTA_4 to LTB_4.[9] The mechanism of inhibition by oxygen is not clear, but control incubations showed that simple oxidation of substrate or products is not the reason for the observed strong inhibition.

Formation of LTA_4 from 5-HPETE by model systems

It is clear that an enzyme or enzyme complex is responsible for the transformation of 5-HPETE into LTB_4 in hepatocyte homogenates for two reasons. First, LTB_4 is formed with a 6-cis double bond which is essential for biological activity but thermodynamically unfavorable. An acid catalyzed non-enzymatic hydrolysis of either 5-HPETE or LTA_4 would result in the formation of LTB_4 with a 6-trans double bond (i.e., 5,12-dihydroxy-6,8,10-trans-14-cis-eicosatetraenoic acid) since the all-trans conjugated triene system forms the thermodynamically most stable transition state.[26] Second, the formation of LTB_4 was completely abolished when the incubations were performed with heat-inactivated or trypsin-treated rat hepatocyte homogenates. However, in keeping with observations made in the presence of inhibitors (Table 1), the formation of all-trans-LTB_4 proved rather insensitive to heat or trypsin inactivation (Table 2). Furthermore, 5-HPETE can be transformed to all-trans-LTB_4, but not to LTB_4, by cytochrome c (2 μM), partially purified bovine catalase, and hematin (5 μM, even after heat-treatment at 80°C, 30 min). Similarly, when 100 μM 5-HPETE was incubated in the presence of 200 μM Fe^{3+}/Fe^{2+} in 0.9% NaCl, pH 7.0, small amounts of all-trans-LTB_4 were formed. The presence of the iron-chelating agent Desferal (1 mM), however, did not lower the amount of all-trans-LTB_4 formed in hepatocyte homogenates, excluding free iron as a major catalyst of that transformation. In contrast to hematin, protoporphyrin IX, which lacks the central ferric iron, does not support 5-HPETE transformation to all-trans-LTB_4. Taken together, these findings seem to indicate that a complexed iron (probably a heme center, although not in its ferrous state as discussed above) plays a crucial role in the transformation of 5-

Table 2. Comparison of Enzymatic/Nonenzymatic Formation of All-trans-LTB_4 and LTB_4 from 5-HPETE by Hepatocyte Homogenates and Other Catalysts

System	All-trans-LTB_4		LTB_4	
	native	heat-denatured[a]	native	heat-denatured[a]
Hepatocyte homogenate	31.0	21.7 (70%)[a]	11.4	—
Cytochrome c (2 μM)	87.0	21.4 (82%)[a]	—	—
Hematin (5 μM)	43.1	34.4 (80%)[a]	—	—
Catalase (17250 U)	67.5	n.m.	[b]	n.m.
Protoporphyrin IX (5 μM)	—	n.m.	—	n.m.

Note. Rat hepatocyte homogenates as well as all other catalysts were incubated under an argon atmosphere in presence of 100 μM 5-HPETE for 15 min at room temperature. Incubation in 10 mM potassium phosphate buffer, pH 8.5, extraction of products, separation on HPLC and quantitation of all-trans LTB_4 and LTB_4 were carried out as described in Methods. Figures are means of at least three experiments and represent ng product obtained per 0.8 mg protein in 400 μl incubation volume. Proteins were heat-denatured at 80°C for 30 min.

[a]Figures in parentheses indicate % residual activity.

[b]Traces of LTB_4 could be detected; however, the ratio of all-trans-LTB_4/LTB_4 was > 6.0 (see Table 3 for comparison).

—Indicates a lack of formation of the respective reaction products (detection limit on HPLC <2 ng).

n.m.—Not measured.

HPETE into a common intermediate such as LTA_4 and that this intermediate subsequently gives rise to LTB_4 and all-trans-LTB_4 by separate pathways. Furthermore, apart from a suitable catalytic site, no further stringent requirements with respect to a putative substrate binding site seem to be necessary for the initial formation of the LTA_4-like intermediate from 5-HPETE, since the formation of this intermediate is insensitive to protein denaturing conditions as shown by the subsequent production of all-trans-LTB_4.

