Metabolic Transformations of Leukotriene B₄ in Primary Cultures of Human Hepatocytes

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ABSTRACT

Leukotriene B₄ (LTB₄) is a potent lipid mediator of the inflammatory response whose biological half-life is believed to be mediated principally by metabolism to inactive forms either in the tissue of origin or in the liver. Pathways of metabolic degradation of LTB₄ along with structural identification of metabolites have been elucidated previously in isolated rat liver cells, human keratinocytes, human polymorphonuclear leukocytes, and cultured HepG2 cells. Research advances in human liver transplantation and preservation have made isolated human hepatocytes available for studying the metabolism of LTB₄ in vitro. LTB₄ was added to plated human hepatocytes from three different subjects for 24-h periods whereupon the substrate was analyzed by high-performance liquid chromatography coupled with scintillation counting, UV spectroscopy, and negative ion electrospray ionization tandem mass spectrometry. Each set of hepatocytes yielded a different distribution of metabolites, but several metabolites appeared in all three sets of cells. These central metabolites included the previously identified 20-carboxy-LTB₄ and 18-carboxy-LTB₄, implicating the presence in the liver of specific P-450-mediated ω-oxidation as well as the enzymes involved in β-oxidation from the ω-terminus. Each set of hepatocytes produced the metabolite 10,11-dihydro-20-COOH-LTB₄, a product of the 12-hydroxyeicosanoid dehydrogenase/Δ¹⁰ reductase pathway. Glucuronides of LTB₄ and several metabolites were found, which represents the first description of glucuronidation as a pathway of LTB₄ metabolism. Finally, a series of novel metabolites were observed corresponding to β-oxidation from the carboxyl terminus of LTB₄.

Leukotriene B₄ (LTB₄) is thought to play an important role as a lipid mediator of the inflammatory response and to be a potent chemotactic factor stimulating the human polymorphonuclear leukocyte through a G protein-linked receptor (Yokomizo et al., 1997). LTB₄ is formed from arachidonic acid (released from cell membrane phospholipids by phospholipase A₂) through a series of enzymatic transformations. The direct product of 5-lipoxigenase metabolism of arachidonic acid is leukotriene A₄ (LTA₄) (Ford-Hutchinson et al., 1994; Drazen et al., 1995), which is enzymatically hydrated by LTA₄ hydrolase (EC 1.1.1.1.) (Mueller et al., 1995). It is now recognized that many cells export LTA₄, such as the human polymorphonuclear leukocyte, and that other cells in the immediate vicinity, which contain LTA₄ hydrolase, generate the biologically active LTB₄. The biological half-life of LTB₄ is mediated by metabolic degradation either in the tissue of origin or through hepatic metabolism if LTB₄ escapes into the circulatory system. The rat liver is known to extract LTB₄ from the blood with a high-affinity uptake system (Keppler, 1992) and then metabolize LTB₄ into a variety of products (Shirley and Murphy, 1990). Considerably less is known about the metabolism of LTB₄ in the human subject or from cells derived from human liver.

The metabolism of LTB₄ has been studied in several human cell types including the human polymorphonuclear leukocyte (Shak and Goldstein, 1984; Soberman et al., 1988), keratinocyte (Wheelan et al., 1993), and cultured HepG2 (Wheelan and Murphy, 1995b) among others. Several pathways of LTB₄ metabolism now have been recognized. The first pathway to be characterized was the ω-oxidation of LTB₄ into 20-hydroxy-LTB₄ by cytochrome P-450. The human neutrophil was shown to express a specific NADPH-dependent microsomal P-450 (Kikuta et al., 1993), which now has been termed CYP4F3 and found on human chromosome 19 (Kikuta et al., 1998). This enzyme is also thought to catalyze the formation of 20-carboxy-LTB₄ by further oxidation of the ω carbon atom. Interestingly, this specific cytochrome CYP4F3 is not expressed in rat or human hepatocytes (Romano et al., 1987), but rather a closely related...
cytochrome P-450 of the CYP4F superfamily is in these hepatocytes (Kikut 
a et al., 1994 and Kawashima et al., 1997). LTB₄ is metabolized by P-450-mediated ω-oxidation in the rat hepatocyte to 20-hydroxy-LTB₄, but is then oxidized further by alcohol dehydrogenase- and aldehyde dehydrogenase-dependent reactions into 20-carboxy-LTB₄ (Baumert et al., 1989; Shirley et al., 1992). Detailed studies of the further metabolism of 20-carboxy-LTB₄ in the isolated rat hepatocyte revealed that a second major metabolic pathway, β-oxidation, proceeded after ω-oxidation and after formation of the CoA ester at the carboxyl group at carbon-20, ultimately leading to a chain-shortened and reduced 16-carboxy-LTB₃ (Shirley and Murphy, 1990).

