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ACCELERATED COMMUNICATION

Conversion of 5-Hydroperoxyeicosatetraenoic Acid into Leukotriene B_4 by Rat Hepatocytes: A Novel Cellular Source for Leukotriene B_4

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SUMMARY

Rat hepatocyte homogenates converted 5-hydroperoxyeicosatetraenoic acid into leukotriene B_4 (LTB_4). The reaction was dependent on time and protein and substrate concentration, did not require NADPH or oxygen, and was not supported by heat-inactivated hepatocyte homogenates. The authenticity of the biologically generated LTB_4 that eluted at the position of synthetic LTB_4 during high performance liquid chromatography was established by UV spectrophotometry, mass spectral analysis, radioimmunoassay, and a LTB_4 receptor displacement assay. In addition, a leukotriene bioassay is described in which transient increases in cytosolic Ca^{2+} within human neutrophils are measured by means of fura-2 fluorescence. Biologically generated LTB_4 was 40, 40, and 33% as active as synthetic LTB_4 in the radioimmunoassay, receptor displacement assay, and cytosolic calcium bioassay, respectively. This activity is consistent with the biologically derived LTB_4 being an epimeric mixture of (5*S*),(12*R*)-LTB_4 and the much less active (5*S*),(12*S*)-LTB_4. The formation of LTB_4 was inhibited by 5,8,11,14-eicosatetraynoic acid (1 mM), 5,6-dehydro-arachidonic acid (50 μM), propanethiol (1 mM), and 0_2 (100%) to the extent of 53, 42, 48, and 66%, respectively. No inhibition was observed in the presence of diethylcarbamazine (1 mM) and desferal (1 mM). A possible contribution towards LTB_4 formation by contaminating Kupffer cells was excluded (< 0.2%). These results suggest that hepatocytes can convert lipid peroxides into potent chemoattractants that may alter the homeostasis of immunomediators within the liver.

Leukotrienes are a group of endogenous mediators active in hypersensitivity reactions and inflammation. 5-HPETE, a lipoxygenase product of arachidonic acid, is the direct precursor of LTA_4, the key intermediate in leukotriene biosynthesis (1). In the presence of glutathione, glutathione-*S*-transferase converts LTA_4 into LTC_4, which, after sequential peptid/olysis, gives rise to LTD_4 and LTE_4. These peptido-leukotrienes are potent vasoactive mediators and are increasingly implicated in the bidirectional communication between the immune system and the central nervous system (2). Hydrolysis of LTA_4 by LTA_4-hydrolases 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, as well as regulation of immune responses (3). Depending on the particular cell type it interacts with, LTB_4 can induce suppressor cell activity, stimulate lymphocyte proliferation, induce monokine production (i.e., interleukin-1 and tumor necrosis factor-α), regulate cytotoxic activities of lymphocytes and monocytes, and act as a calcium ionophore (4–6).

Few reports describe the biosynthesis of chemotactic factors of the leukotriene family by other than specialized peripheral cells (i.e., macrophages, neutrophils, mast cells, basophils). Preliminary experiments in this laboratory (7) indicated that rabbit cytochrome $P\text{-}450_{LM2}$ reconstituted into phospholipid vesicles could catalyze the conversion of 5-HPETE to a product with UV absorption and HPLC retention time similar to that of LTB_4. In addition, formation of the peptido-leukotrienes and LTB_4 from LTA_4 was recently measured in cytosolic frac-

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ABBREVIATIONS: 5-HPETE, 5-hydroperoxy-6,8,11,14-eicosatetraenoic 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 C_4; LTD_4, leukotriene D_4; LTE_4, leukotriene E_4; RP HPLC, reversed phase high performance liquid chromatography; RIA, radioimmunoassay; desferal, *N*-[[5-(*N*-hydroxyacetamido)-pent)]carbamoyl]propionohydroxamic acid monomethanesulfonate; NPC, nonparenchymal cells; ETYA, 5,8,11,14-eicosatetraynoic acid; 5-HETE, (5*S*)-hydroxy-6-*trans*-8,11,14-*CIS*-eicosatetraenoic acid; PC, parenchymal cells; EGTA, [ethylenebis(oxyethylenenitrilo)] tetraacetic acid.

tions obtained from whole livers of both guinea pig and rat (8, 9). 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 those of LTB_4 (10), indicating a possible alternative pathway in hepatocytes for generation of leukotrienes and/or their precursor molecules (i.e., 5-HPETE, LTA_4).

Leukotrienes have been implicated in the pathophysiology of hepatotoxicity. The possible interplay of distinct hepatic cell types that could produce leukotrienes led us to investigate the role of rat hepatocytes and Kupffer cells in the transformation of arachidonic acid-derived lipid hydroperoxides (particularly 5-HPETE, which *in situ* might arise intra- or trans-cellularly by means of lipoxygenase action or via lipid peroxidation), into endogenous mediators of the leukotriene family. This laboratory has very recently demonstrated that the epoxide intermediate LTA_4 is a substrate for both cytosolic and microsomal LTA_4 hydrolase activity of rat and human liver. LTA_4 hydrolysis gives rise, among other products, to the formation of biologically fully active LTB_4 (11). Maximal formation rates were achieved under an argon atmosphere; molecular oxygen inhibited LTB_4 formation by about 60%. We have also shown that the extent of metabolism of LTB_4 to biologically less potent mediators is strongly dependent on the oxygen concentration experienced by cultured rat hepatocytes (12). Extending this line of investigations, we here report that homogenates of isolated rat hepatocytes enzymatically transform 5-HPETE into biologically active LTB_4, as demonstrated by HPLC, UV spectrophotometry, stereospecific receptor displacement, and a bioassay measuring transient increases in the cytosolic calcium concentrations in human neutrophils. The conversion of 5-HPETE into LTB_4 is strongly inhibited by molecular oxygen and, to a lesser extent, by classical inhibitors of leukotriene biosynthetic pathways. The observed activity can not be accounted for by the presence of residual Kupffer cells.

Experimental Procedures

Preparation of rat hepatocytes and Kupffer cells. Single cell suspensions of hepatocytes and Kupffer cells were prepared from fed adolescent male Sprague-Dawley rats by *in situ* perfusion of collagenase as described (13). Separation of liver PC and NPC was performed as described by Smedsrod and Pertoft (14). Briefly, cell suspensions were filtered through nylon mesh to remove cellular aggregates and cell debris. The filtrate was then centrifuged for 4 min at 50 × *g*, yielding a pellet enriched in PC and a supernatant fraction enriched in NPC. The pellet, containing mostly hepatocytes, was then washed another four times in Earle's salts, pH 7.4, and diluted aliquots (25 ml) of resuspended hepatocytes were immediately 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. Only viable hepatocytes (as measured by trypan blue exclusion) penetrated the Percoll layer and were recovered for use in the subsequent experiments. The supernatant fraction, containing mainly NPC, was recentrifuged twice at 50 × *g* for 4 min and aliquots were layered on top of a preformed discontinuous Percoll gradient, of which the bottom cushion had a density of 1.066 g/ml and the top layer had a density of 1.037 g/ml. The gradients were centrifuged at 800 × *g* for 15 min at 4°. A fraction, highly enriched in Kupffer cells, was dispersed in the 1.066 g/ml density band and separated from pelleted hepatocytes and erythrocytes as well as from liver endothelial cells, which concentrated at the interface of the two density layers, and cell debris, which did not penetrate the Percoll layer. Kupffer cells were collected, diluted with equal volumes of iso-Hanks solution, and recentrifuged at 800 × *g* for 10 min. For a final purification step, the pellets of Kupffer cells were resuspended in Earle's balanced salt solution, and aliquots of cell suspensions were allowed to attach to Lux Permanox Contur (Miles, Kankakee, IL) culture dishes for 30 min at 37° in 5% CO_2/95% air. The attached cells were washed twice and culture was continued overnight in M199 plating medium as described (13), with the exception that dexamethasone was omitted. After 24-hr, the cells were harvested and used in the subsequent experiments.

