Biochemical Characterization of Hepatic Microsomal Leukotriene B₄ Hydroxylases*

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ω-Hydroxylation of leukotriene B₄ (LTB₄) has been reported in human and rodent polymorphonuclear leukocytes; preliminary information indicates that this metabolism is cytochrome P-450 dependent. Therefore, these studies were initiated to characterize the cytochrome P-450-dependent metabolism of LTB₄ in other tissues. LTB₄ was metabolized by rat hepatic microsomes to two products, 20-hydroxy(ω)-LTB₄ and 19-hydroxy(ω-1)-LTB₄. The formation of these metabolites was both oxygen and NADPH dependent indicating that a monooxygenase(s) was responsible for these reactions. The apparent Kₘ and Vₘₐₓ for LTB₄ ω-hydroxylase were 40.28 μM and 1202 pmol/min/mg of protein, respectively. In contrast, the apparent Kₘ and Vₘₐₓ for LTB₄ (ω-1)-hydroxylase were 61.52 μM and 73.50 pmol/min/mg of protein, respectively. Both LTB₄ ω- and (ω-1)-hydroxylases were inhibited by metyrapone in a concentration-dependent fashion. However, SK&F 525A inhibited LTB₄ (ω-1)- but not ω-hydroxylation. In contrast, α-naphthoflavone decreased LTB₄ ω- but not (ω-1)-hydroxylase activities. The differences in the Kₘ apparent for substrate as well as the differential inhibition by inhibitors of cytochrome P-450 suggest that the ω- and (ω-1)-hydroxylations of LTB₄ in hepatic microsomes are mediated by different isoforms of P-450. Furthermore, several additional characteristics of LTB₄ ω-hydroxylases indicate that these isoforms of P-450 may be different from those which catalyze similar reactions on medium-chain fatty acids, such as laurate and prostaglandins.

In leukocytes, oxygenation of arachidonic acid at the 5-position to produce 5-hydroperoxy-5,8,11,14-eicosatetraenoic acid is the initial step leading to production of a class of compounds collectively known as leukotrienes (1). Among them, LTB₄ is considered an important mediator of inflammation. In vitro at nanomolar concentrations, LTB₄ induces chemotaxis and chemokinesis of polymorphonuclear leukocytes (2, 3) and stimulates oxidative metabolism, with consequent production of superoxides, in both neutrophils and eosinophils (4). At higher concentrations, it causes degranulation of rabbit (5) and human (6) polymorphonuclear leukocytes and cellular aggregation (7). These potent biological effects can be reduced by metabolism. ω-Oxidation has been reported to be the major route of catabolism of LTB₄ in polymorphonuclear leukocytes (8). ω-Hydroxy LTB₄ is less potent than LTB₄ in stimulation of chemotactic and proaggregatory responses (6, 8–11). The subsequent oxidation product, ω-carboxy LTB₄, is almost devoid of these activities (6). Therefore, it is reasonable to assume that ω-oxidation is the first step in the inactivation of LTB₄.

ω- and (ω-1)-hydroxylation of other lipids such as prostaglandins and medium chain fatty acids has been demonstrated in hepatic (12–14) and extrahaepatic (15–17) microsomal preparations. Fatty acid and prostaglandin hydroxylases are cytochrome P-450-dependent enzymes (18–20). However, the specific isoforms of cytochrome P-450 which mediate hydroxylation of fatty acids constitute only a small fraction of total hepatic cytochrome P-450 (21). Several investigators, utilizing prostaglandins and medium chain fatty acids, have demonstrated that ω- and (ω-1)-hydroxylations are catalyzed by different isoforms of cytochrome P-450 (12, 16, 22, 23). In addition, the exact isoforms which catalyze a specific reaction (i.e., ω-hydroxylation) may be different depending upon the fatty acid.

Recently, we presented preliminary evidence for the existence of LTB₄ ω- and (ω-1)-hydroxylases in hepatic microsomes (24). Furthermore, Murphy and co-workers (25) have demonstrated that LTB₄ can be hydroxylated at the ω position in isolated hepatocytes. These studies were, therefore, directed at characterizing hepatic LTB₄ ω- and (ω-1)-hydroxylases; their primary focus was the hypothesis that differences exist in the isozyme specificity for LTB₄ ω- and (ω-1)-hydroxylations. In addition, the information obtained will allow for comparison of LTB₄ hydroxylases to the prostaglandin and laurate hydroxylases, an effort which may indicate whether LTB₄ hydroxylases are unique among hepatic microsomal lipid hydroxylases. Finally, these studies will provide a framework for the further characterization of polymorphonuclear leukocyte LTB₄ ω-hydroxylases.

