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# IDENTIFICATION AND BIOLOGICAL ACTIVITY OF DIHYDRO-LEUKOTRIENE $B_4$: A PROMINENT METABOLITE OF LEUKOTRIENE $B_4$ IN THE HUMAN LUNG

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Exogenous [3H]leukotriene $B_4$ ($LTB_4$) was converted into several polar and non-polar metabolites in the chopped human lung. One of the major metabolites was identified as 5(S),12-dihydroxy-6,8,14-eicosatrienoic acid (10,11-dihydro-$LTB_4$) by means of co-chromatography with authentic standards, ultraviolet spectrometry and gas chromatography-mass spectrometry. Analysis on chiral straight phase HPLC revealed the presence of both the 12(S) and 12(R) epimers of dihydro-$LTB_4$. Dihydro-$LTB_4$ was also formed from endogenously generated $LTB_4$ in ionophore A23187 stimulated incubations. The dihydro metabolites were approximately 100 times less potent than $LTB_4$ in causing guinea pig lung strip contraction and leukocyte-dependent inflammation in the hamster cheek pouch in vivo. 

In a previous study on the metabolism of leukotrienes (LT) in human lung (1), we described the conversion of the inflammatory mediator $LTB_4$ (2) into ω-oxidized products and two unknown metabolites. The structure and some selected biological activities of one of these metabolites are described here.

The metabolism of $LTB_4$ into ω-oxidation products, which is catalyzed by a cytochrome P-450 enzyme, has been documented in human polymorphonuclear leukocytes (3-7), but this conversion may not always lead to biological inactivation (3, 8, 9). More recently, an alternative pathway for the catabolism of $LTB_4$ was described in different rodent cells (10, 11). That particulate metabolite of $LTB_4$ was formed via a reductase pathway, and a similar metabolite was identified in porcine leukocytes as 5,12-dihydroxy-6,8,14-eicosatrienoic acid (10,11-dihydro-$LTB_4$) (12). Dihydro-$LTB_4$ has not been found in human leukocytes, but its formation was implicated in lung macrophages (13). However, dihydro-$LTB_4$ has not been fully characterized in human tissues.

## MATERIALS AND METHODS

Total synthesis of 5(S),12(S)- and 5(S),12(R)-dihydroxy-6(Z),8(E),14(Z)-eicosatrienoic acids was performed by Wittig coupling of segments derived from 2-deoxy-D-ribose and L-glutamic acid as described (14). [5,6,8,9,11,12,14,15-$^3$H]$LTB_4$ (200 Ci/mmol) was from NEN, U.K. and arachidonic acid was purchased from Nu-Chek Prep., Elysian, MN, U.S.A.

Human lung tissue were from patients undergoing surgery and the specimens were processed and incubated as described (1). Products were extracted on Sep-Pak $C_{18}$ cartridges (eluted with methanol, *cf.* Ref. 15) or analyzed directly by reverse phase (RP)- HPLC (Nova-

0006-291X/90 $1.50

Pak $C_{18}$, 150 x 4.6 mm, flow rate 1 ml/min). The mobile phases were mixtures of methanol / water / acetic acid (58/42/0.01, v/v/v, system A) or acetonitrile / water / acetic acid (38/62/0.01, v/v/v, system B). Separation of the two C-12 epimers of dihydro-$LTB_4$ as methyl esters was performed on straight phase (SP)-HPLC using a Bakerbond chiral column (250 x 4.6 mm), comprised of (R)-N-3,5-dinitrobenzoylphenylglycine ionically bonded to 5 μ aminopropyl silica, eluted with isopropanol / hexane (4/96, v/v, 1 ml/min). Mass spectra of the methyl ester trimethylsilyl ether (Me-$Me_3Si$) derivatives were obtained with a HP model 5970B mass selective detector connected to a HP model 5890 gas chromatograph.

Spasmogenic activity was assayed in the guinea-pig lung parenchyma under non-flow conditions (16). All compounds were added as single doses and the responses were expressed in percent of the tissue maximum. Microvascular responses were studied using in vivo microscopy of the hamster cheek pouch as described (17). Briefly, vascular permeability was quantitated by counting FITC-dextran (given i.v.) extravasation and expressed as leakage sites per $cm^2$. The number of extravascularly emigrated leukocytes was counted in the tissue before and 40 min after challenge The compounds were given topically in the presence of the vasodilator prostaglandin $E_2$ (30 nM ).