Microscopy was done with a Nikon Diaphot inverted phase contrast microscope. Cell numbers in solutions were estimated by microscopy using a hemocytometer. Attached cells were counted by observing four fields of approximately 200 cells on two individuals dishes at 200 × magnification. Kupffer cells were identified by peroxidase staining using 3,3-diaminobenzidine tetrahydrochloride as a substrate (15).

Preparation of cell homogenates. Hepatocytes or Kupffer cells were pelleted, transferred into ice-cold homogenization buffer containing 10 mM potassium phosphate, pH 8.5, 20% (v/v) glycerol, and 50 μg/ml L-α-dilauroyl-phosphatidylcholine, disrupted by four stokes in a Potter Elvehjem homogenizer at 4° followed by sonication for 10 min in a Branson bath type sonifier in an ice-water bath. The homogenates were stored in aliquots at −20° and thawed only once, immediately before the experiment.

Incubation with 5-HPETE and HPLC analysis of product formation. Under standard conditions, aliquots of both hepatocytes and Kupffer cell preparations were brought to 200 μl 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 10 μl. The incubations were continued at room temperature under argon for the specified time. Incubations were stopped by addition of 10 μl of 1 N formic acid, followed by 400 μl of methanol/water (1:1, v/v), containing 25 ng/400 μl of prostaglandin B_1 as an internal standard. Samples were extracted with 5 ml of dichloromethane, washed twice with 1 ml of water, taken to dryness under nitrogen, resuspended in 100 μl of eluent, and injected onto the HPLC column. Isocratic product separation was achieved on an Ultrasphere ODS HPLC column (C-18, 0.46 × 25 cm, 5 μm; Beckman, Fullerton, CA; operated with a guard column) in methanol/water/acetic acid (72:28:0.02, v/v) adjusted to pH 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; losses during sample preparation were corrected for and the amounts of LTB_4 generated were determined by external standardization with synthetic LTB_4. Recovery of prostaglandin B_1 was typically 91 ± 4% (seven experiments) and that of LTB_4 93 ± 3% (six experiments). Retention times were 9.5 min ± 0.3 min (eight determinations) for prostaglandin B_1 and 14.0 min ± 0.2 min (eight determinations) for synthetic LTB_4. Material eluting with the same retention time as synthetic LTB_4 was collected, rechromatographed in the same eluent system, taken to dryness, and, before further structural and biological characterization, transferred into the suitable assay buffers.

Binding of [^{3}H]LTB_4 to human neutrophils. The equilibrium binding of [^{3}H]LTB_4 to human neutrophils in the absence and presence of nonradioactive preparations of LTB_4 was assessed as previously described (16). Neutrophils of 95% or greater purity were prepared from human peripheral blood (16). Neutrophils (1×10^7) were incubated with 0.1 nM [^{3}H]LTB_4 in the absence and presence of different concentrations (0.5–100 nM) of biologically derived LTB_4 for 1 hr in an ice water bath. The total amount of bound radioactivity was determined by filtering the binding suspension on GF/F glass fiber filters (Whatman, Clifton, NJ), washing the filters with several aliquots of ice-cold isotonic saline, and then determining the amount of radioactivity retained by the filters. Nonradioactive LTB_4 was continuously present during the binding incubation. The binding of [^{3}H]LTB_4 in the presence of 1 μM synthetic LTB_4 was defined as nonspecific binding and sub-

tracted from the binding determinations performed at the other LTB_4 concentrations to yield the specific binding. The specific binding in the absence of nonradioactive LTB_4 was used to define the 100% binding level. The IC_{50} was estimated from a Hill plot of the specific binding data.

Bioassay for LTB_4. 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 was compared. Purified peripheral blood neutrophils were suspended at 5×10^6 per ml of Hank's balanced salt solution (without phenol red) containing 10 mM Tris·HCl, 0.1 g/100 ml ovalbumin, pH 7.2 (HBSS-OA) and incubated with 5 μM fura-2/AM, 0.1% diemthylsulfoxide for 20 min at 37°. The fura-2-loaded neutrophils were then washed twice and resuspended in HBSS-OA. The cytosolic calcium concentration was monitored in a Deltascan fluorimeter (Photon Technology International, Inc., Princeton, NJ). Suspensions of 2×10^5 neutrophils/ml of HBSS-OA were incubated for 15 min at 37° to establish the baseline fluorescence. The fluorescence signals obtained at excitation wavelengths of 335 and 380 nm were measured at an emission wavelength of 508 nm for 2 min before and 5 min after the addition of the leukotriene stimulus. The ratio of the fluorescence signals obtained at the two excitation wavelengths was used to calculate the cytosolic calcium concentration according to the formula of Grynkiewicz *et al.* (17). Autofluorescence was determined from a matched sample containing neutrophils without fura-2. A standard solution of 2 μM fura-2, pentapotassium salt, in 100 mM KCl, 10 mM HEPES, 1 mM EGTA, pH 7.05, without and with 10 mM $CaCl_2$ was used to determine the signal ratios for fura-2 in the absence and presence of saturing calcium, respectively. A dissociation constant of 220 nM was used for calcium ion binding to fura-2. The difference between the maximal concentration of cytosolic calcium achieved after the addition of stimulus and the baseline concentration was determined for each dose of LTB_4. Concentrations of synthetic LTB_4 greater than 1 nM yielded maximal increases in cytosolic calcium. The concentration of LTB_4 that gave an increase in cytosolic calcium that was half of the increase observed in response to 1 nM LTB_4 was determined by interpolation from the dose/response curve and defined as the EC_{50}.

Other methods. Mass spectra of biologically generated LTB_4 were obtained by direct chemical ionization MS as previously described (18). 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 5985 A quadrupole mass spectrometer. [^{3}H]LTB_4 RIAs were performed according to the manufacturers guidelines. The cross-reactivity (at 50% B/B_0 replacement) of the LTB_4 antiserum used 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 LTD_4. Protein concentrations were estimated by the Bio-Rad assay procedure using bovine serum albumin as a standard.

Materials. 5-HPETE was generously provided by Dr. C. C. Reddy, Pennsylvania State University. Synthetic LTB_4 was a gift by Dr. J. Rokach of Merck-Frosst Canada. [5,6,8,9,11,12,14,15,(*N*)-^{3}H]LTB_4 (180–220 Ci/nmol) and [^{3}H]LTB_4 RIA kits were obtained from Amersham Corp. (Arlington Heights, IL). ETYA and 5,6-dehydro-arachidonic acid were purchased from Cayman Chemicals (Ann Arbor, MI). Prostaglandin B_1 and bovine serum albumin, fraction V, were from Sigma Chemical Co. (St. Louis, MO). Fura-2/AM and the pentapotassium salt of fura-2 (Molecular Probes, Eugene, OR), ovalbumin, and Hank's balanced salt solution without phenol red (Sigma) were obtained from the designated suppliers. HPLC-grade solvents were purchased from Baker Chemical Company (Phillipsburg, NJ). All HPLC eluents were prepared daily and sparged with helium throughout. All glassware was silanized before use.