MATERIALS AND METHODS

Chemicals—Unlabeled LTB₄ (5S,12R)-dihydroxy-6,14-cis-8,10-trans-eicosatetraenoic acid) was synthesized in 99.3% purity according to Corry et al. (26). Reagents were obtained from sources as indicated: [14,15-3H]LTB₄ (New England Nuclear); NADPH, prostaglandin B₅, metyrapone (Sigma); α-naphthoflavone (Aldrich); glucose-6-phosphate and glucose-6-phosphate dehydrogenase (Boehringer Mannheim); 3-methylcholanthrene (Eastman Kodak Co., Rochester, NY); phenobarbital (Gaines Chemicals, Pennsville, PA); Wyeth...
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14,643 (Wyeth Laboratories, Philadelphia, PA); N-O-bis-(trimethyl-
silyl)trifluoroacetamide and trimethylchlorosilane (Supelco, Belle-
fonte, PA); SK&F 525A (Smith Kline & French). All other reagents
used were of the highest purity available commercially.

Experimental Procedure—Male Fisher rats (CDF (F344/Cr Br),
Charles River Laboratories, Wilmington, MA) were kept in Biocon-
rooms (Hazelton Systems, Aberdeen, MD). Temperature was main-
tained at 22 ± 1 °C, relative humidity of 50 ± 10%, with a 12-h light/
dark cycle (0700–1900 h). Rats were provided laboratory chow (Cert-
ified Rodent Chow 5002, Ralston Purina Co., St. Louis, MO) and
lasp water ad libitum. Rats were delivered and maintained virus free
as determined by monitoring of sentinel animals for rat corona virus/
silolodacryoadenitis. After 10 days of adaptation, some animals were
-treated intraperitoneally with either phenobarbital in saline at a dose
of 80 mg/kg daily for 3 days or 3-methylcholanthrene in corn oil at a
dose of 20 mg/kg daily for 3 days. Another group of animals received
WK 14,643 in dimethyl sulfoxide orally at a dose of 50 mg/kg daily
for 3 days. The respective controls were treated with the vehicle
alone. The animals were killed by cervical dislocation, and organs
were excised immediately. Microsomes were prepared as reported (27)
and stored at −75 °C until used. Rat liver microsomes were prepared
according to Bressnick et al. (28) and used immediately. Protein
concentrations were determined according to Gornall et al. (29).

Determination of LTB₄ Metabolism—Reaction vessels contained
microsomes (0.4–0.5 mg/ml), Tris-HCl (0.05 M), pH 7.5, MgCl₂ (5
mM), LTB₄ (25 μM), and a NADPH-regenerating system consisting
of glucose-6-phosphate (12.5 mM) and glucose-6-phosphate dehydro-
genase (2.2 units/ml) in a volume of 0.2 ml. After 1 min of preincu-
batation, the reaction was initiated by addition of 0.2 μM of NADPH. Protein
concentration and time of incubation were adjusted such that
less than 10% of substrate was metabolized. In inhibition experi-
ments, progaglandins and 2-dithethylaminoethyl-2,2-diphenylvalerate
(SK&F 525A) were dissolved in Tris-HCl (0.05 M), pH 7.5; α-naph-
thol/fluorone was dissolved in aceton, coated on the walls of the
incubation vessel, and dried prior to adding the microsomal sus-
ension. Samples were incubated in a shaking water bath at 37 °C, and
the reaction was terminated by addition of 0.1 ml of acetic acid (10%).
Prostaglandin B₂ (1.24 μg) was added to each sample as an internal
standard. The incubations were extracted twice with fresh ether and
the ether was evaporated under nitrogen. The samples were resus-
pended in 0.1 ml of acetonitrile (30%) in ammonium acetate (50 mM).
Greater than 95% of LTB₄ and metabolites are recovered from
incubations using this procedure. LTB₄ and its metabolites were
quantitated on a HPLC system consisting of two model M6000-A
solvent delivery systems, a model 721 system controller, a model 481
thoflavone was dissolved in acetone, coated on the walls of the
incubation vessel, and dried prior to adding the microsomal sus-
ension, metyrapone and