## RESULTS AND DISCUSSION

***Metabolism of exogenous [$^3$H]LTB$_4$.*** The time course of the conversion of [$^3$H]$LTB_4$ (0.1 μCi) was studied. The results were similar using tracer amounts (0.2 nM) or 600 nM substrate concentration. After precipitation of proteins with ethanol, 86 ± 2 % (mean ± SE, n=17) of the radioactivity added to the incubation (initial radioactivity) was recovered in the supernatants. Essentially all of this eluted with methanol from Sep-Pak $C_{18}$ cartridges. On RP-HPLC (system A, see Fig. 1) about 75 % of the initial radioactivity was associated with five main peaks with a profile changing with time (Fig. 1). The remainder of the radioactivity consistently eluted in the solvent front and the methanol eluate from the HPLC column.

The amount of radioactivity associated with the respective peaks, expressed as percent of total radioactivity eluting in these fractions, changed considerably during 90 min of incubation. Thus, $LTB_4$ decreased from 84 (72-90)% to 7 (4-9)% (means and range from 3 experiments), whereas two less polar metabolites appeared (met II and met III, see Fig. 1). None of these metabolites were present at time zero, but at 90 min met II made up 19 (18-20)% and met III 13 (11-14)%. An earlier eluting peak (met I, Fig. 1) accounted for 11 (3-21)% at 0 min and 23 (19-28)% at 90 min. A polar fraction, corresponding to the retention time of ω–oxidized metabolites of $LTB_4$ (Fig. 1), increased from 2 (0-4)% to 38 (31-42)%. Some of this polar radioactivity could be due to non-enzymatic conversion, since it was was not always clearly distinguishable from the solvent front.

In solvent system A, $LTB_4$ was the only peak absorbing at 270 nm (Fig. 1). The ω-oxidation products were imperfectly separated from the solvent front, but using a more polar solvent system (with increased content of water), this material could be separated into several peaks. One of these showed a characteristic leukotriene spectrum with $\lambda_{max}$ at 270 nm and shoulders at 260 and 280 nm, indicating that some ω-oxidation of $LTB_4$ occurred in the human lung. This fraction was not further characterized. However, in contrast to the rapid metabolism of $LTB_4$ into ω-hydroxylated metabolites in polymorphonuclear leukocytes (5), the polar products in the human lung did not exceed $LTB_4$ until after 60 min of incubation (Fig. 1). The other metabolites all showed single uv-absorption maxima at 230 nm, indicating the presence of a diene structure due to a saturation of one of the three conjugated double bonds in $LTB_4$. Met I eluted before $LTB_4$ in