Human cells also have been found to express a third pathway of LTB₄ metabolism termed the 12-hydroxyeicosanoid dehydrogenase/Δ¹¹ reductase pathway (Wainwright and Powell, 1991; Yokomizo et al., 1993), which leads to the initial oxidation of a hydroxy group at carbon-12 to the 12-oxo derivative, which then is reduced at the adjacent double bond, yielding a series of Δ¹⁰,¹¹ dihydro-LTB₄ metabolites (Powell and Gravelle, 1989). This pathway appears to be in competition with the cytochrome P-450 pathway for the LTB₄ substrate. In cells that do not express a specific cytochrome P-450 isozyme capable of acting on LTB₄, the 12-hydroxyeicosanoid dehydrogenase pathway can account for the majority of metabolic products.

Recent developments in human hepatic cell isolation and tissue culture from livers obtained through organ donation have made possible studies of in vitro metabolic transformations under controlled conditions. Such human hepatocyte cultured cells were used to investigate the biochemical conversion of LTB₄ and provide some insight into the potential competent metabolic pathways in human liver cells integrating enzymatic systems located in cytosol, endoplasmic reticulum, mitochondria, and peroxisomes.

**Experimental Procedures**

**Materials.** LTB₄ and [6,7,14,15-²H₄]LTB₄ (d₄ LTB₄) were purchased from Biomol Research Laboratories (Plymouth, PA). [5,6,8,9,11,12,14,15-²H₄]LTB₄ (195 Ci/mol) was purchased from DuPont/New England Nuclear (Boston, MA). All solvents were high-performance liquid chromatography (HPLC) grade and obtained from Fisher Scientific (Paisley, Scotland, UK). Ethanol (99.5%) was obtained from Fisher Scientific (Fair Lawn, NJ).

**Isolation and Culture of Human Hepatocytes.** Human liver samples were obtained through a collaboration with the Organ Bank International for the Advancement of Medicine (Exton, PA). Human livers used for hepatocyte isolation were procured under the United Network for organ-sharing guidelines but determined unsuitable for orthotopic liver transplantation because of physical trauma to a portion of the organ, anatomy, or a high interstitial fat content. On occasion, lobes also were made available for isolation when, because of size considerations, the entire liver was not given to the recipient.

Hepatocytes were isolated by modification of Seglen’s perfusion technique (Seglen, 1976). Briefly, human liver pieces were cannulated and perfused with Hanks’ balanced salt solution (HBSS) without calcium or magnesium ions. This HBSS solution contained 0.46 mM NaCl, 0.4 mM KCl, 0.38 mM CaCl₂, 0.44 mM MgSO₄, and 10 mM HEPES buffer (pH 7.4). The liver was gently teased apart and the suspension was filtered through a 100-μm nylon mesh and washed three times in ice-cold L-15. Hepatocytes were plated at densities of 3 to 5 × 10⁴ cells/cm² on modified culture plates with a minimal essential medium/Waymouth's medium, 3:1 (Sigma, St. Louis, MO) containing 10% fetal bovine serum (HyClone Laboratories, Inc., Logan, UT). These modified culture plates consisted of surface-modified polystyrene containing a high density and variety of nitrogen and oxygen containing functional groups (Primera; Becton Dickinson, Franklin Lakes, NJ). These functional groups on the plate surface are believed to enhance cell adhesion onto the plate (Chilkti et al., 1995). After an 18- to 24-h incubation, cells were placed in a serum-free, defined medium to maintain the stable differentiation of normal human hepatocytes (Ledley et al., 1993). Modified culture dishes and tyrosine-free media were used to select against the proliferation of fibroblasts and nonparenchymal cells. Hepatocytes were incubated in a gas mixture of 95% air/5% CO₂ in humidified 37°C. Once confluent, the media were changed to enhance cell adhesion onto the plate (Chilkti et al., 1995).