The series of incubations performed in the presence of the agents in Table 1 and the studies of the ability of model systems to form all-trans-LTB_4 shown in Table 2 provide some information about the nature of the enzyme or enzyme complex and the catalytic step involved in 5-HPETE transformation in hepatocyte homogenates. The differential inhibition of the formation of LTB_4 and all-trans-LTB_4 would be consistent with a mechanism involving two reaction steps which divide after formation of a common intermediate. For example, if only formation of LTB_4 requires constraint of a rotational conformer on the enzyme surface (Fig. 2), then after 5-HPETE transformation is initiated on the enzyme, part of the reaction intermediate could leave the enzyme surface (in the form of LTA_4) and, upon acidic hydrolysis, yield all-trans-LTB_4. The second part could remain attached to the active site in the correct conformation to form LTB_4. This second part of the reaction would then be susceptible to inhibition by substances that bind near the catalytic site. Alternatively, catalysis could be initiated before 5-HPETE is tightly bound to the enzyme surface; a portion of the reaction intermediate (i.e., LTA_4) could then bind to the enzyme catalytic site in a conformation that leads to formation of LTB_4 during bond migration, and the remainder could form all-trans-LTB_4 without binding to the surface. The formation of the former product would be inhibited by compounds that bind to the catalytic surface of the enzyme while the formation of the latter product would remain almost unaffected by inhibitors.

Subcellular localization of 5-HPETE transforming activity

Our experimental findings suggest that formation of LTB_4 from 5-HPETE depends on a heme-containing enzyme. In fact, this laboratory has previously reported that reconstituted rabbit liver cytochrome $P450_{LM2}$ was associated with formation from 5-HPETE of a product with UV absorption and HPLC retention time similar to that of LTB_4.[6] However, the 5-HPETE transforming activity (per mg protein) in the supernatant of the hepatocyte homogenates is much greater than in the reconstituted cytochrome P450 vesicles. In addition, as shown in Table 3, the bulk of the LTB_4-forming activity in rat hepatocyte homogenates does not behave like a normal endoplasmic reticulum-bound protein. Approximately 20% of the activity is found in the cytosolic fraction obtained after a high speed centrifugation (105000 × g) of rat hepatocyte homogenates prepared in either 155 mM KCl at pH 7.4 or 10 mM potassium phosphate buffer at pH 8.5. In contrast, when homogenization is carried out in either 100 mM potassium pyrophosphate at pH 8.2 (in presence or absence of 1 mM EGTA) or 155 mM KCl and 1 mM EGTA at pH 7.4, approximately 70% of the activity is in the cytosolic fraction. Ca^{2+} might play a crucial role in the subcellular distribution of this enzyme. Therefore, it is unlikely that the enzyme with the greatest LTB_4-forming activity is a normal endoplasmic reticulum-bound cytochrome P450. These properties appear