Results

LTB_4 formation by rat hepatocyte homogenates. When acidified dichloromethane extracts of rat hepatocyte incubations with 5-HPETE were analyzed on RP HPLC, three prominent UV-absorbing peaks (280 nm) were reproducibly detected (Fig. 1). Peaks I and II co-chromatographed with and exhibited identical retention times as Δ^6-*trans*-LTB_4 and Δ^6-*trans*-12-epi-LTB_4, respectively, corresponding to products formed in model systems upon nonenzymatic mild acid hydrolysis of synthetic LTA_4 free acid (19). Thus, the presence of peak I and peak II in the chromatograms of organic extracts of incubations of rat hepatocyte homogenates with 5-HPETE suggests that LTA_4 is an intermediate in the conversion process. Peak III co-eluted with synthetic LTB_4 and, after rechromatography, showed an UV-absorption spectrum identical to that of synthetic LTB_4 (i.e., relative absorption maxima at 261, 270, and 280 nm, directly measured in the HPLC eluent comprising methanol/water/acetic acid, ((72:28:0.02, v/v, adjusted to pH 5.7 with triethylamine) indicating the presence of a conjugated triene moiety (spectrum not shown). When reacted with ethereal diazomethane, the methyl ester of peak III eluted as a homogeneous peak with the same retention time as the methyl ester of synthetic LTB_4. The formation of peak III was completely abolished when the incubations were done with either heat-inactivated (80°, 30 min) or trypsin-treated [36.4 units trypsin (bovine pancreas) for 15 min at 37°] rat hepatocyte

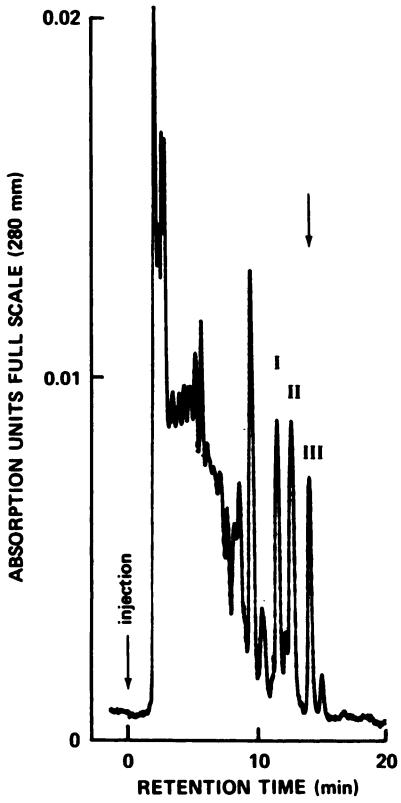

Fig. 1. RP HPLC profile of dichloromethane extracts of 5-HPETE incubations with rat hepatocyte homogenates. The analyses were performed at room temperature on an Ultrasphere ODS column (C-18, 0.46 × 25 cm, 5 μm) in methanol/water/acetic acid (72:28:0.02, v/v), adjusted to pH 5.7 with triethylamine, at 1.0 ml/min and absorbance changes were recorded at 280 nm. The *arrow* denotes the elution position of synthetic LTB_4. Peaks I and II coeluted with Δ^6-*trans*-LTB_4 and Δ^6-*trans*-12-epi-LTB_4, respectively. Material under peak III was collected, rechromatographed, and used for further characterization; the concentration of LTB_4 was estimated with UV spectrophotometry using an E_{max} of 51,000 (44). The peak eluting with a retention time of 9.5 min is prostaglandin B_1 which was used as an internal standard.

homogenates (data not shown). However, the formation of peaks I and II did not cease under these conditions. As compared with control incubations, approximately 70% residual all-*trans*-LTB$_4$ (i.e., Δ^6-*trans*-LTB$_4$ and Δ^6-*trans*-12-epi-LTB$_4$) was still detected, pointing to a possible nonenzymatic transformation of 5-HPETE to all *trans*-LTB$_4$. In control experiments, we have found that 5-HPETE can be transformed to all-*trans*-LTB$_4$s but not to LTB$_4$, by both cytochrome c (5 μM) and hematin (5 μM), even after heat treatment (e.g., 80°, 30 min). Similarly, when 100 μM 5-HPETE was incubated in presence of 200 μM Fe^{2+}/Fe^{3+} in 0.9% NaCl, pH 7.0, small amounts of all-*trans*-LTB$_4$ were formed.[1] However, the presence of the iron-chelating agent desferal (1 mM) in hepatocyte homogenates did not lower the amount of nonenzymatically formed all-*trans*-LTB$_4$, excluding free iron as a major catalyst of that transformation.

In a separate set of experiments, we have established that 5-HPETE remains intact throughout the extraction procedure used and does not spontaneously decay to any detectable amounts (limit, <2 ng of LTB$_4$) of all-*trans*-LTB$_4$. Furthermore, 89 ± 4.9% and 79 ± 15% (five experiments) of 5-HPETE could be recovered intact from 15 and 30 min incubations, respectively, with hepatocyte homogenate (0.8 mg of protein per 400 μl) at 100 μM starting substrate concentration. After 30-min incubation, a total of 2.2 ± 0.4% (five experiments) of the consumed substrate were recovered as all-*trans*-LTB$_4$s and LTB$_4$. Therefore, substrate depletion should not be a factor in the enzymic characterization of LTB$_4$ formation, despite the partial instability of 5-HPETE in hepatocyte homogenates. In fact, the formation of LTB$_4$ (i.e., Fig. 1, peak III) was dependent on both time and protein concentration. The formation rates for all-*trans*-LTB$_4$s (i.e., Fig. 1, peak I and II combined) as well as LTB$_4$ were constant up to 30 min incubation time (Fig. 2A) and up to 1.2 mg of protein/200-μl incubation volume (Fig. 2B). Moreover, Δ^6-*trans*-LTB$_4$, Δ^6-*trans*-12 epi-LTB$_4$, and LTB$_4$ were all generated from 5-HPETE in a concentration-dependent manner up to about 100 μM 5-HPETE, whereas inhibition of product formation was observed at 200 μM substrate concentration (Fig. 2C). The double reciprocal analysis of these data in Lineweaver/Burk plots revealed a complex relationship between substrate concentration used and product (i.e., all-*trans* LTB$_4$s or LTB$_4$) formed. This finding is to be expected in the case of studies of the decay of 5-HPETE in cell homogenates rather than with isolated enzymes, when the many possible enzymatic and nonenzymatic pathways of 5-HPETE conversion are taken into account (20). Therefore, because the requirements for Michaelis-Menten type of behavior are not fulfilled, no attempts were made at this point to estimate K_m and V_{max} values for the transformation of 5-HPETE into LTB$_4$ by rat hepatocyte homogenates.

Mass spectral elucidation of peak III. Material collected under peak III (Fig. 1) was rechromatographed and subjected to ammonia direct chemical ionization mass spectral analysis as described previously (18). The newly developed technique allowed measurement of chemical ionization mass spectra without need for conversion of the LTB$_4$ to the methyl ester and silyl ether derivatives. The major mass fragments observed with both synthetic LTB$_4$ and hepatocyte-derived peak III were *m/z* 354 $[M + NH_4]^+$, *m/z* 336 $[M + NH_4 - H_2O]^+$, *m/z* 319 $[M + H - H_2O]^+$, and a base peak of *m/z* 301 $[M + H - 2 H_2O]^+$ (spectra not shown). Furthermore, the ratio of fragment ions *m/z* 354, 336, 319, and 301 in the spectra of synthetic LTB$_4$ and hepatocyte-derived peak III were almost identical.