**Incubation of Human Hepatocytes with LTB₄.** LTB₄ and [³H]LTB₄ were evaporated to dryness under nitrogen and reconstituted in HBSS to a final concentration of 12 μM LTB₄ (0.2 μCi/ml). Culture media were removed from the plated hepatocytes and replaced with HBSS containing LTB₄ media (3 ml for hepatocytes in wells and 10 ml for hepatocytes in 75-cm² culture flasks) and then incubated at 37°C for 24 h. The HBSS incubation buffer had been used in previous rat hepatocyte studies (Shirley and Murphy, 1990; Wheelan and Murphy, 1995a) to reduce binding of LTB₄ to extracellular proteins in serum-containing buffers. Light microscopic examination of cells after the 24-h LTB₄ light incubation did not reveal any morphologic alternations. Supernatants were decanted and the hepatocytes were washed with ice-cold ethanol (3–5 ml) several times, with the ethanol washes added to supernatants. The supernatant/ethanol solution was brought to 80% ethanol and kept at 0°C for at least 3 h to allow precipitation of protein. Samples were centrifuged and supernatants were decanted and evaporated to near dryness by rotary evaporation. Samples were reconstituted in reversed-phase (RP)-HPLC solvents at proportions of initial gradient, filtered, and kept at 0°C until analysis.

**Metabolite Purification.** Samples were analyzed by RP-HPLC using an Ultrapac C₁₈ column (4.6 × 250 mm; Phenomenex, Torrance, CA). The initial mobile phase consisted of methanol/aqueous 0.05% acetic acid (8.7 mM), with pH adjusted to pH 5.0 with ammonium hydroxide (10/90) at a flow rate of 1 ml/min. A linear gradient was started immediately to 70% methanol at 60 min followed by a second linear gradient to 95% methanol at 75 min. Column effluent was monitored using UV detection and on-line radioactivity monitoring. For analysis of samples by electrospray mass spectrometry, an Ultrapac C₁₈ column (1.0 × 150 mm) was used with the above mobile phase conditions at a flow rate of 50 μl/min, and the effluent was introduced into the mass spectrometer by a 0.5-m, 50-μm fused silica capillary.

**Mass Spectrometry.** Mass spectrometry was performed on a Scien Sci API III® triple quadrupole mass spectrometer (Perkin-Elmer Sciex, Ontario, Canada) operating in the negative ion mode with a spray voltage of −2600 to −3400 V and an orifice voltage of −60 V. Highly purified air was used as nebulizer gas to reduce the glow discharge at these negative voltages. Collisionally induced decomposition (CID) spectra were obtained using an offset potential of 20 eV and argon as the collision gas at a thickness equivalent to 200 × 10¹³ molecules/cm². Mass spectra were obtained by scanning from m/z 100 to 1000 in 3 s as previously described (Wheelan and Murphy, 1995b).

**Results**

Incubation of LTB₄ (12 μM in 3 ml of media) for 24 h with isolated human hepatocytes (1 × 10⁶) resulted in recovery of
60.7% of LTB₄ or LTB₄ metabolites in the supernatant as determined by recovery of radioactivity. RP-HPLC analysis with radioactivity monitoring revealed numerous LTB₄ metabolites (Fig. 1A). In one series of experiments with a single human hepatocyte preparation, eight separate flasks were prepared with identical aliquots of cells plated into the serum-free, defined media. After an initial 24-h incubation, one flask was taken for an LTB₄ incubation and metabolite profile analysis. At 24-h sequential intervals for 1 week, an additional flask was taken for LTB₄ metabolite profiling. There was no difference observed in the metabolic profile for any of the time points, suggesting no major cellular differentiation altered LTB₄ metabolism while culturing cells in the defined media.

Several metabolites displayed a UV absorption spectrum indicative of a conjugated triene structure with an absorption maximum at 270 nm (Fig. 1B), whereas others contained a conjugated diene structure as evidenced by an absorption maximum at 230 nm (Fig. 1C). Negative ion electrospray mass spectral analysis of metabolites resulted in identification of several previously known metabolites as well as elucidation of several novel structures.

**Metabolite A, 18-COOH-LTB₄.** The RP-HPLC retention time, triene chromophore, and molecular anion at m/z 337 for metabolite A suggested the previously identified LTB₄ metabolite, 18-COOH-LTB₄. The CID mass spectrum of the molecular anion generated by electrospray ionization has not been reported previously and contained many of the fragment ions observed in the CID mass spectrum of LTB₄ (Wheelan et al., 1996a), including abundant ions at m/z 195, m/z 71, and m/z 59 and a lesser abundant ion at m/z 129 (Fig. 2A). A fragment ion observed at m/z 141, analogous to m/z 169 observed in the CID mass spectrum of 20-COOH-LTB₄, was most likely a result of fragmentation of the C(11)-C(12) bond with formation of an aldehyde and charge retention on the α-terminus. Fragmentation of the same bond with charge retention on the carboxyl terminus results in formation of the ion at m/z 195. An abundant ion at m/z 206 was most likely formed by an oxy-Cope rearrangement with loss of H₂O as observed previously in the formation of odd electron fragment ions containing the 3-OH 1,5-diene moiety (Fig. 3) (Wheelan et al., 1996b).