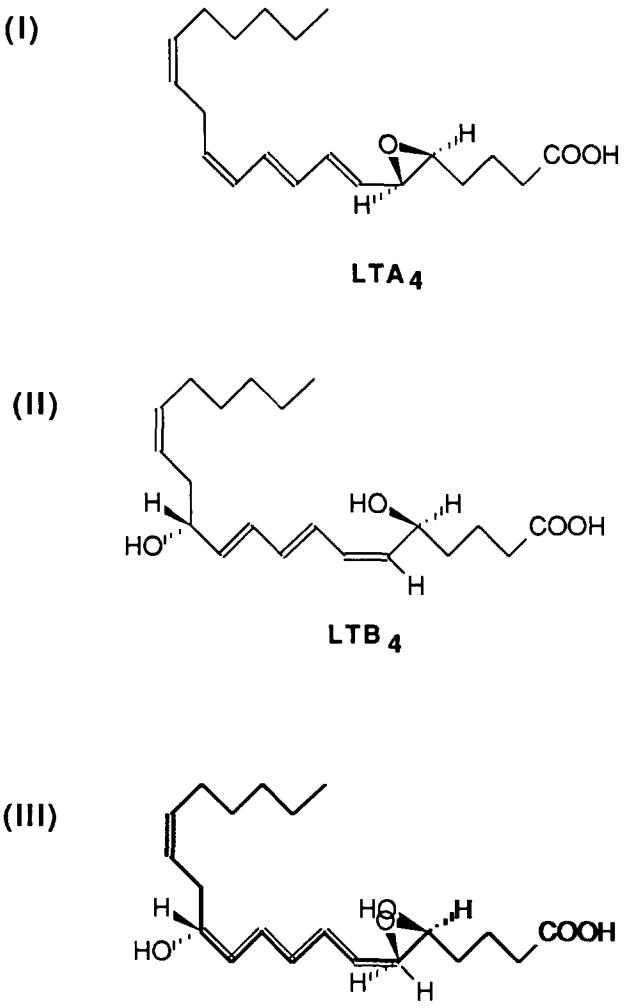

Fig. 2. Molecular modeling of conformers of LTA_4 and LTB_4 by XICAMM. A fragment of LTA_4 consisting of C5 to C12 was entered into the molecular modeling program XICAMM. Rotations about the single bonds of the fragment were performed to search for rotational conformers that might allow conversion into LTB_4 without motion or rotation of the carbon skeleton. In the example (I) carbon atoms C5 to C8 were defined to lie in the same plane. A similar fragment of LTB_4 was then constructed (II) and both conformers were modeled to have correct bond angles and bond lengths as well as to minimize steric strain. Then the two modeled structures were superimposed (III) to see if it would be possible to effect catalysis of the epoxide of (I) while retaining the positions of C5 to C8 and directing the formation of a 6-cis double bond in (II). The actual structures shown were drawn with the Chemintosh drawing program from those produced by XICAMM.

similar to those reported for human leukocyte 5-lipoxygenase[27] and might point to structural similarities of these enzymes.

Proposed reaction mechanism of 5-HPETE transformation into LTA_4

A number of hydroperoxide decompositions have been shown to be catalyzed by Fe^{2+}-cysteine, protic acids, or Lewis acids. They result in cleavage of the —O—O— bond to form allylic epoxides that are subsequently hydrolyzed to conjugated diols, as best described for 13—OOH—18:2.[28] Hematin (hydroxo-(phorphyrinato)iron(III)), the prosthetic group of several hydroperoxide-metabolizing enzymes, has been shown to catalyze a similar hydrolysis of 13—OOH—18:2, although the source of the hydroxyl oxygen and the stereochemistry of the hematin-catalyzed reaction are different from the acid-catalyzed reaction.[29] A reaction sequence similar to that of the hematin-catalyzed decay of 13—OOH—18:2 could also be involved in the transformation of 5-HPETE to LTA_4, the first half of the reaction sequence that eventually yields all-trans-LTB_4 and LTB_4. The mechanism we propose for LTA_4 formation by a putative ferric heme enzyme in rat hepatocyte homogenates (as well as in heme-containing model systems) is shown in Figure 1. The first step is a one-electron reduction of 5-HPETE by the central ferric iron to yield an alkoxyl radical (I) and a ferryl-hydroxo complex, a rather strong oxidant. The direct formation of this ferryl-hydroxo complex as a consequence of Fe^{3+}-porphyrin-peroxide interaction has been suggested by a number of investigators. For example, Cadenas proposed this intermediate in the decomposition of tert-butyl-hydroperoxide by ferricytochrome c[30]. In addition, White and coworkers[31] and Blake and Coon[32] have suggested that cytochrome P450 reduces organic hydroperoxides by a similar mechanism involving the ferryl-hydroxo complex. The formation of a stable carbon-oxygen bond results in generation of the 5,6-epoxide-allylic carbonyl radical intermediate (Fig. 1, II). A one-electron oxidation of the allylic carbonyl radical at C-7 of the carbon chain gives rise to the proposed carbonium ion (Fig. 1, III). A likely oxidant that could accomplish this step is the ferryl-hydroxo complex. Loss of a proton from the carbonium ion III and migration of the pi-electrons in a caged reaction sequence would give rise to the conjugated triene system of LTA_4. This proton could react with the hydroxo complex to form H_2O and restore the catalytic ferric iron center.