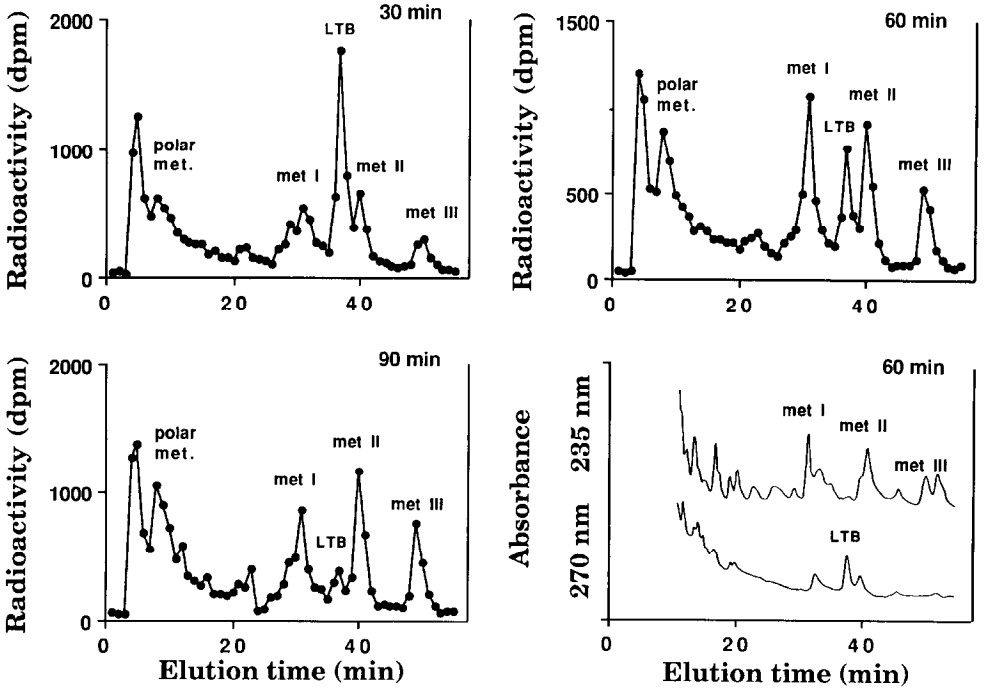



Fig.1. RP-HPLC (system A) of products formed in incubations of chopped lung with 600 nM [$^3$H-LTB4] for 30, 60 and 90 min. Radioactivity in chromatographic fractions was determined by liquid scintillation counting. Lower right panel shows uv-absorbance at 235 nm and 270 nm of products formed during 60 min of incubation, monitored with a HP 1040A diode array detector with uv-spectra recorded consecutively during runs.

solvent system A (Fig. 1), but in solvent system B (not shown), this metabolite eluted after $LTB_4$ with a retention time in accordance with the 12-oxo-dihydro metabolite of $LTB_4$ reported in porcine leukocytes (12). Therefore, met I was tentatively identified as 12-oxo-10,11-dihydro-$LTB_4$. The further identification of met II as dihydro-$LTB_4$ is described below. Met III eluted after $LTB_4$ on RP-HPLC in both solvent system A (Fig. 1) and B, and did not share characteristics with any other known metabolite of $LTB_4$. It was not possible to obtain sufficient amount of material for mass spectrometric analysis of either met I or met III.

Interestingly, when lung homogenates (800 x g supernatants) were used for incubations instead of chopped lung, there was no conversion of [$^3$H]$LTB_4$ into the metabolites I, II or III, and a proportionally larger part of the radioactivity was recovered as intact $LTB_4$ after 90 min of incubation. These results indicate that metabolites I-III are formed via similar mechanisms. In line with these observations, Kaever et al. (11) found that dihydro-$LTB_4$ was formed in whole cell preparations but not in cytosolic fractions of rat mesangial cells.

***Identification of metabolite II as 5(S),12(R,S)-dihydroxy-6,8,14-eicosatrienoic acid (10,11-dihydro-$LTB_4$).*** Met II coeluted with both the synthetic 5(S),12(S)- and 5(S),12(R)-dihydroxy-6,8,14-eicosatrienoic acid in two RP-HPLC systems ( A and B). The biologic material showed uv-absorbance with $\lambda_{max}$ at 230 nm as did the synthetic C-12 epimers of dihydro-$LTB_4$. Furthermore, GC/MS analysis of the Me-Me$_3$Si derivative of met II gave results similar to those of the authentic 12(S)-dihydro-$LTB_4$ standard. The findings were also

consistent with previously published data for dihydro-$LTB_4$ (12). The mass spectrum of met-II-Me-$Me_3Si$ showed prominent ions at 496 (M), 481 (M-15), 406 (M-90), 395 (M-101, loss of $\cdot CH_2$-$(CH_2)_2$-$COOCH_3$)), 385 (M-111, loss of $\cdot CH_2$-CH=CH-$(CH_2)_4$-$CH_3$), 305 (M-(101+90)), 295 (M-(111+90)), 269 (M-227, loss of $\cdot CH_2$-CH($OSiMe_3$)-$CH_2$-CH=CH-$(CH_2)_4$-$CH_3$), 229, 205 (M-(111+90+90)), 203 ($Me_3SiO^+$= CH-$(CH_2)_3$-$COOCH_3$), 149, 129, and 73 (base peak) (Fig. 2). The ion at 339 was also seen in the background. It was however not seen in the spectrum of the standard and varied in relative intensity between runs of the biologic sample (from 20 to 80 %), whereas the relation between other ions remained constant. This ion was thus interpreted as due to contamination. Finally, the met II-Me chromatographed as two peaks on chiral SP-HPLC (Fig. 3), each of which coeluted with synthetic 12(S)- and 12(R)-dihydro-$LTB_4$ methyl ester, respectively (Fig. 3).