**Metabolite B, 10,11-Dihydro-18-COOH-LTB₄.** A relatively minor yet novel metabolite was observed eluting with slightly longer HPLC retention time than 18-COOH-LTB₄ (Fig. 1A). This metabolite retained a diene chromophore and a molecular anion at m/z 339, two mass units higher than that for 18-COOH-LTB₄. This suggested saturation of one double bond present in the triene unit of 18-COOH-LTB₄. In addition to ions at m/z 321 (loss of H₂O), m/z 303 (loss of 2H₂O), and m/z 277 (loss of H₂O and CO₂), the CID mass spectrum of metabolite B contained abundant ions at m/z 115 and m/z 141 (Fig. 2B). The ion at m/z 141 likely was identical...
with that formed after collisional activation of the 18-COOH-LTB₄ anion whereas the presence of an abundant ion at m/z 115 had been recognized previously as indicative of 10,11-dihydro LTB₄ metabolites (Wheelan et al., 1996a). This ion was reported to arise from the cleavage of the C(5)-C(6) bond with transfer of the hydroxyl proton, formation of a C-5 aldehyde, and charge retention at C(1). These data suggested that metabolite B differed from 18-COOH-LTB₄ by saturation of the 10,11 double bond and that metabolite B was 10,11-dihydro18-carboxy-LTB₄.

Metabolite C, Glucuronide Conjugate of 20-COOH-LTB₄. With an observed molecular anion at m/z 541 and the presence of a conjugated triene chromophore, the structure of metabolite C was consistent with a novel glucuronide conjugate of 20-COOH-LTB₄. The CID mass spectrum showed characteristic ions at m/z 347 (loss of H₂O), m/z 329 (loss of 2H₂O), and m/z 303 (loss of H₂O and CO₂) as well as significant ions resulting from C(11)-C(12) bond fragmentation with charge retention on the carboxyl terminus (m/z 195) or on the ω-carboxyl terminus (m/z 169). Abundant ions also were observed at m/z 141 [C(12)-C(13) bond fragmentation, ω-carboxyl terminus charge] and at m/z 129, 115, 347, 329, and 303.

Metabolite D, 12-Oxo-10,11-Dihydro-20-COOH-LTB₄. Metabolite D eluted by RP-HPLC approximately 3 min earlier than standard 20-COOH-LTB₄ yet the observed molecular anion, m/z 365, was identical with that of 20-COOH-LTB₄. The presence of a conjugated diene chromophore for this metabolite instead of a triene chromophore suggested a 10,11-dihydro metabolism and, in combination with the molecular weight, also suggested the presence of the C(12) oxo moiety. Also, a 12-oxo-10,11-dihydro modification of 20-COOH-LTB₄ would be expected at an earlier RP-HPLC retention time when only methanol was used as the organic phase (Wheelan et al., 1993). The CID mass spectrum displayed fragment ions at m/z 347 (loss of H₂O) and m/z 303 (loss of H₂O and CO₂) (Fig. 4A). A prominent fragment ion at m/z 115 was consistent with a 10,11-dihydro modification resulting in fragmentation of C(5)-C(6) with charge retention at C(1), and the fragment ion at m/z 249 was likely a result of the same fragmentation but with charge retention at C(20).

Metabolite E, 20-COOH-LTB₄. The RP-HPLC retention time, UV triene chromophore, and molecular anion at m/z 365 for the radioactive metabolite eluting at 39 min were identical with authentic 20-COOH-LTB₄. The CID mass spectrum showed characteristic ions at m/z 347 (loss of H₂O), m/z 329 (loss of 2H₂O), and m/z 303 (loss of H₂O and CO₂) as well as significant ions resulting from C(11)-C(12) bond fragmentation with charge retention on the carboxyl terminus (m/z 195) or on the ω-carboxyl terminus (m/z 169). Abundant ions also were observed at m/z 141 [C(12)-C(13) bond fragmentation, ω-carboxyl terminus charge] and at m/z 129, 115, 347, 329, and 303.

Fig. 3. Mechanism for the formation of m/z 206 after collisional activation of the molecular anion (m/z 337) of 18-COOH-LTB₄ (metabolite A). Initial rearrangement of the 3-OH 1,5-diene structure by an oxy-Cope rearrangement followed by homolytic fragmentation and loss of water would produce the odd electron fragment ion at m/z 206.

Fig. 4. Product ions obtained from the collisional activation of the carboxylate anion of metabolite D (m/z 365), identified as 12-oxo-10,11-dihydro-20-COOH-LTB₄ (A), and the carboxylate anion of metabolite F (m/z 367) (B).
which also is observed in the CID mass spectrum of LTB₄ (Wheelan et al., 1996a).