RESULTS

Identification of Microsomal Metabolites of LTB₄. Incuba-
tion of [³H]LTB₄ with rat liver microsomes, in the presence of
oxygen and NADPH, produced two polar metabolites. The two
metabolites were separated by HPLC and analyzed by mass spectrometry. Both mass spectra (Figs. 1 and 2) were consistent with a leukotriene structure with 3 hydroxyl groups
located at C-5, C-12, and the third one at a position beyond
C-13. The trimethylsilyl methyl ester derivative of meto-
ablone I (Fig. 1) had a C-value of 29.1; prominent ions were
found at m/z 492 (M-90), 383 (M-199), 327, 317, 293 (M-
15), 267, 217, 203, 129, and 117 (α-cleavage product,
Me₂SiO⁴ = CH₂–CH₂). The trimethylsilyl methyl ester deriv-
ate of metabolite II (Fig. 2) and a C-value of 30.0; prominent ions
were found at m/z 582 (M), 567 (M-15), 551 (M-31), 492, 383,
327, 317, 290, 287, 217, 203, and 129. On the basis of C-value
and mass spectra, metabolite I was identified as 5,12,19-
trihydroxy-6,8,10,14-eicosatetraenoic acid (ω-1)-hydroxy-

Fig. 1. Electron impact mass spectrum of the trimethylsilyl
(TMS) ether methyl ester of (ω-1)-hydroxylated microsomal
metabolite of LTB₄. (ω-1)-Hydroxy LTB₄ was isolated and derivat-
ized as described under “Materials and Methods.”

Fig. 2. Electron impact mass spectrum of the trimethylsilyl
(TMS) ether methyl ester of (ω-1)-hydroxylated microsomal
metabolite of LTB₄. ω-Hydroxy LTB₄ was isolated and derivatized
as described under “Materials and Methods.”
LTB₄ and metabolite II as 5,12,20-trihydroxy-6,8,10,14-eicosatetraenoic acid (ω-hydroxy-LTB₄).

Subcellular Distribution and Co-substrate Dependence of Hepatic LTB₄ Hydroxylase—Subcellular distribution experiments indicated that the primary site of LTB₄ hydroxylation was associated with the microsomal fraction (Table I).

Rat liver cytosol was completely devoid of LTB₄ hydroxylase activity. Detectable amounts of LTB₄ ω-hydroxylase activity were observed in rat liver nuclei (Table I). The purity of the nuclei preparation was estimated to be greater than 95%. Therefore, due to the low LTB₄ hydroxylase activity observed in nuclei incubations (less than 1% of microsomal activity), we cannot exclude the possibility that microsomal contamination was responsible for the apparent nuclear LTB₄ hydroxylase activity.

The rate of formation of ω- and (ω-1)-hydroxy LTB₄ by hepatic microsomes was linear with time (up to 30 min) and protein (up to 2 mg/ml). However, both LTB₄ ω- and (ω-1)-hydroxylases were sensitive to temperature inactivation in the absence of substrate. Both enzymes were reduced to 60% of the control after 1 h at 50 °C (Fig. 3). Substrate versus velocity plots of the ω- and (ω-1)-hydroxylases indicated that these enzymes had different Michaelis-Menten constants (Fig. 4). The apparent Kₘ and Vₘₐₓ for (ω-1)-hydroxylation of LTB₄ were 61.52 μM and 73.50 pmol/min/mg of protein, respectively. In contrast, the apparent Kₘ and Vₘₐₓ for ω-hydroxylation of LTB₄ were 40.28 μM and 1202 pmol/min/mg of protein, respectively. These kinetic constants, primarily Vₘₐₓ, are substantially different from those reported previously (24). The lower values for Vₘₐₓ obtained previously were the result of an inhibitor of LTB₄ hydroxylases that was present in the regenerating system. The regenerating system used in the present study did not inhibit LTB₄ hydroxylases.

The hydroxylation of LTB₄ by hepatic microsomes was supported by both NADPH and NADH (Table II). However, the rates of ω- and (ω-1)-hydroxylations supported by NADH were only 20% of those observed when NADPH was used as a reducing equivalent. The apparent Kₘ values of LTB₄ ω- and (ω-1)-hydroxylases for NADPH were nearly identical, 66 and 57 μM, respectively. On the contrary, the apparent Kₘ values of LTB₄ ω- and (ω-1) hydroxylases for NADH were different, 868 and 154 μM, respectively (Table II). Synergism by NADH of the NADPH-supported hydroxylations was not observed. Addition of cytochrome c (250 μM) to the incubation mixture reduced ω- and (ω-1)-hydroxylations to 30 and 20% of the control activity, respectively (data not shown).