Formation of LTB_4 from LTA_4

Since a substantial fraction of either the acid- or metal-catalyzed decomposition of 5-HPETE would pass through a transition state equivalent to the 5,6-trans-oxido-7,9-trans-11,14-cis structure of LTA_4 (Fig. 1, IV-VI), the key question is how a putative enzyme could cause a fraction of the LTA_4 intermediate to assume the 6-cis structure essential for activity in LTB_4. In this paper we propose that there is a particular low energy conformation of LTA_4 that will result in formation of the required 6-cis double bond if the C5 to

Table 3. Subcellular Localization of 5-HPETE Transforming Activity in Rat Hepatocytes

Buffer system	Cytosol % Activity	105000 × g pellet % Activity
1.15% KCl, pH 7.4	22	78
10 mM KPi, pH 8.5, 20% (v/v) glycerol	19	81
100 mM potassium pyrophosphate, pH 8.2	77	23
1.15% KCl, 1 mM EGTA, pH 7.4[a]	66	34
100 mM potassium pyrophosphate, pH 8.2, 1 mM EGTA[a]	63	37

Note. Rat hepatocytes were homogenized in the indicated buffer systems and both a cytosolic fraction and a pellet were obtained after centrifugation at 105000 × g. The pellet was washed twice and resuspended in the respective buffer. Incubations (0.8 mg protein/400 μl incubation volume) were performed in the respective buffer systems in presence of 100 μM 5-HPETE for 15 min at room temperature under an argon atmosphere. All buffers contained 1 mM EDTA and 1 mM DTT. The formation of LTB_4 was measured as described in Methods. The values are means of two experiments and are given as percentage of activity obtained with subcellular fractions as compared to the total activity of a preparation obtained with the corresponding hepatocyte homogenate. The ratio of all-trans-LTB_4 to LTB_4 in hepatocyte homogenates was 2.2 ± 0.15 (n = 7), irrespective of buffer systems used for homogenization, and this ratio was not significantly different in any of the cytosol or pellet fractions.
[a]Single determination.

C8 carbon atoms of the fatty acid chain are restricted in movement during the opening of the epoxide ring and the migration of the double bonds. It is unlikely that a nonspecific binding site on a heme group would be capable of inducing any rotation or distortion of the carbon chain of LTA_4 in order to force the transition state into the essential, but thermodynamically less stable, 6-cis-8,10-trans-triene conformation. The suggestion that the enzyme does not input energy into the molecule is based on our finding that the conversion of 5-HPETE or LTA_4 to LTB_4 does not require NADPH, ATP, or oxygen.[9,15] We propose that these conditions can be met with the single constraint that the C5 to C8 carbon atoms do not move appreciably during the transition state of the hydrolysis. Otherwise, if the carbon atoms of the fatty acid chain were not constrained during the opening of the epoxide ring and migration of the double bonds, the thermodynamically most stable 6,8,10-trans-triene would be formed.

It was therefore necessary to search for a conformation of LTA_4 that would result in formation of the required 6-cis double bond without any specific rotation or input of energy by the enzyme during the transition state. We have used the computer molecular modeling program XICAMM (XIRIS Corporation, Middletown, NY), which uses a classical molecular mechanics algorithm, to search for pairs of low energy conformations of LTA_4 and LTB_4 in which the C5 to C8 segments of the fatty acid chain could be overlaid. These conformers were sought by making suitable rotations about the 4–5, 6–7, and 8–9 single bonds of LTA_4 (Fig. 1, IV), modeling these conformers into a low energy conformation with XICAMM, then testing whether it was possible to make suitable rotations about the 4–5, 5–6, and 7–8 single bonds of LTB_4 that, when also modeled into a low energy conformation, could be overlaid with that of the LTA_4 conformer. This type of analysis revealed a pair of conformers of LTA_4 and LTB_4 in which all carbon atoms C5 to C8 lie in a plane and which fit the criteria that they could be held in place during the opening of the epoxide ring and the migration of the double bonds (Fig. 2, I and II). When these two conformers are individually put in their lowest energy configuration with respect to bond length, bond angle, and steric hindrance by the XICAMM program, they are nearly superimposable (Fig. 2, III). Although other pairs of conformers with more complex shapes were found, the pair shown in Fig. 2, I and II, was selected for display because all C5 to C8 carbon atoms lie in a plane, an appropriate starting conformation for placing LTA_4 on a binding site of an enzyme. Moreover, the planar arrangement of carbon atoms C5 to C8 is desirable for migration of the pi-electrons of the conjugated triene system from the 7,9,11-triene of LTA_4 to the 6,8,10-triene system of LTB_4. The excellent superposition of the low energy conformers of LTA_4 and LTB_4 as shown in Figure 2, III, demonstrates that the hydrolysis would result in formation of the required 6-cis-8,10-trans-triene conformation rather than the biologically less active 6,8,10-trans-triene conformation.