Metabolite F, 10,11-Dihydro-20-COOH-LTB₄. Metabolite F, at a slightly longer RP-HPLC retention time than 20-COOH-LTB₄, displayed a diene chromophore and a molecular anion at m/z 367, 2 Da higher than 20-COOH-LTB₄. This suggested the saturation of one double bond in the conjugated triene of 20-COOH-LTB₄. The CID mass spectrum revealed a prominent ion at m/z 115 [C(5)-C(6) bond fragmentation], which is characteristic of the 10,11-dihydro-LTB₄ structure (Fig. 4B). The fragment ion at m/z 251 also was likely due to the same bond fragmentation but with charge localized on the ω-terminus. Fragment ions at m/z 141 and m/z 169 also were observed in the CID mass spectrum of 20-COOH-LTB₄ and result from charge localization at the ω-carboxyl terminus (Wheelan and Murphy, 1995b). These data were consistent with a structure of metabolite F as 10,11-dihydro-20-carboxy-LTB₄.

Metabolite G, 18-COOH-10-Oxo-4,6,12-Octadecatrienoic Acid. The molecular anion for metabolite G at m/z 321, 44 atomic mass units (amu) less than for metabolite D, suggested a chain-shortened metabolite resulting from β-oxidation at the carboxyl terminus with loss of the C(5) hydroxyl substituent. The diene chromophore for this metabolite also was consistent with a chain-shortened analog of 12-ooxo-10,11-dihydro-20-COOH-LTB₄. The most prominent ion in the CID mass spectrum, m/z 277, most likely resulted from loss of CO₂, and a second loss of CO₂ was suggested by the ion at m/z 233 (Fig. 5A). The facile loss of 44 amu for the chain-shortened metabolites, observed earlier in the CID spectra of 10-hydroxy-4,6,8,12-octadecatetraenoic acid (10-HOTE) and 10-hydroxy-4,6,12-octadecatrienoic acid (10-HOTrE) (Wheelan and Murphy, 1995b), also was apparent in the low-mass ions at m/z 135 and 123. The ion at m/z 135 was likely due to fragmentation of the C(10)-C(11) bond of the C(1) carboxylated anion whereas the ion at m/z 123 may have arisen by fragmentation of the C(9)-C(10) bond of the ω-terminus carboxylated anion. This metabolite, identified as 18-carboxy-10-ooxo-4,6,12-octadecatrienoic acid, has not been observed in previous in vitro metabolic studies of LTB₄.

Metabolite H, 18-COOH-10-hydroxy-4,6,12-octadecatrienoic Acid. Even though it is a minor component, the molecular anion of metabolite H at m/z 323 was readily observed, suggesting β-oxidation from the carboxyl terminus and a structure similar to metabolite G. The two additional protons in this molecule suggested reduction of the ketone moiety in metabolite G and the presence of a C(10) hydroxyl substituent. Product ions observed in the CID mass spectrum were consistent with fragmentation of the C(10)-C(11) bond and formation of a terminal alkene and an ω-terminal carboxylate anion (m/z 141). Cleavage of the C(9)-C(10) bond would lead to formation of a terminal aldehyde and the ω-terminal carboxylate anion (m/z 169) (Fig. 5B). The UV data (λ_max = 230 nm) and electrospray tandem mass spectrometry were consistent with identification of this previously unidentified metabolite as 18-carboxy-10-hydroxy-4,6,12-octadecatrienoic acid.

Metabolites I and K, Glucuronide Conjugates of LTB₄. Metabolites I and K both displayed triene chromophores and a molecular anion at m/z 511. Decomposition of the molecular anion revealed loss of 176 Da with formation of a fragment ion at m/z 335. This neutral ion loss, vide supra, UV spectra, and molecular weight were consistent with isomeric glucuronide conjugates of LTB₄. The exact position of the glucuronide attachment could not be ascertained in this experiment.

Metabolite J, Glucuronide Conjugate of 10,11-Dihydro-18-COOH-LTB₄. Metabolite J displayed a molecular anion during electrospray ionization at m/z 513, which, upon collisional activation, fragmented to m/z 337. This was consistent with the glucuronide conjugate of the previously identified metabolite, 10,11-dihydro-LTB₄. No further structure information was possible for this metabolite because of the inability to yield structurally relevant ions indicative of the position of glucuronide conjugation.