Effect of Cytochrome P-450 Inducers and Inhibitors on LTB₄ Hydroxylases—Several classical inhibitors of cytochrome P-450 (ω-naphthoflavone, metyrapone, and SK&F 525A) had differential effects on LTB₄ ω- and (ω-1)-hydroxylations (Fig. 5). Metyrapone inhibited both ω- and (ω-1)-hydroxylases in a

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### TABLE I

**Subcellular distribution of hepatic LTB₄ hydroxylases**

<table>
<thead>
<tr>
<th>Micromes / Nuclei</th>
<th>LTB₄ hydroxylase activities</th>
<th>ω-1</th>
<th>ω</th>
</tr>
</thead>
<tbody>
<tr>
<td>Microsomes</td>
<td>23.3 ± 1.1*</td>
<td>396.4 ± 25.0*</td>
<td></td>
</tr>
<tr>
<td>Cytosol</td>
<td>ND*</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>Nuclei</td>
<td>3.1 ± 0.2*</td>
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*Mean ± S.E. of triplicate determinations.

ND, not detectable.

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**Fig. 3. Temperature-dependent inactivation of ω- and (ω-1)-LTB₄ hydroxylases.** Hepatic microsomes (2-3 mg/ml) and Tris-HCl (50 μM) pH 7.5 buffer, in a volume of 1 ml, were preincubated at 25, 37, and 50 °C for 0, 10, 20, 30, and 60 min. Then 30 μl of preincubation mixture was used to determine LTB₄ metabolism as described under “Materials and Methods.” Data represent the mean ± S.E. of 3 different determinations and are expressed as percent of control. ○, ω-LTB₄; □, (ω-1)-LTB₄.

**Fig. 4. Michaelis-Menten plots of ω- and (ω-1)-LTB₄ hydroxylases in rat hepatic microsomes.** ω- and (ω-1)-LTB₄ hydroxylase activities were measured as reported under “Materials and Methods.” The data represent the mean of triplicate determinations. Michaelis-Menten constants, apparent Kₘ and Vₘₐₓ, were calculated by the method of nonlinear regression as described by Cleland (41).
concentration-dependent manner; at 10⁻⁴ M, inhibition of both enzymes activities was approximately 50%. α-Naphthoflavone inhibited only ω-hydroxylation of LTβ₄ at 10⁻⁴ M; inhibition of LTβ₄ ω-hydroxylation was approximately 45%. In contrast, SK&F 525A decreased (ω-1)- but not ω-hydroxylation of LTβ₄; at 10⁻⁴ M inhibition of (ω-1)-hydroxylation was approximately 60%.

Several cytochrome P-450 inducers were evaluated for their effect on LTβ₄ hydroxylation. Hepatic LTβ₄ ω- and (ω-1)-hydroxylases were not induced after either phenobarbital, 3-methylcholanthrene, or WY 14,643 pretreatment (Fig. 6). Renal microsomes also displayed significant ω- (17% of activity in hepatic microsomes) and (ω-1)- (23% of activity in hepatic microsomes) hydroxylase activity. However, renal LTβ₄ ω-hydroxylase, but not (ω-1)-hydroxylase, activity was induced 2-fold by pretreatment with WY 14,643 (Fig. 6). Pretreatment with phenobarbital or 3-methylcholanthrene had no effect on renal LTβ₄ hydroxylases.

DISCUSSION

LTβ₄ is metabolized in microsomal preparations to two products, ω- and (ω-1)-hydroxy LTβ₄. In polymorphonuclear leukocytes, LTβ₄ is extensively metabolized to ω-hydroxy LTβ₄ (10, 30), which is subsequently metabolized to ω-carboxy LTβ₄ (10). In contrast, rat mononuclear cells metabolize LTB₄ to (ω-1)-hydroxyl LTβ₄ (31). Therefore, microsomes represent the only system reported, thus far, in which LTβ₄ is hydroxylated at both the ω- and (ω-1)-carbon. Polymorphonuclear leukocyte LTβ₄ hydroxylases appear to play a major role in regulating LTB₄ concentrations at the inflammatory site. However, the physiologic importance of hepatic LTβ₄ hydroxylases is unknown. Hepatic LTβ₄ hydroxylases may act to clear hepatic tissue and peripheral blood of LTB₄. This may be especially important in inflammatory hepatic diseases. Alternatively, the (ω-1)- and ω-hydroxy metabolites of LTβ₄ may be important in the regulation of normal hepatic physiology.

