Analysis of leukotriene B₄ metabolism in human promyelocytic HL-60 cells

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We previously reported that human alveolar macrophages rapidly metabolize the chemotactic active lipid mediator leukotriene B₄ (LTB₄) into the dihydro-LTB₄ by reduction of one of the conjugated double bonds. We herein report that human HL-60 cells (a myeloid precursor which can be differentiated into granulocyte- as well as monocyte-like cells by dimethyl sulphoxide or phorbol myristate acetate) express a highly active LTB₄ reductase in the undifferentiated state. Differentiation by dimethyl sulphoxide (1.3%) along the granulocyte lineage, as confirmed by light microscopy, conversion of NitroBlue Tetrazolium into formazan, failed to induce a substantial capacity for ω -oxidation of LTB₄; this reaction is exclusively found in mature granulocytes. Studies with the cell homogenate of undifferentiated HL-60 cells indicated that the activity of the enzyme depends on the presence of NADPH, Ca²⁺ and Mg²⁺, with a pH optimum of 7.5 at 37 °C. The enzyme was not released into the supernatant after stimulation of HL-60 cells with phorbol myristate acetate (100 ng) or Ca²⁺ ionophore (7.5 μ M). Subcellular fractionation revealed evidence that the LTB₄ reductase is located within the membrane fraction. Purification of the enzyme by gel filtration and gel electrophoresis suggests an apparent molecular mass of 40 kDa.

INTRODUCTION

The lipoxygenase products derived from arachidonic acid are important lipid mediators and play a major role in host defence against bacterial infection and in allergic processes (Borgeat & Samuelsson, 1979; Lewis & Austen, 1984; Parker, 1987; Rola-Pleszczynski, 1989; König *et al.*, 1990). In this regard, leukotriene B₄ (LTB₄; 5*S*,12*R*-dihydroxyicosatetra-6,8,10,14-enoic acid) exerts potent effects on a variety of target cells, such as neutrophils (chemotaxis and activation), lymphocytes and monocytes [proliferation of CD8⁺ lymphocyte population; modulation of cytokine production (Parker, 1984; Rola-Pleszczynski *et al.*, 1987; Lewis & Austen, 1988)].

To control the action of this mediator within the microenvironment, a rapid metabolism into biologically less active products exists: LTB₄ is converted into 20-hydroxy-LTB₄ by a specific cytochrome *P*-450-dependent 20-hydroxylase, and into 20-carboxy-LTB₄ by a 20-OH-LTB₄ dehydrogenase. This pathway is almost exclusively present in polymorphonuclear granulocytes (Hansson *et al.*, 1981; Shak & Goldstein, 1985; Soberman *et al.*, 1985; Brom *et al.*, 1988).

We have identified a second metabolic pathway for LTB₄ in human tissue macrophages such as tonsil and lung macrophages (Schönfeld *et al.*, 1988; Schlüter *et al.*, 1988). After stimulation of purified lung macrophages with the Ca²⁺ ionophore A23187, a time-dependent decrease in the synthesized LTB₄ was observed. Chromatographic analysis provided evidence that LTB₄ was converted exclusively into non-polar metabolites; the main metabolite co-chromatographed with the LTB₄ metabolite observed in mouse fibroblast cells (L929), i.e. dihydro-LTB₄, which resulted from the loss of one of the conjugated double bonds of LTB₄. This pathway was originally identified in rat polymorphonuclear granulocytes (Powell, 1987) and rat mesangium cells (Kaeffer *et al.*, 1987) as well as mouse fibroblasts. Dihydro-LTB₄, when studied in various biological assays (such

as chemotaxis of granulocytes), is significantly less chemotactic than LTB₄ (Kaeffer *et al.*, 1988; Schönfeld *et al.*, 1988).

We have also provided evidence that the LTB₄ reductase activity within the monocytic lineage depends on the differentiation of the monocytes into macrophages (Schönfeld *et al.*, 1990). In this study we observe that a high reductase activity is present in undifferentiated HL-60 cells, a human promyelocytic cell line. Additional studies were carried out to partially characterize the enzymic properties of the LTB₄ reductase.

MATERIALS AND METHODS

Materials

The human promyelocytic leukaemia cell line HL-60 was generously provided by Professor N. Seemayer, Institut für Umwelthygiene, Düsseldorf, Germany. All media and supplements were purchased from Gibco (Karlsruhe, Germany) unless stated otherwise. [14,15-³H]LTB₄ (specific radioactivity 1.11–2.22 TBq/mmol) was supplied by New England Nuclear (Dreieich, Germany). NADPH, leupeptin, thermolysin (protease from *Bacillus thermoproteolyticus*) and sulphanilic acid were purchased from Sigma (Deisenhofen, Germany). Organic solvents were from J. T. Baker (Deventer, The Netherlands). All other chemicals were from Merck (Darmstadt, Germany). Triton X-100 was supplied by Serva (Heidelberg, Germany).