Human hepatocytes in two additional hepatocyte preparations were incubated with [6,7,14,15-²H₄]LTB₄, unlabeled LTB₄, and the stable isotopically labeled [6,7,14,15-²H₄]LTB₄ (d₄-LTB₄). Recovery of LTB₄ in the cell supernatant (based on radioactivity) was 90.8%. Analysis of cell supernatants by RP-HPLC with radioactivity monitoring showed more extensive metabolism than observed in the first experiment, with most of the radioactivity eluting within the first 10 min. Many of the metabolites identified in the first experiment...
were also present, and the ratio of $d_0:d_4$ of the molecular anions was used to verify these metabolites as derived from LTB$_4$. The ratio of $d_0:d_4$ LTB$_4$ used in the incubation was approximately 100:75 as assessed from the ratio of molecular anions at $m/z$ 335 and 339 after mass spectral analysis of the initial incubation media. An identical ratio was observed for $m/z$ 337:341 at the appropriate RP-HPLC retention time for metabolite A, 18-COOH-LTB$_4$. Likewise, 10,11-dihydro-18-COOH-LTB$_4$ ($m/z$ 541:545) and two peaks at $m/z$ 365:369 corresponding to metabolites D and E, and ions at $m/z$ 367:371 for 10,11-dihydro-20-COOH-LTB$_4$, also showed the expected ratio of $d_0:d_4$ for LTB$_4$-derived metabolites. Two peaks also were observed for the glucuronides conjugates of LTB$_4$ and displayed the same ratio of ions at $m/z$ 511:515 as the starting $d_0:d_4$ LTB$_4$. Collisional activation of both of these ions resulted in the expected loss of 176 amu with formation of ions at $m/z$ 335 and 339, respectively, corresponding to the molecular anions of LTB$_4$ and $d_4$-LTB$_4$ (Fig. 6).

Metabolites L and M, 6-Cysteiny1-5,12-Dihydroxy-7,9,14-Eicosatrienoic Acid, e-LTB$_3$. Two additional metabolites, not identified in the first experiment, were identified by the ratio of $d_0:d_4$ molecular anions at RP-HPLC retention times slightly longer than the two glucuronide conjugates of LTB$_4$. Both peaks contained the molecular anion at $m/z$ 456, with $m/z$ 460 for the deuterated analog, suggesting the addition of cysteine to LTB$_4$. The presence of two such metabolites separated approximately 2 min in the RP-HPLC analysis (Table 1) was consistent with stereoisomeric compounds, and both fragmented after collisional activation to identical mass spectra. The CID mass spectra revealed prominent ions resulting from loss of 87 amu, fragmentation of the cysteine moiety, from the molecular anions resulting in ions at $m/z$ 369 and $m/z$ 373 for the unlabeled and $d_4$-labeled metabolites, respectively (Fig. 7. Loss of H$_2$O from these two ions was also observed, resulting in ions at $m/z$ 351 and $m/z$ 355. Loss of the cysteine moiety likely was responsible for the observed ion at $m/z$ 335 with additional losses of H$_2$O ($m/z$ 317) and CO$_2$ ($m/z$ 273) as observed in the CID mass spectrum of LTB$_4$. The collisional mass spectrum of the $d_4$ metabolite revealed an ion at $m/z$ 339, consistent with the expected molecular anion of $d_4$ LTB$_4$, and a loss of 19 amu from this ion at $m/z$ 320, suggesting the loss of H$_2$O also involved one of the deuterated positions. Loss of the cysteine moiety with charge retained on the amino acid rather than on the LTB$_4$ backbone also was apparent as evidenced by the ion at $m/z$ 120. A fragment ion at $m/z$ 229 also was observed in the CID mass spectrum of a previously identified dipeptide conjugate of LTB$_4$, $d$-LTB$_3$ (Wheelan et al., 1993). Formation of this ion likely was a result of fragmentation of the C(11)-C(12) bond of the initially formed $m/z$ 369 ion. This ion was shifted by 2 amu to $m/z$ 231 in the CID mass spectrum of the deuterated analog, reflecting the presence of two deuterium atoms, at positions C(6) and C(7), and loss of two deuterium atoms, at positions C(14) and C(15). The ion at $m/z$ 195 also was present in the CID mass spectrum of LTB$_4$ and likely resulted from initial loss of the cysteine moiety followed by fragmentation of the C(11)-C(12) bond as for LTB$_4$ (Wheelan et al., 1996a). This ion was shifted to $m/z$ 197 in the CID mass spectrum of the deuterated analog, consistent with the C(1)-C(11) backbone structure.