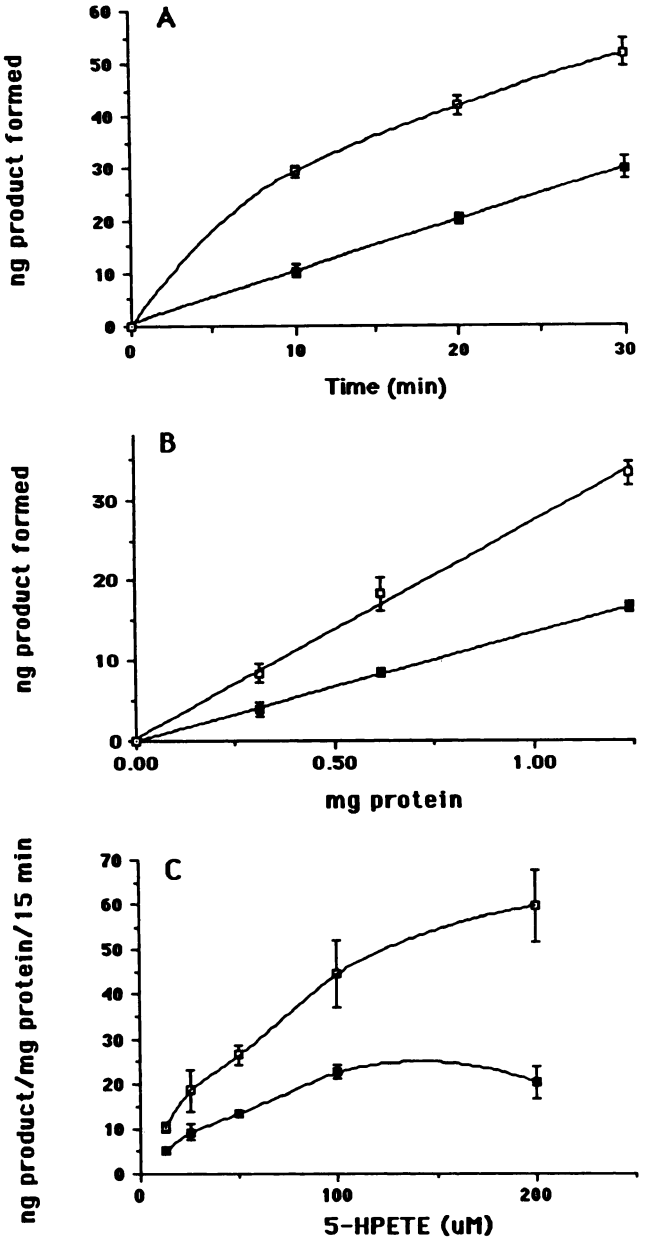

Fig. 2. Enzymatic characterization of the transformation of 5-HPETE into LTB$_4$ by rat hepatocyte homogenates. All incubations were performed at room temperature under an argon atmosphere. A, The time-dependent formation of all *trans*-LTB$_4$ (i.e., Δ^6-*trans*-LTB$_4$ and Δ^6-*trans*-12-epi-LTB$_4$) combined (□) and that of LTB$_4$ (■) by rat hepatocyte homogenates was assayed. Hepatocyte homogenates (0.8 mg of protein/ml in 200-μl final volume) were incubated with 100 μM 5-HPETE for various length of time. Values are means ± standard deviations, three experiments. B, The protein concentration-dependent conversion of 5-HPETE into all-*trans*-LTB$_4$ (□) and LTB$_4$ (■) by rat hepatocyte homogenates was assayed. 5-HPETE concentration and incubation time were 100 μM and 15 min, respectively. Values represent means ± standard deviations, three experiments. C, Rat hepatocyte homogenates (0.8 mg of protein/ml in 400-μl final volume) were incubated with increasing amounts of 5-HPETE. Incubation time was 15 min. The substrate concentration-dependent formation of LTB$_4$ (■) and that of Δ^6-*trans*-LTB$_4$ and Δ^6-*trans*-12-epi-LTB$_4$ combined (□) are shown.

[1] Josef Gut, unpublished observation.

These results suggest that these four major fragment ions all arise from the same molecule and strengthen the identification of the HPLC peak III as LTB_4.

Functional elucidation of peak III. Although two classes of receptors for LTB_4 have been defined by quantifying the binding affinity of [^{3}H]LTB_4 to human neutrophils, the high affinity site has the greatest stereochemical selectivity (21). Here, we compared the functional identity of the material collected under peak III (Fig. 1) with authentic LTB_4 by measuring the inhibitory effect of 0.1–100 nM of this material on the binding of [^{3}H]LTB_4 to the high affinity receptors for LTB_4 on human neutrophils (Fig. 3). Synthetic LTB_4 [i.e., (5*S*),(12*R*)-dihydroxy-6,14-*cis*-8,10-*trans*-eicosatetraenoic acid] displaced [^{3}H]LTB_4 with an inhibitory dissociation constant IC_{50} = 4.0 nM (calculated from Hill plot, three experiments) (Table 1). Material obtained under peak III from incubations of 5-HPETE with rat hepatocyte homogenates revealed an IC_{50} value of 10.0 nM (three experiments) (Table 1) indicating a capacity approximately half that of synthetic LTB_4 to displace [^{3}H]LTB_4 from these high affinity receptors. These findings are in keeping with our recent report (11) that LTB_4 obtained from incubations of LTA_4 with human and rat liver microsomes exhibits about a 2-fold higher IC_{50} than synthetic LTB_4. Using the same receptor displacement assay, (5*S*),(12*S*)-dihydroxy-6,14-*cis*-8,10-*trans*-eicosatetraenoic acid [i.e., (5*S*),(12*S*)-LTB_4] exhibited an IC_{50} value of about 20–30 nM (21). Based on the assumption that a nonstereocontrolled attack of a hydroxyl group at C-12 takes place during the conversion of 5-HPETE to LTB_4 by rat hepatocyte homogenates, we propose that the material of peak III is a mixture of highly active (5*R*),(12*R*)-LTB_4 and the 10-fold less active (5*R*),(12*S*)-LTB_4. This interpretation is supported by the finding (data not shown) that 40% (three experiments) of the material collected under peak III is recognized as (5*S*),(12*R*)-LTB_4 in the highly stereoselective RIA, the cross-reactivity of which with (5*S*),(12*S*)-LTB_4 at 50% B/B_o replacement is less than 0.2% (according to manufacturers instructions provided by Amersham Corp. with the [^{3}H]LTB_4 assay reagent system, code TRK.840).

In order to further characterize biological activity, the capacity of synthetic and hepatocyte-derived preparations of LTB_4 to elicit a transient increase in the cytosolic calcium concentration of human neutrophils were compared (Fig. 4). This bioassay has a marked specificity for LTB_4 when compared with the other stereochemical isomers of 5,12-diHETE that have been tested (22). Whereas synthetic LTB_4 exhibited an EC_{50} of 0.09 nM, hepatocyte-derived LTB_4 was a factor of 3 (i.e., EC_{50} = 0.3 nM) less active in inducing the observed transient cytosolic calcium increases (Table 1). Again, this finding is in keeping with the notion that the hepatocyte-derived material collected under peak III (Fig. 1) is a mixture of the biologically fully active (5*S*),(12*R*)-LTB_4 and the less active (5*S*),(12*S*)-LTB_4.

Inhibition of LTB_4 formation in rat hepatocyte homogenates. We have shown that some inhibitors of arachidonic acid metabolism (23, and references therein) are able to moderately inhibit, in a concentration-dependent manner (data not shown), the formation of LTB_4 from 5-HPETE as catalyzed by rat hepatocyte homogenates (Table 2). Thus, 1 mM ETYA, a structural analogue of arachidonic acid in which all the double bonds are replaced with acetylenic bonds, inhibits LTB_4 formation by 53%. After a preincubation time of 15 min, 50 μM 5,6-dehydro-arachidonic acid, a more specific inhibitor of 5-lipoxygenase, also inhibited LTB_4 formation by 43%. We have previously shown that propanethiol inhibited the human and rat liver microsome-catalyzed conversion of LTA_4 into LTB_4 (11). In the present experiments, 1 mM propanethiol inhibited the conversion of 5-HPETE into LTB_4 by rat hepatocyte homogenates by 49%. However, due to the low potency of these inhibitors and the complexity and variety 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); we were able to recognize a preferential inhibi-

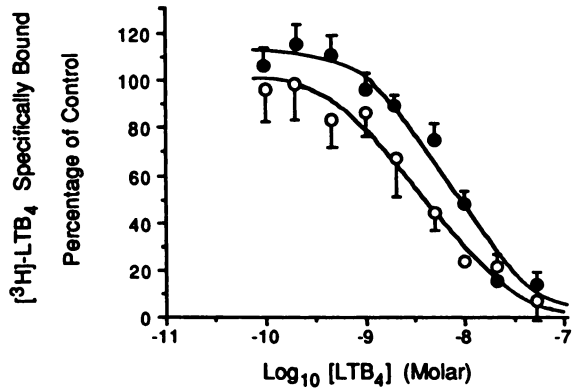

Fig. 3. Inhibition of [^{3}H]LTB_4 binding to human neutrophils by synthetic and hepatocyte homogenate-derived preparations of LTB_4. The amount of [^{3}H]LTB_4 specifically bound to human neutrophils in the presence of different concentrations of either synthetic (○) or hepatocyte homogenate-derived (●) LTB_4 is expressed as a percentage of the specific binding determined in the absence of nonradioactive LTB_4. Each data point is the mean ± standard error of three experiments.