Taken together, based on these analytical data met II was identified as a mixture of 12(S)- and 12(R)-10,11-dihydro-$LTB_4$. A direct saturation of the $\Delta^{10}$-double bond in $LTB_4$ would result in the formation of the 12(S)-epimer of dihydro-$LTB_4$. The absolute configuration of C-12 in unchanged during this transformation, although the rules of the Cahn-Ingold-Prelog system require that the designation of the stereochemistry is changed from (R) to (S). However, if a ketone, e.g. 12-oxo-dihydro-$LTB_4$, serves as an intermediate in the conversion, a subsequent reduction may result in the generation of both C-12 epimers of dihydro-$LTB_4$. Therefore, the presence of 12(R)- as well as 12(S)-dihydro-$LTB_4$ in the human lung supports the tentative identification of met I as 12-oxo-dihydro-$LTB_4$ (see above). Furthermore, it was recently shown in porcine leukocytes that 10,11-dihydro-12-epi-$LTB_4$ (here named 12(R)-dihydro-$LTB_4$) may be formed either directly from 10,11-dihydro-$LTB_4$ (here 12(S)-dihydro-$LTB_4$) or through 12-oxo-dihydro-$LTB_4$, both pathways appearing to be enzymatic (18).

***Formation of dihydro-$LTB_4$ from endogenously generated $LTB_4$.*** Dihydro-$LTB_4$ was also formed in incubations of chopped lung with arachidonic acid (70 μM) and calcium ionophore A23187 (2.5 μM). After an initial increase in $LTB_4$ formation (up to 10 min), the levels of $LTB_4$ decreased with a concomitant increase in the amounts of dihydro-$LTB_4$ (Fig. 4). This indicates that also endogenously formed $LTB_4$ in the human lung was metabolized via

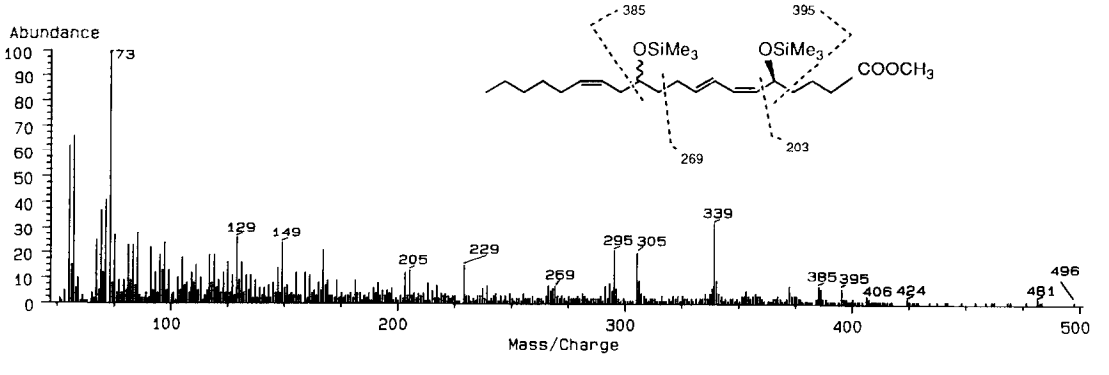
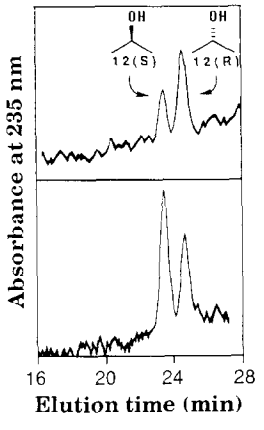



Fig.2. Mass spectrum of the Me-$Me_3Si$ derivative of met II after treatment with ethereal diazomethane followed by trimethylchlorosilane and hexamethyldisilazane in pyridine.

the reductase pathway. In addition, small peaks corresponding to the retention times of met I and met III with uv-absorbance maxima at 230 nm were observed, as well as a few polar compounds with $\lambda_{max}$ at 270 nm. These metabolites could not be quantitated in this study due to lack of authentic standards.