An additional incubation in 75-cm$^2$ flasks of LTB$_4$ with hepatocytes from a third donor resulted in recovery of 76.6% of radioactivity in the supernatant after a 24-h incubation. Several metabolites that were identified in the first experiments were present, including 20-COOH-LTB$_4$ (metabolite E), 10,11-dihydro-20-COOH-LTB$_4$ (metabolite F), 18-COOH-10-oxo-4,6,12-octadecatrienoic acid (metabolite G), and glucuronide conjugates of LTB$_4$ (metabolites I and K). More abundant metabolites were observed at longer RP-HPLC retention times including unmetabolized LTB$_4$. Mass spectral analysis of these metabolites revealed structures that likely were intermediates in the metabolic process and were identical with previously identified 12-oxo-10,11-dihydro-LTB$_4$ (metabolite O), 10,11-dihydro-LTB$_4$ (metabolite P), 10-HoTrE (metabolite Q), and 10-HoTrE (metabolite R). One chain-shortened metabolite eluting at 20 min was identified as 4-hydroxy-12-carboxy-6-dodecenoic acid (N, data not shown). A summary of the metabolites observed in the three separate incubations is shown in Table 1.

**Discussion**

A number of specific metabolic pathways have been described in animal as well as human cells that can participate in the metabolism of LTB$_4$. These include pathways
mediated by specific cytochrome P-450 isozymes, alcohol dehydrogenase, 12-hydroxyeicosanoid dehydrogenase, as well as β-oxidation from both the C-1 carboxyl and methyl terminus of LTB 4. The combination of these numerous pathways results in a complex profile of metabolites observed during in vitro incubations of LTB4 with isolated cells (Murphy and Wheelan, 1997; Wheelan and Murphy, 1998). A central role for hepatic metabolism of LTB4 has emerged with studies of a high-affinity uptake system for LTB 4 (Keppler, 1992). Initial studies of the metabolic transformation of LTB 4 in isolated rat hepatocytes suggested a predominant pathway for cytochrome P-450-dependent metabolism because all metabolites were derived from initial β-oxidation with no evidence of an operational 12-hydroxy dehydrogenase eicosanoid pathway. The majority of metabolites in rat hepatocytes were the result of a combination of cytochrome P-450-dependent oxidation followed by β-oxidation from the ω-terminus. Detailed metabolic studies with human cells such as human keratinocytes, macrophages, and lung-derived cells have suggested that the 12-hydroxyeicosanoid dehydrogenase pathway may play a more important role in nonhepatic human cells.

Investigation of the metabolic disposition of LTB4 in human subjects or even within human hepatic tissue has been somewhat difficult to pursue. Recent developments in isolation and maintenance of isolated human hepatocytes (Seglen, 1976; Ledley et al., 1993) have enabled studies of LTB4 metabolism in vitro with human hepatocytes. The results of these studies suggest that some variability exists in ultimate metabolism of LTB4 between each hepatocyte preparation. Significant interindividual variation in biotransformation of xenobiotics has been reported in primary human hepatocyte cultures (Straub et al., 1987). Such differences had been attributed to natural polymorphisms in enzymatic pathways and specific enzymes that arise in outbred populations. Such variations have been suggested to be responsible for individual differences in responses to toxicological and environmental exposures including particular adverse reactions. Nonetheless, it is clear that these hepatocytes express many of the

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<th>TABLE 1</th>
<th>Summary of observed metabolites of LTB4 formed after incubation (12 μM) with isolated human hepatocytes (1 × 10⁶ cells) for 24 h²</th>
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<td>Metabolite</td>
<td>Incubation</td>
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<tr>
<td>A 18-COOH-LTB4</td>
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<td>B 10,11-Dihydro-18-COOH-LTB4</td>
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<td>C 20-COOH glucuronide</td>
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<td>E 20-COOH-LTB4</td>
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<tr>
<td>F 10,11-Dihydro-20-COOH-LTB4</td>
<td>I, II, III</td>
</tr>
<tr>
<td>G 18-COOH-10-oxo-4,6,12-octadecatetraenoic acid (18-COOH-10-oxo-OctE)</td>
<td>I, II, III</td>
</tr>
<tr>
<td>H 18-COOH-10-hydroxy-4,6,12-octadecatetraenoic acid (18-COOH-10-hydroxy-OctE)</td>
<td>I</td>
</tr>
<tr>
<td>I LTB4 glucuronide</td>
<td>I, II</td>
</tr>
<tr>
<td>J 10,11-Dihydro-LTB4 glucuronide</td>
<td>I, III</td>
</tr>
<tr>
<td>K LTB4 glucuronide</td>
<td>I, II, III</td>
</tr>
<tr>
<td>L 6-Cysteinyl-5,12-dihydroxy-7,9,14-eicosatrienoic acid</td>
<td>II</td>
</tr>
<tr>
<td>M 6-Cysteinyl-5,12-dihydroxy-7,9,14-eicosatrienoic acid</td>
<td>II</td>
</tr>
<tr>
<td>N 4-Hydroxy-12-COOH-6-dodecenoic acid (12-COOH-4-HDME)</td>
<td>III</td>
</tr>
<tr>
<td>O 12-Oxo-10,11-dihydro-LTB4</td>
<td>III</td>
</tr>
<tr>
<td>P 10,11-Dihydro-LTB4 (10,11-DH-LTB4)</td>
<td>III</td>
</tr>
<tr>
<td>Q 10-HOTE</td>
<td>III</td>
</tr>
<tr>
<td>R 10-HOTRE</td>
<td>III</td>
</tr>
</tbody>
</table>