TABLE 1

Biological activity of hepatocyte-derived LTB_4

The bioassay for hepatocyte-derived LTB_4 (transient increase in cytosolic Ca^{2+} of human neutrophils), the binding of [^{3}H]LTB_4 to human neutrophils, and the RIA for LTB_4 were carried out as described in Experimental Procedures.

Source of LTB_4*	Ca^{2+} influx (EC_{50})	[^{3}H]LTB_4 binding (IC_{50})	RIA
	M	*M*	*% binding*
Synthetic	9×10^{-11}	4×10^{-9}	100
Hepatocyte-derived	3×10^{-10}	1×10^{-8}	40
Fold difference	3.0	2.5	2.5

* Hepatocyte-derived LTB_4 concentration determined by external standardization on HPLC, followed by UV spectrophotometry.

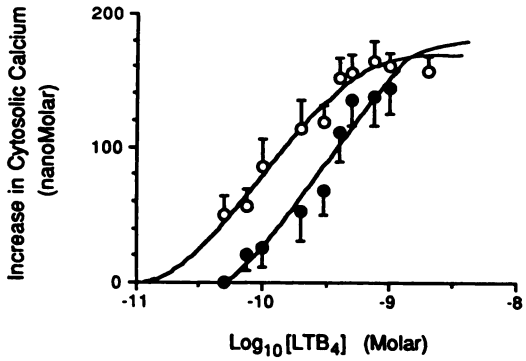

Fig. 4. Capacity of synthetic and hepatocyte homogenate-derived preparations of LTB_4 to elicit an increase in cytosolic calcium from human neutrophils. The maximal increase in cytosolic calcium concentration was determined for each dose of synthetic (○) and hepatocyte homogenate-derived (●) LTB_4. Each data points is the mean ± standard error of five experiments.

TABLE 2

Inhibition of 5-HPETE conversion to LTB_4

Rat hepatocyte homogenates (0.8 mg of protein/ml) were incubated under an argon atmosphere in the 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 Experimental Procedures. The control activity, determined in the presence of 0.8 mg of protein/400-μl incubation volume and 2 μl vehicle (typically methanol), was 31.0 ± 5.2 ng of all-*trans*-LTB_4 and 11.4 ± 1.8 ng of LTB_4 formed, respectively, per 15 min (eight experiments). Inhibitors were added in vehicle from stock solutions which were kept under argon. Values are means ± standard deviations and given as percentage of activity remaining in presence of inhibitor. Statistical analysis (unpaired *t* test) was done with Statview 512+ software on a Macintosh II computer

Inhibitor[a]	all-*trans*-LTB_4	LTB_4	No. of expts.
	% *residual activity*		
Diethylcarbamazine, 1 mM	85.2 ± 7.6[b]	83.9 ± 15.7[b]	4
Desferal, 1 mM	103.4 ± 1.2	98.1 ± 2.0	3
ETYA, 1 mM	99.9 ± 18.2	46.9 ± 12.4[b,c]	3
5,6-Dehydro-arachidonic acid, 50 μM	88.1± 14.9[b]	57.4 ± 7.8[b,c]	3
Propanethiol, 1 mM	73.4 ± 4.1[b]	51.1 ± 10.1[b,c]	3
O_2, 100%[d]	37.5 ± 9.1[b]	33.8 ± 5.8[b]	5

[a] Inhibitors and 5-HPETE were added simultaneously, except for 5,6-dehydro-arachidonic acid, which was added 15 min before the substrate.
[b] Indicates significant difference from control value, $p < 0.05$.
[c] Indicates significant difference from value of all-*trans*-LTB_4, $p < 0.05$.
[d] Argon atmosphere was replaced by 100% O_2.

tion of LTB_4 formation with respect to either Δ^6-*trans*-LTB_4 or Δ^6-*trans*-12-epi-LTB_4 formation. In fact, with the exception of O_2 (see below), the formation of the all-*trans*-LTB_4s proved to be rather insensitive (Table 2) to inhibition by all the inhibitors used. Diethylcarbamazine (1 mM), which has been shown to inhibit LTA_4 synthesis in mastocytoma cells (23) and which exerts protective action in *in vivo* models of leukotriene-mediated hepatotoxicity (24), had almost no inhibitory effect on 5-HPETE conversion into LTB_4. Also, the iron-chelating agent desferal (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 dependent on free iron.

We have previously reported that both the cytosolic and the microsome-associated formation of LTB_4 from LTA_4 in human and rat liver were inhibited in the presence of 100% O_2 (11). In the present study, the formation of LTB_4 as well as that of all-*trans*-LTB_4s from 5-HPETE was inhibited by 66% and 62.5%, respectively, when the argon atmosphere was replaced by 100% O_2. In control experiments, we established that, in the presence of O_2, neither metabolism (25) of LTB_4 (possibly due to endogenous NADPH) nor degradation (due to instability of LTB_4) took place. Thus, rat hepatocyte homogenate (0.8 mg/ml) was incubated with 1 μM synthetic LTB_4 under an 100% O_2 atmosphere in the absence of NADPH, and the recovery of intact LTB_4 after a 15-min incubation period was determined. Under these conditions, the recovery of synthetic LTB_4 was >98% (four experiments).

A slight temperature dependence of the LTB_4-forming activity in rat hepatocyte homogenates was also noted. Thus, as compared with the control activity established at 21°, only 73.0 ± 4.5% (four experiments) and 58.4 ± 10.2% (four experiments) of residual activity were detected at 4° and at 37°, respectively.

Cellular origin of hepatic LTB_4-forming activity. The mammalian liver comprises several functionally different cell types. PC constitute about 90% of the total cell mass, but only about 65% of total cell number (26). The remainder corresponds mainly to nonparenchymal sinusoidal cells, including Kupffer cells, epithelial cells, fat-storing cells, and pit cells. They account for about 10% of total cell mass but for about 35% of total cell number. Kupffer cells, endothelial cells, and fat-storing cells contribute about 54, 29, and 17%, respectively, of total sinusoid cells, whereas pit cells are rare (27). Kupffer cells have been shown to be a rich source of prostaglandins and peptidoleukotrienes (i.e., LTC_4, LTD_4, etc.) and a very recent report indicated that LTB_4 is also released by rat Kupffer cells upon stimulation by frog virus-3 (28).

In order to assess the possible contribution of enzymes derived from contaminating Kupffer cells to the observed formation of LTB_4 from 5-HPETE in rat hepatocyte homogenates, we have incubated isolated Kupffer cells (1.0×10^6 cells/200-μl incubation volume) in the presence of 100 μM 5-HPETE under argon for 15 min and compared with the extent of LTB_4 formation with that of identical incubations containing 7.2×10^5 hepatocytes/200-μl incubation volume. Hepatocytes, purified and enriched by passage through a Percoll layer of a density of 1.070 g/ml (14), contained <1.5% nonhepatocytes, mostly Kupffer cells (i.e., maximal 1.125×10^4 Kupffer cells). It was found that 7.2×10^5 hepatocytes produced 885.0 ± 141.1 pg of LTB_4/15 min (two experiments) and 228.5 ± 101.1 pg of LTB_4/15 min (two experiments) in the absence and presence of O_2, respectively. In contrast, only 137.5 ± 53.0 pg of LTB_4/15 min were produced by 1.0×10^6 Kupffer cells (two experiments) under an argon atmosphere (Table 3). These results indicate that only a marginal contribution (i.e., <0.2%) could be made by Kupffer cell-derived enzymes to the formation of LTB_4 from 5-HPETE in this *in vitro* assay system. Accordingly, no differences were found in the amounts of LTB_4 formed by homogenates (0.8 mg of protein/400-μl incubation, 100 μM 5-HPETE) of either Percoll-purified hepatocytes (essentially free of non-

TABLE 3

Formation of LTB_4 as catalyzed by rat hepatocytes or Kupffer cells

Incubation of cells was done under either an argon (Ar) or oxygen (O_2, 100%) atmosphere in presence of 100 μM 5-HPETE for 15 min at room temperature in 200-μl final incubation volume. Extraction of reaction products and separation on HPLC were done as described in Experimental Procedures. Material with the same retention time as synthetic LTB_4 was collected, taken to dryness, and subjected to RIA analysis for LTB_4. Values represent means ± standard deviation of two separate experiments.