A functionally equivalent formation of LTB_4 would also occur if the binding site did not hold atoms C5 to C8, but rather selected for particular conformations of LTA_4 (such as IV in Fig. 1 and I in Fig. 2) and allowed the epoxide oxygen of only those conformations to approach the central ferric iron. As soon as a delocalized carbonium ion is created, the conformation would be locked in position and migration of the pi-bonds would occur faster than reorientation of C5 to C8 into a 6-trans conformation could occur,[33] thus forming the 6-cis double bond.

The approximately 2% yield of total LTB isomers from 5-HPETE and the observed ratio of 37% LTB_4

and 63% all-trans-LTB_4 formed could result from a combination of several factors: i) binding and catalysis of non-superimposable conformers of LTA_4; ii) weak binding of carbon atoms C5 to C8 that allows some formation of the thermodynamically favored 6-trans double bond of all-trans-LTB_4; iii) weak selectivity of those conformers that are allowed to approach the binding site; and iv) the presence of a mixture of isozymes that catalyze the transformation.

The binding affinities of the LTB_4 generated upon hydrolysis of 5-HPETE or LTA_4 in the radioimmunoassay for LTB_4, the receptor displacement assay for LTB_4 on human neutrophils, and the bioassay measuring the transient increase of cytosolic calcium in human neutrophils upon exposure to LTB_4[9,15] were only half of those expected based on the extinction coefficient of synthetic LTB_4. In that the 12(S) isomer of LTB_4 is biologically much less active than the 12(R) isomer, the binding results are consistent with the suggestion that the attack by OH^- at the C12 position is not stereochemically controlled (Fig. 1, VI), therefore yielding an epimeric mixture of 5(S),12(R)-LTB_4 and the less active 5(S),12(S)-LTB_4.

SUMMARY

The results cited above support the hypothesis that a loosely membrane-associated enzyme is involved in the transformation of 5-HPETE to biologically fully active LTB_4 in rat hepatocyte homogenates. This protein or protein complex has properties distinct from those of the previously described LTA_4-synthetase or LTA_4-hydrolase. Previous studies have confirmed that an epimeric mixture of 5(S),12(R)-LTB_4 and 5(S),12(S)-LTB_4 is obtained in incubations of 5-HPETE with hepatocyte homogenates (unlike the sole product 5(S),12(R)-LTB_4 formed by LTA_4-hydrolase in macrophages). These results suggest that attack by the hydroxyl group at carbon C12 of the reaction intermediate is not sterically controlled. Furthermore, we have found that heme groups such as those found in cytochrome *c*, catalase or hematin (but not iron-free protoporphyrin IX) could catalyze the formation of all-trans-LTB_4 but not that of LTB_4. The collective evidence from these data allowed us to formulate a hypothesis of the reaction mechanism and the minimal requirements of the enzyme transforming 5-HPETE to LTB_4 in rat hepatocyte homogenates. We propose that a central ferryl-hydroxo complex is essential in the conversion of 5-HPETE to the LTA_4 intermediate. The substrate binding site should be capable of selecting a particular conformation of the carbon atoms C5 to C8 of the transition state LTA_4, thereby facilitating the transition from the 5,6-trans-oxido form of LTA_4 to the 6-cis-8,10-trans-triene form of biologically active LTB_4.

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ABBREVIATIONS

5-HPETE—5(S)-hydroperoxyeicosatetraenoic acid
BHT—Butylated hydroxytoluene
Desferal—*N*-[[5-(N-hydroxyacetamido)-pentyl]carbamoyl]propionohydroxamic acid monomethanesulfonate
ETYA—5,8,11,14-eicosatetraynoic acid
LTA_4—5(S)-5,6-oxido-7,9-trans-11,14-cis-eicosatetraenoic acid
LTB_4—5(S),12(R)-dihydroxy-6,14-cis-8,10-trans-eicosatetraenoic acid
LTC_4—Leukotriene C4
LTD_4—Leukotriene D4
LTE_4—Leukotriene E4
NDGA—Nordihydroguaiaretic acid
RIA—Radioimmunoassay