Several similarities exist between the LTβ₄ ω-hydroxylases found in peripheral blood cells and hepatic microsomes. Both appear to be cytochrome P-450 dependent since LTβ₄ metabolism in both peripheral blood cells (32) and hepatic microsomes (33) is inhibited by carbon monoxide and P-450 inhibitors. Furthermore, the maximal velocities for LTβ₄ ω-hydroxylation in polymorphonuclear leukocytes (34) and hepatic microsomes were nearly identical, 1.2 nmol/min/mg of protein. However, the apparent Kₘ for hepatic LTβ₄ ω-hydroxylases was 40 μM while the apparent Kₘ for polymorphonuclear leukocyte ω-hydroxylases was 0.6 μM (34). There appear to be differences, however, in the subcellular localization of the LTβ₄ ω-hydroxylases. The ω-hydroxylase found in polymorphonuclear leukocyte appears to be associated with the soluble fraction of the cell (35, 36) while hepatic LTβ₄ ω-hydroxylase activity is entirely microsomal. However, recent evidence (34) suggests that subcellular fractionation techniques in polymorphonuclear leukocytes may not have been adequate to conclusively demonstrate that polymorphonuclear leukocyte LTβ₄ ω-hydroxylase was not localized to the microsomal fraction. The LTβ₄ ω-hydroxylases in polymorphonuclear leukocyte and hepatic microsomes appear to respond differentially to inhibitors of cytochrome P-450. For instance, the SK&F 525A inhibited LTβ₄ ω-hydroxylation in polymorphonuclear leukocyte (34) but not hepatic microsomes.

In addition to the distinct differences noted in LTβ₄ ω-hydroxylases between cell types, it appears that the ω- and (ω-1)-hydroxylases act to clear hepatic tissue and peripheral blood of LTB₄. This may be especially important in inflammatory hepatic diseases. Alternatively, the (ω-1)- and ω-hydroxy metabolites of LTβ₄ may be important in the regulation of normal hepatic physiology.

**TABLE II**

Apparent kinetic constants of hepatic ω- and (ω-1)-LTβ₄ hydroxylases for NADPH and NADH

<table>
<thead>
<tr>
<th></th>
<th>NADPH</th>
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<th>NADH</th>
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<tbody>
<tr>
<td></td>
<td>Kₘ</td>
<td>Vₘₐₓ</td>
<td>Kₘ</td>
<td>Vₘₐₓ</td>
</tr>
<tr>
<td></td>
<td>μM</td>
<td>pmol/min/mg protein</td>
<td>μM</td>
<td>pmol/min/mg protein</td>
</tr>
<tr>
<td>ω-LTβ₄</td>
<td>66.1</td>
<td>308.8</td>
<td>867.7</td>
<td>75.9</td>
</tr>
<tr>
<td>(ω-1)-LTβ₄</td>
<td>57.4</td>
<td>18.7</td>
<td>153.7</td>
<td>2.6</td>
</tr>
</tbody>
</table>

**FIG. 5.** In vitro effect of metyrapone, SK&F 525-A, and α-naphthoflavone on rat hepatic microsomal ω- and (ω-1)-LTβ₄ hydroxylases. ω-(○) and (ω-1)-(○) LTβ₄ hydroxylases were measured as reported under "Materials and Methods." Data are expressed as percentage of the respective control. Control values were ω-LTβ₄ = 371 ± 41 and (ω-1)-LTβ₄ = 19.6 ± 2 pmol/min/mg protein.
ysis of LTBl hydroxylation by separate forms of cytochrome P-450 provides the most conclusive evidence in this study for catalysis by different isozymes of cytochrome P-450. Furthermore, the isozymes of P-450 responsible for LTBl hydroxylation superficially appear to be different than those which mediate the hydroxylation of laurate and possibly prostaglandins. The characterization of hepatic LTBl hydroxylases may provide an approach toward the further characterization of LTBl, omega-hydroxylases in polymorphonuclear leukocytes which could eventually lead to the isolation of specific inducers of LTBl, omega-hydroxylases. As first noted by Shak and Goldstein (34), the induction of LTBl, hydroxylases in polymorphonuclear leukocytes, by reducing steady state concentrations of LTBl at the inflammatory site, may be therapeutically beneficial in the treatment of inflammatory disease.

REFERENCES
Hepatic Leukotriene B4 Hydroxylases