***Biological activity of 12(R)- and 12(S)-dihydro-LTB$_4$.*** Both synthetic epimers of dihydro-LTB$_4$ produced contractions of the guinea-pig lung parenchyma with a time-course which was identical to that of LTB$_4$ (Fig. 5a). However, the compounds were approximately two log orders of magnitude less potent than LTB$_4$. The 12(S) isomer appeared somewhat more potent, but the difference was not significant in this initial series of experiments. The contraction response to the dihydrogenated metabolites was blocked by pretreatment with indomethacin (10 μM, 30 min) Furthermore, preparations desensitized to LTB$_4$ failed to respond to the dihydrogenated metabolites, and, likewise, by pretreatment with 12(S)-dihydro-LTB$_4$, the response to subsequent stimulation with LTB$_4$ was markedly depressed (Fig. 5b). Together, the data support that in the lung strip, the metabolites had the same mode of action as LTB$_4$ (16), i. e. to cause a thromboxane-dependent contraction, but the substances were approximately 100 times less potent than LTB$_4$ on a molar basis.

Concerning inflammatory responses, the 12(S) epimer was selected for evaluation in the hamster cheek pouch. In this in vivo model, the actions of LTB$_4$ have been extensively

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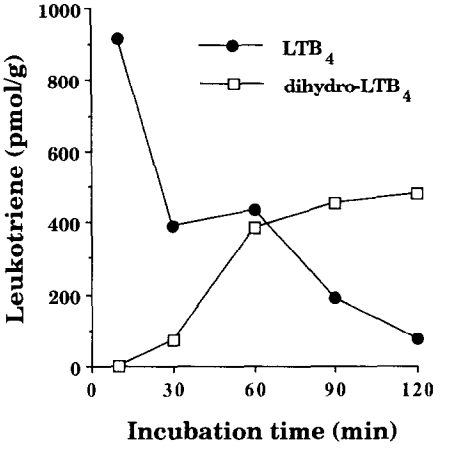


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Fig.3. SP-HPLC (for details see Materials and Methods) of dihydro-LTB4 as methyl esters after treatment with ethereal diazomethane. *Upper panel:* material collected as met II on RP-HPLC from incubations of 60-90 min duration. *Lower panel:* mixture of synthetic 5(S),12(S)- and 5(S),12(R)-dihydroxy-6,8,14-eicosatrienoic acid (12(S)- and 12(R)-dihydro-LTB4, respectively).

Fig. 4. Amounts of LTB4 and dihydro-LTB4 formed in incubations of chopped human lung in the presence of arachidonic acid (70 μM) and ionophore A23187 (2.5 μM). Quantitations were performed on RP-HPLC by comparing peak areas in the samples with the ones obtanied with authentic compounds, using prostaglandin $B_1$ as internal standard and 27,000 as extinction coefficient for dihydro-LTB4. Results are mean of duplicate determinations in one specimen of human lung.

characterized (17). 12(S)-Dihydro-$LTB_4$ increased microvascular permeability in an apparent dose-dependent manner (Fig. 5c). It was also in this system almost two log orders of magnitude less potent than $LTB_4$. In the same experiments, the number of emigrated leukocytes after challenge with 12(S)-dihydro-$LTB_4$ was 3 (10 nM), 75 (100 nM), and 128 (300 nM), respectively, as compared with 230 ± 54 emigrated leukocytes after challenge with 10 nM of $LTB_4$ (mean ± SD, n=3).