Cells	No. of cells	LTB_4 formed
		pg/15 min
Kupffer cells + Ar	1.0×10^6	137.5 ± 53.0
Hepatocytes[a] + Ar	7.2×10^5	885.0 ± 141.1
Hepatocytes + O_2	7.2×10^5	228.5 ± 101.1

[a] Less than 1.5% contamination by nonhepatocytes.

hepatocytes) or whole liver (containing the natural abundance of nonhepatocytes); 11.4 ± 1.8 ng of LTB_4 (eight experiments) and 12.1 ± 2.1 ng of LTB_4 (four experiments) were formed, respectively. These results do not exclude, however, mutually stimulatory or inhibitory interactions in the biosynthesis of leukotrienes by distinct hepatic cell types in the intact liver.

Discussion

In this report we show that rat hepatocyte homogenates convert 5-HPETE into biologically fully active LTB_4, possibly via the epoxide intermediate LTA_4. Several lines of evidence support this conclusion. First, the hepatocyte homogenate-derived LTB_4 shows the same retention time on HPLC and a UV spectrum identical to synthetic LTB_4 with characteristic relative maxima at 261, 270, and 280 nm. Second, mass spectral analysis reveals the same characteristic fragment ions as those obtained with synthetic LTB_4. Third, in a sensitive and highly stereoselective receptor displacement assay on human neutrophils, LTB_4 generated by rat hepatocyte homogenates from 5-HPETE exhibits IC_{50} values comparable to those noted for synthetic LTB_4. Finally, in the cytosolic calcium bioassay, hepatocyte homogenate-derived LTB_4 induces a transient increase in cytosolic Ca^{2+} in human neutrophils exactly the way synthetic LTB_4 does, with comparable EC_{50} values. We have ruled out the possibility that an almost unavoidable contamination by nonhepatocytes (i.e., Kupffer cells) significantly contributes to the formation of LTB_4 from 5-HPETE in rat hepatocyte homogenates. Thus, the collective evidence from these data indicates that hepatocytes contain enzymes that are capable of catalyzing the biosynthesis of leukotrienes from peroxidized arachidonic acid.

Here one should note that the structural and functional characterization of biologically generated LTB_4 was performed throughout on RP HPLC-purified and rechromatographed material collected under peak III (Fig. 1). This material, either as the free acid or the methyl ester derivative, showed no heterogeneity on HPLC when co-chromatographed with either synthetic LTB_4 free acid or the methyl ester of synthetic LTB_4 (i.e., (5*S*),(12*R*)-LTB_4). Although biologically generated LTB_4 is almost identical to synthetic LTB_4 in its biological functions, we observed that in the highly stereoselective receptor displacement assay for LTB_4 on human neutrophils (21), hepatocyte homogenate-derived LTB_4 exhibited an IC_{50} about 2.5 times higher than synthetic (5*S*),(12*R*)-LTB_4 (i.e., 10.0 nM versus 4 nM). In the same receptor displacement assay, (5*S*),(12*S*)-LTB_4 exhibited IC_{50} values of 20–30 nM, whereas (5*S*),(12*S*)-Δ^6-*trans*-LTB_4, LTD_4, and 5-HETE bound with affinities that are orders of magnitude lower than that of (5*S*),(12*R*)-LTB_4 to these receptors (21). Therefore, we propose that the hepatocyte homogenate-derived LTB_4 is a mixture of the biologically fully active (5*S*),(12*R*)-LTB_4 and the less active (5*S*),(12*S*)-LTB_4 brought about by a non-stereocontrolled attack of a hydroxyl group at C-12 in a transition state between 5-HPETE and LTB_4. This interpretation is in keeping with the finding that the stereoselective RIA recognizes 40% of the material collected under peak III (Fig. 1) as the stereochemically correct (5*S*),(12*R*)-LTB_4.

In specialized peripheral cells, arachidonic acid is transformed enzymatically into a variety of hydroperoxy intermediates by cyclooxygenase and various lipoxygenases (1). In particular, the enzyme 5-lipoxygenase exhibits dual enzymic activity, catalyzing the synthesis of 5-HPETE from arachidonic acid as well as the subsequent conversion of 5-HPETE to LTA_4 and hence is the key enzyme in leukotriene biosynthesis (29, 30). However, no reports thus far have indicated the presence of 5-lipoxygenase activity in hepatocytes and Decker has reported that isolated hepatocytes do not convert exogenous arachidonic acid into leukotrienes (31). We have performed preliminary experiments to test for lipoxygenase activity in rat hepatocyte homogenates. However, using up to 500 μM arachidonic acid as a substrate, we have not been able to detect the formation of either 5-HPETE or LTA_4 (by measuring the formation of the acid hydrolysis products Δ^6-*trans*-LTB_4 and Δ^6-*trans*-12-epi-LTB_4 within the detection limits of our HPLC-analysis (i.e., about 2 ng for all-*trans*-LTB_4). As of yet no efforts were made with respect to optimizing possible regulatory factors such as CA^{2+}, ATP, or proteinaceous intracellular factors that may be necessary for activation of hepatocyte 5-lipoxygenase activity (29, 30). Nevertheless, to us it seems unlikely that hepatocytes would exhibit considerable constitutive 5-lipoxygenase activity. Our studies of the effect of inhibitors of 5-lipoxygenase on the transformation of 5-HPETE to LTB_4 as catalyzed by hepatocyte homogenates (Table 2) suggest that the pathways and the catalytic sites involved in the formation of LTB_4 are different from those of the all-*trans*-LTB_4s. Neither ETYA (1 mM) nor 5,6-dehydro-arachidonic acid (50 μM), at concentrations about 10-fold higher than those that cause complete inhibition of 5-lipoxygenase activity in peripheral cells (23), inhibited the formation of the all-*trans*-LTB_4s. The lack of inhibition of all-*trans*-LTB_4 formation by these 5-lipoxygenase inhibitors in hepatocyte homogenates (Table 2), as well as the relative insensitivity (about 70% residual all-*trans*-LTB_4 detected) of this activity towards heat and proteolytic inactivation, suggests that there are nonenzymatic routes of transformation of 5-HPETE to all-*trans*-LTB_4s within these cells.