² Incubation in three separate preparations of human hepatocytes.

² Percentage of initial [3H]LTB4 added to isolated hepatocytes. The numbers represent the observed range in two to three experiments or the percentage from one experiment.

c Carboxylate anion (negative ion) obtained by electrospray ionization.

d Abbreviation used for oxidized, unsaturated fatty acids follows previously published nomenclature rules (Smith et al., 1990).

Fig. 7. Product ions obtained after collisional activation of the carboxylate anion of metabolite L, 6-cysteinyl-5,12-dihydroxy-7,9,14-eicosatrienoic acid (e-LTB₄), from the unlabeled metabolite precursor ion at m/z 456 (A) and from the deuterated metabolite precursor ion at m/z 460 and deuterium label (d) at carbon atoms 6,7,14, and 15 (B).
enzymes responsible for LTB₄ metabolism observed in other animal as well as human cell types. Therefore, some qualitative insight into the potential for LTB₄ metabolism within human hepatocytes was obtained after detailed structural analysis of the metabolites formed in these experiments. For this purpose, electrospray tandem mass spectrometry proved to be of great value in providing critical information to suggest structural assignment of these metabolites. This information in combination with UV absorption and UV retention time was used to identify more than 20 different metabolites of LTB₄ formed by cultured human hepatocytes after in vitro incubation (Table 1).

One of the features of LTB₄ metabolism in cultured human hepatocytes was that overall metabolism was relatively slower than it was in freshly isolated hepatocytes. This might suggest the loss of enzymatic activity in the human hepatocyte preparations and uncontrolled variables that could contribute to this included cell density in plating, cell viability, and preparation delays before suspension experiments. The LTB₄ metabolite profile in the rat was largely determined by cytochrome P-450-dependent ω-oxidation. There are numerous examples of the loss of specific cytochrome P-450 isozymes from cells in culture (Glatt et al., 1987; Utesch and Oesch, 1992), and possibly a loss of specific cytochrome P-450 isozymes from cells in culture (Kumlin and Dahleñ, 1990). This has been found to be of great value in providing critical information to suggest structural assignment of these metabolites. For this purpose, electrospray tandem mass spectrometry proved not to be ascertained, several isomers were observed, suggesting that each hydroxyl group could be conjugated as well as the possibility of formation of acyl glucuronides. These results suggest that glucuronide conjugation may play a more important role in the elimination of LTB₄ and its metabolites into the urine of humans. Interestingly, primary cultures of hepatocytes have been found to retain both glucuronide and sulfate conjugation pathways of xenobiotics and possibly provide a faithful picture of phase II reactions that occur in vivo (Mertes et al., 1985; Mennes et al., 1994).

Another pathway for LTB₄ metabolism is β-oxidation from the carboxyl terminus. Previous studies of LTB₄ metabolism with HepG2 cells (Wheelan and Murphy, 1995b) revealed this route of β-oxidation as the most prevalent with formation of 10-HOTE. The metabolic events leading to this metabolite are poorly understood and involve loss of the oxygen substituent at C-5. As summarized in Fig. 8, some of the more abundant metabolites (Table 1, 18-COOH-10-oxoOTrE and 18-COOH-10-HOTrE) must arise from involvement of ω-oxidation, the 12-hydroxyeicosanoid dehydrogenase pathway, as well as β-oxidation from the carboxyl terminus with loss of the C-5 oxygen atom. Thus, human hepatocytes express multiple enzymatic pathways to metabolically transform LTB₄. Although a quantitative picture of LTB₄ metabolism in the human liver in vivo cannot be ascertained from these studies, it is clear that in the human hepatocyte, LTB₄ is processed sequentially by multiple oxidative pathways as well as by glucuronidation.

References
Chilkoti A, Schmierer AE, Perez-Luna VH and Ratnir BD (1995) Investigating the relationship between surface chemistry and endothelial cell growth: Partial least-


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