Taken together, in two systems where $LTB_4$ is known to act via its proposed high-affinity receptor, the dihydrogenated metabolites proved to be almost 100 times less active than $LTB_4$. This would seem to be approximately the same degree of inactivation as previously suggested for the influence of a murine dihydro metabolite of $LTB_4$ on human leukocyte functions in vitro (19). Interestingly, at least in the guinea-pig lung parenchyma, the ω-oxidized metabolites are as potent as $LTB_4$ (4). Therefore, the reductase pathway may represent an important first step in the

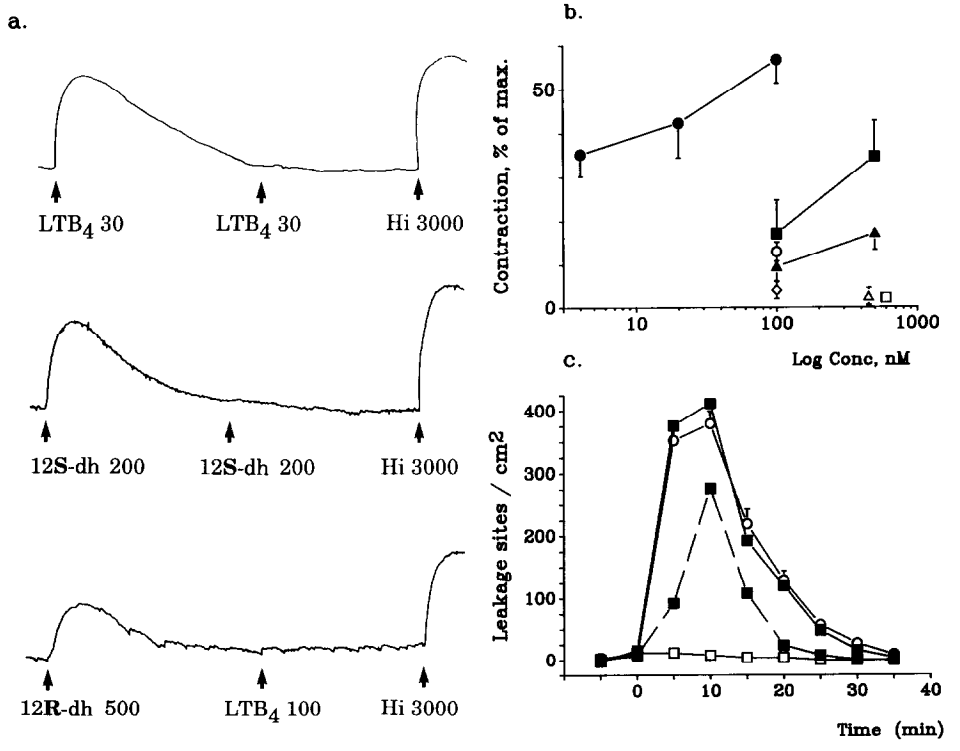


Fig. 5. Biological activities of 12(S) and 12(R)-dihydro-LTB4 (12(S)dh and 12(R)dh, respectively) compared with those of LTB4. *Panel a:* Contraction responses in three different strips of guinea-pig lung parenchyma. Note the desensitization to repeated administration of LTB4 or 12(S)-dh, as well as the cross-tachyphylaxis between 12(S)-dh and LTB4. Hi=histamine, concentrations in nM. *Panel b:* Dose response relations for LTB4 (filled circles), 12(S)-dh (filled squares) and 12(R)-dh (filled triangles) in the guinea-pig lung parenchyma (n=4-6 at each dose). Open circle and diamond represent response to LTB4 in preparations previously exposed to 12(S)-dh and 12(R)-dh, respectively. Pretreatment with indomethacin (10 μM, 30 min, n=3) blocked the response to 12(S)-dh (open square) and 12(R)-dihydro-LTB4 (open triangle). *Panel c:* Time course for plasma leakage (FITC-dextran extravasation) in the hamster cheek pouch after a 10 min topical application of LTB4 (open circles, mean±SD, n=3) or 12(S)-dh (squares, one experiment at each of the concentrations 10, 100 and 300 nM). Experiments were performed in the presence of the vasodilator prostaglandin $E_2$.

process of bioinactivation of $LTB_4$. Considering the role for $LTB_4$ in recruitment of neutrophils into the lung as a primary event in airway inflammation (20), a route for bioinactivation of $LTB_4$ within the lung may be of considerable importance. Since a dihydrogenated metabolite of 12(R)-hydroxyeicosatetraenoic acid (12(R)-HETE) was found to exhibit biological activity which was different from its parent compound (21), it remains to evaluate if dihydro-$LTB_4$ possesses biological activities which are not shared by $LTB_4$.

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