Here, one might speculate that heme groups, either enzyme bound, as in the case of cytochrome *c*, or free as in the case of hematin, could be responsible, at least in part, for the transformation of 5-HPETE to products with HPLC retention times and UV absorption properties similar to those of all-*trans*-LTB_4 (7). The possible interaction of 5-HPETE with a heme group is substantiated by the inhibition of activity by propanethiol (Table 2), a compound that has been shown to interact with the iron of heme groups (32). On the other hand, the lack of effect of desferal in hepatocyte homogenates suggests that free iron is not important in this conversion. By contrast, LTB_4 formation from 5-HPETE was inhibited 47 and 57% by ETYA (1 mM) and 5,6-dehydro-arachidonic acid (50 μM), respectively. In addition, LTB_4 formation was readily abolished upon heat and trypsin treatment, which is in keeping with earlier findings (8, 9, 11) that conversion of LTA_4 to LTB_4 in liver microsomal and cytosolic fractions is heat sensitive. This laboratory previously reported that reconstituted rabbit cytochrome $P\text{-}450_{LM2}$ catalyzed formation of LTB_4 from 5-HPETE (7). However, the inhibitor studies in Table 2 and in our report of the formation of LTB_4 from LTA_4 (11), as well as preliminary studies on subcellular localization, suggest that cytochromes P-450 make only a small contribution to the total formation of LTB_4 in rat hepatocyte homogenates. On the other hand, the enzyme responsible for the activity must be rather similar to a cytochrome P-450. As discussed above, inhibition of activity with propa-

nethiol is consistent with a heme group as a catalytic site. We suggest that the essential difference between the enzyme that produces LTB_4 and either hematin or cytochrome *c* is a binding site that is capable of holding the 5-HPETE in a fixed stereochemistry during the transition state to LTB_4. If the bonds between C-5 and C-9 are not free to rotate during the hydrolysis, then some 6-*cis*-LTB_4 will be formed rather than the thermodynamically favored all-*trans*-LTB_4. The suggestion that such a binding site exists is substantiated by the complete inhibition of LTB_4 formation, but only partial inhibition of all-*trans*-LTB_4 formation, after trypsin or heat treatment. Both ETYA and 5,6-dehydro-arachidonic acid are arachidonic acid analogues and could bind to the same site as 5-HPETE, thereby inhibiting its transformation. Clearly, these inhibitor studies provide only indirect evidence for the nature and number of enzymes involved in LTB_4 synthesis from 5-HPETE in hepatocytes; only their purification and thorough characterization will reveal their true properties and their relatedness to known enzymes of the leukotriene biosynthetic pathways in peripheral cells.

Hepatocytes do not need a lipoxygenase to produce 5-HPETE on their own but could obtain it through intercellular transfer processes, resembling those occurring in platelet-endothelial cell interactions that provide vascular prostacyclin synthetase with platelet-derived endoperoxides as substrates (33). Similarly, it has been shown that transcellular exchange of hydroperoxy or hydroxy acids can lead to a variety of modulators and/or formation of new products in responsive target cells (34). Earlier reports indicated that neutrophil-derived LTA_4 could serve as a trans-cellular substrate for leukotriene biosynthesis by erythrocytes (35), endothelial and mast cells (36), and human platelets (37). We (11) and others (8, 9) have recently shown that liver subcellular fractions are also capable of transforming LTA_4 into active leukotrienes.

In addition to transport from other cells, lipid peroxidation in the hepatocyte is a possible contributing factor to the formation of arachidonic acid-derived hydroperoxides as precursors of biologically active leukotrienes. Both 5-HPETE and 15-HPETE were among the prominent peroxidation products of arachidonic acid in an *in vitro* model system using 1-chloro-2,2,2-triflouroethyl free radical as a model for drug-derived free radical-initiated lipid peroxidation (38). Under conditions of physiologically (39) low O_2 tensions, the hepatotoxins halothane and carbon tetrachloride markedly increased the extent of lipid peroxidation in hepatocyte suspensions and rat liver microsomes (40). However, one should be aware of the possibility that lipid peroxidation *in vivo* might yield a distinctly different profile in peroxidation products, both qualitatively and quantitatively (41), thereby affecting the availability of lipid hydroperoxides as the source for a wide variety for secondary reactions.

There are other routes of lipid hydroperoxide decay that may be important within the hepatocyte and in its communications with neighboring cells. For example, hematin catalyzed the conversion of linoleic acid hydroperoxide (i.e., 13-OOH-18:2) to hydroxy-, epoxyhydroxy-, and trihydroxy-fatty acid derivatives (20). Epoxyols were also formed from 13-OOH-18:2 by Lewis acid catalysis (42). Epoxyols, arising via hydroperoxide intermediates, have additionally been reported as products of both arachidonic acid oxidation in lung tissue and metabolism by hematin of 12-hydroperoxyarachidonate (43). However, a role of these epoxyols in the etiology of hepatotoxicity remains to be elucidated. The capacity of hepatocyte homogenates to convert 5-HPETE into LTB_4, described herein, points to a presumably potent role, at least *in vitro*, of hepatocytes in leukotriene biosynthesis, possibly using intermediates of leukotriene biosynthesis (i.e., 5-HPETE and LTA_4) as preferential primary substrates. We have demonstrated that under low oxygen tension the formation of LTB_4 from both LTA_4 (11) and 5-HPETE (this report) is enhanced whereas the conversion of LTB_4 to biologically less active compounds is diminished (12). In that hepatocyte homogenates are capable of transforming 5-HPETE into biologically active LTB_4 as well as other mediators of the leukotriene family, hepatocytes may have an important role in the presumably delicate equilibrium of hepatic leukotriene homeostasis.

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References

1. Samuelsson, B. Leukotrienes: mediators of immediate hypersensitivity reactions and inflammation. *Science (Wash. D. C.)* **220:** 568–575 (1983).
2. Goetzl, E. J. Neuromodulation of immunity and hypersensitivity. *J. Immunol.* **135**(suppl.):739s–863s (1985).
3. Rola-Pleszcynski, R. Immunoregulation by leukotrienes and other lipoxygenase metabolites. *Immunol. Today* **6**:302–307 (1985).
4. Payan, D. G., and E. J. Goetzl. Specific suppression of human lymphocyte functions by leukotriene B_4. *J. Immunol.* **131**:551–553 (1983).
5. Rola-Pleszczynski, M. Differential effects of leukotriene B_4 on $T4^+$ and $T8^+$ lymphocyte phenotype and immunoregulatory functions. *J. Immunol.* **135**:1357–1360 (1985).
6. Serhan, C. N., J. Fridovich, E. J. Goetzl, P. B. Dunham, and G. Weissmann. Leukotriene B_4 and phosphatidic acid are calcium ionophores. *J. Biol. Chem.* **257**:4746–4752 (1982).
7. Bosterling, B., and J. R. Trudell. Evidence for leukotriene A_4 as an intermediate in the conversion of 5-HPETE to leukotriene B_4 catalyzed by cytochrome P-450. *Biochem. Biophys. Res. Commun.* **115**:995–1001 (1983).
8. Haeggstroem, J., O. Radmark, and F. A. Fitzpatrick. Leukotriene A_4-hydrolase activity in guinea pig and human liver. *Biochim. Biophys. Acta* **835**:378–384 (1985).
9. Pace-Asciak, C. R., J. Klein, S. Lombard, J. Torchia, and J. Rokach. Catabolism of leukotriene A_4 into B_4, C_4, and D_4 by rat liver subcellular fractions. *Biochim. Biophys. Acta* **836**:153–156 (1985).
10. Roll, F. J., D. M. Bissell, and H. D. Perez. Human hepatocytes metabolizing ethanol generate a nonpolar chemotactic factor for human neutrophils. *Biochem. Biophys. Res. Commun.* **137**:688–694 (1986).
11. Gut, J., D. W. Goldman, G. C. Jamieson, and J. R. Trudell. Conversion of leukotriene A_4 to leukotriene B_4: catalysis by human liver microsomes under anaerobic conditions. *Arch. Biochem. Biophys.* **259**:497–509 (1987).
12. Gut, J., A. K. Costa, and J. R. Trudell. Oxygen concentration-dependent metabolism of leukotriene by hepatocyte monolayers. *Biochim. Biophys. Acta* **878**:194–199 (1986).
13. Costa, A. K., D. F. Heffel, T. M. Schieble, and J. R. Trudell. Toxicity of *t*-butylhydroperoxide in hepatocyte monolayers exposed to hypoxia and reoxygenation. *In Vitro* **23**:501–506 (1987).
14. Smedsrod, B., and H. Pertoft. Preparation of pure hepatocytes and reticuloendothelial cells in high yield from a single rat liver by means of Percoll centrifugation and selective adherence. *J. Leukocyte Biol.* **38**:213–230 (1985).
15. Wisse, E. Observations on the fine structure and peroxidase cytochemistry of normal rat liver Kupffer cells. *J. Ultrastruct. Res.* **46**:393–426 (1974).
16. Gifford, L. A., T. Chernov-Rogan, J. P. Harvey, C. H. Koo, D. W. Goldman, and E. J. Goetzl. Recognition of human polymorphonuclear leukocyte receptors for leukotriene B_4 by rabbit anti-idiotypic antibodies to a mouse monoclonal antileukotriene B_4. *J. Immunol.* **138**:1184–1189 (1987).
17. Grynkiewicz, G., M. Poenie, and R. Y. Tsien. A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. *J. Biol. Chem.* **260**:3440–3450 (1985).
18. Gut, J., G. E. Jamieson, and J. R. Trudell. Leukotriene biosynthesis: direct chemical ionization mass spectrometry of underivatized arachidonic acid metabolites. *Biomed. Environ. Mass Spectrom.* **15**:509–516 (1988).
19. Radmark, O., C. Malmsten, B. Samuelsson, D. A. Clark, G. Goto, A. Marfat, and E. J. Corey. Leukotriene A: stereochemistry and enzymatic conversion to leukotriene B. *Biochem Biophys. Res. Commun.* **92**:954–961 (1980).
20. Dix, T. A., and L. J. Marnett. Conversion of linoleic acid hydroperoxide to hydroxy, keto, epoxyhydroxy, and trihydroxy fatty acids by hematin. *J. Biol. Chem.* **260**:5351–5357 (1985).

21. Goldman, D. W., and E. J. Goetzl. Heterogeneity of human polymorphonuclear leukocyte receptors for leukotriene B_4: identification of a subset of high affinity receptors that transduce the chemotactic response. *J. Exp. Med.* **159**:1027–1041 (1984).
22. Goldman, D. W., L. A. Gifford, D. M. Olson, and E. J. Goetzl. Transduction by leukotriene B_4 receptors of increases in cytosolic calcium in human polymorphonuclear leukocytes. *J. Immunol.* **135**:525–530 (1985).
23. Bach, M. K. Inhibitors of leukotriene synthesis and action, in *The Leukotrienes, Chemistry and Action.* (L. W. Chakrin and D. M. Bailey, eds.). Academic Press, New York, 163–194 (1984).
24. Keppler, D., C. Forsthove, W. Hagmann, S. Rapp, C. Denzlinger, and H. K. Koch. Leukotrienes and liver injury, in *Trends in Hepatology* (Bianchi, L., Gerok, W., and Popper, H., eds.). MTP Press, Lancaster, England, 137–145 (1985).
25. Newton, J. F., R. Eckhart, P. E. Bender, T. Leonard, and K. Straub. Metabolism of leukotriene B_4 in hepatic microsomes. *Biochem. Biophys. Res. Commun.* **128**:733–738 (1985).
26. Fabrikant, J. I. The kinetics of cellular proliferation in regenerating liver. *J. Cell Biol.* **42**:68–91 (1969).
27. Blouin, A., R. P. Bolender, and E. R. Weibel. Distribution of organelles and membranes between hepatocytes and nonhepatocytes in the rat liver parenchyma: a stereological study. *J. Cell Biol.* **72**:441–455 (1977).
28. Hagmann, W., A. M. Steffan, A. Kirn, and D. Keppler. Leukotrienes as mediators in frog virus 3-induced hepatitis in rats. *Hepatology (Baltimore)* **7**:732–736 (1987).
29. Hogaboom, G. K., M. Cook, J. F. Newton, A. Varrichio, R. G. L. Shorr, H. M. Sarau, and S. T. Crooke. Purification, characterization, and structural properties of a single protein from rat basophilic leukemia (RBL-1) cells possessing 5-lipoxygenase and leukotriene A_4 synthetase activities. *Mol. Pharmacol.* **30**:510–519 (1986).
30. Rouzer, C. A., and B. Samuelsson. Reversible, calcium-dependent membrane association of human leukocyte 5-lipoxygenase. *Proc. Natl. Acad. Sci. USA* **84**:7393–7397 (1987).
31. Decker, K. Eicosanoids as signal molecules between hepatocytes and sinusoidal cells, in *Modulation of Liver Cell Expression* (W. Reutter, H. Popper, I. M. Arias, P. C. Heinrich, D. Keppler, and L. Landmann, eds.). MTP Press, Lancaster, England, 397–409 (1987).
32. Sono, M., L. A. Andersson, and J. D. Dawson. Sulfur donor ligand binding to ferric cytochrome P-450-CAM and myoglobin: ultraviolet-visible absorption, magnetic circular dichroism, and electron paramagnetic resonance spectroscopic investigation of the complexes. *J. Biol. Chem.* **257**:8308–8320 (1982).
33. Marcus, A. J., M. J. Broekman, L. B. Safier, H. L. Ullman, N. Islam, C. N. Serhan, L. E. Rutherford, H. M. Korchak, and G. Weissmann. Formation of leukotrienes and other hydroxy acids during platelet-neutrophil interactions *in vitro*. *Biochem. Biophys. Res. Commun.* **109**:130–137 (1982).
34. Maclouf, J., B. Fruteau de Laclos, and P. Borgeat. Stimulation of leukotriene biosynthesis in human blood leukocytes by platelet-derived 12-hydroperoxyeicosatetraenoic acid. *Proc. Natl. Acad. Sci. USA* **79**:6042–6046 (1982).
35. Fitzpatrick, F., W. Liggett, J. McGee, S. Bunting, D. Morton, and B. Samuelsson. Metabolism of leukotriene A_4 by human erythrocytes: a novel cellular source of leukotriene B_4. *J. Biol. Chem.* **259**:11403–11407 (1984).
36. Dahinden, C. A., R. M. Clancy, M. Gross, J. M. Chiller, and T. E. Hugli. Leukotriene C_4 production by murine mast cells: evidence of a role for extracellular leukotriene A_4. *Proc. Natl. Acad. Sci. USA* **82**:6632–6636 (1985).
37. Maclouf, J. A., and R. C. Murphy. Transcellular metabolism of neutrophil-derived leukotriene A_4 by human platelets: a potential cellular source of leukotriene C_4. *J. Biol. Chem.* **263**:174–181 (1988).
38. Bosterling, B., and J. R. Trudell. Production of 5- and 15-hydroperoxy-eicosatetraenoic acid from arachidonic acid by halothane free radicals generated by UV-irradiation. *Anesthesiology* **60**:209–213 (1984).
39. Jones, D. P. Hypoxia and drug metabolism. *Biochem. Pharmacol.* **30**:1019–1023 (1981).
40. deGroot, H., T. Noll, and B. Rymsa. Alterations of ferrous iron and haloalkane free-radical-mediated lipid peroxidation. *Biochem. Biophys. Acta* **881**:350–355 (1986).
41. Frank, H., M. Wiegand, M. Strecker, and D. Thiel. Monohydroperoxides of linoleic acid in endoplasmic lipids of rats exposed to tetrachloromethane. *Lipids* **22**:689–697 (1987).
42. Gardner, H. W., D. Weisleder, and R. Kleiman. Formation of *trans*-12,13-epoxy-9-hydroperoxy-*trans*-10-octadecenoic acid from 13-L-hydroperoxy-*cis*-9-*trans*-11-octadecadienoic acid catalyzed by either a soybean extract or cysteine-$FeCl_3$. *Lipids* **13**:246–252 (1978).
43. Pace-Asciak, C. R. Arachidonic acid epoxides: demonstration through [^{18}O] oxygen studies of an intramolecular transfer of the terminal hydroxyl group of (12*S*) hydroperoxyeicosa-5,8,10,14-tetraenoic acid to form hydroxyepoxides. *J. Biol. Chem.* **259**:8332–8337 (1984).
44. Miyamoto, T., J. A. Lindgren, and B. Samuelsson. Isolation and identification of lipoxygenase products from the rat central nervous system. *Biochim. Biophys. Acta* **922**:372–378 (1987).

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