Culture conditions

HL-60 cells were maintained in RPMI 1640 medium (Gibco, Eggenstein, Germany) containing 10% fetal calf serum, 100 units of penicillin/ml and 100 ng of streptomycin/ml (Biochrom, Berlin, Germany). The cells were cultured at a density of 1 × 10⁶ cells/ml in a humidified atmosphere containing 5% CO₂. Differentiation of the cells was carried out by treatment with dimethyl sulphoxide (DMSO, 1.3%) for 5–9 days.

Abbreviations used: LTB₄, leukotriene B₄; DMSO, dimethyl sulphoxide; PMA, phorbol 12-myristate 13-acetate; NBT, NitroBlue Tetrazolium; PBS, phosphate-buffered saline.

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Fig. 1. Reversed-phase h.p.l.c. analysis of $[^3\text{H}]\text{LTB}_4$ conversion by HL-60 cells

$[^3\text{H}]\text{LTB}_4$ (3.7 kBq) was incubated with (a) a cell homogenate of 2×10^7 cells in the presence of NADPH (1 mM), and (b) 1×10^7 intact cells for 60 and 20 min respectively at 37°C .

Cell homogenization

HL-60 cells ($1 \times 10^8/\text{ml}$) were suspended in Tris/sucrose buffer (0.34 M/0.05 M, pH 7.5) and disrupted by sonication for 6×10 s (30 W, sonifier W 250; Branson Ultrasonics Co., Danbury, CT, U.S.A.). Complete cell breakage was assessed by light microscopy. In order to avoid proteolytic inactivation, EDTA (1 mM), leupeptin (100 $\mu\text{g}/\text{ml}$) and dithiothreitol (1 mM) were added. The protein content was determined according to Lowry *et al.* (1951) using bovine serum albumin as a standard.

Preparation of the diazonium salt of sulphanilic acid

The salt was synthesized as described by de Pierre & Karnovsky (1974). NaNO_3 (23 mg) and sulphanilic acid (39 mg) were dissolved in 1 ml of water. After the addition of 50 μl of HCl (36%), the tube was gently stirred until the diazonium salt formed a precipitate. The suspension was placed on ice for 15 min, followed by centrifugation at 1000 g for 5 min. The pellet was resuspended in 4 ml of phosphate-buffered saline (PBS).

Chemiluminescence

The production of oxygen radicals was measured by determination of the luminol-enhanced response, as described previously (Scheffer *et al.*, 1985). Briefly, 5×10^6 cells (final volume 300 μl) were incubated for 10 min at 37°C in the presence of luminol (Sigma) and $\text{Ca}^{2+}/\text{Mg}^{2+}$ (1 mM/0.5 mM). Subsequently, phorbol 12-myristate 13-acetate (PMA; final concentration 100 ng) was added and chemiluminescence was studied for an additional 20 min (Lumacounter M 2080; Lumac, Schaesberg, The Netherlands).

NitroBlue Tetrazolium (NBT) reduction was monitored after incubation of the cells (5×10^6 cells) with PMA (100 ng) (Collins *et al.*, 1979); the conversion of NBT (0.2%) into the blue-black formazan complex was observed by light microscopy.

Differential centrifugation

Disrupted cells were centrifuged at 10000 g for 20 min at 4°C (JL-20 centrifuge, rotor JA-20; Beckman, Palo Alto, CA, U.S.A.). The resulting supernatant was subsequently centrifuged at 100000 g for 60 min (4°C , ultracentrifuge L8-70, rotor 40 Ti; Beckman). The pellets were resuspended in Tris/sucrose buffer (0.34 M/0.05 M, pH 7.5) and the protein concentration was determined in all fractions.

Solubilization

The homogenate of 6×10^8 cells was treated with Triton X-100 (0.3% in PBS) for 40 min on ice. After centrifugation at 100000 g

for 60 min, the supernatant, which contained 80% of the total amount of protein, was analysed by gel filtration. For gel electrophoresis, membranes of HL-60 cells (the resulting 100000 g pellet after differential centrifugation) were treated with Triton X-100 (0.3% in PBS) for 40 min on ice. After subsequent centrifugation at 100000 g for 60 min, 300 μg of protein from the resulting supernatant was analysed by gel electrophoresis.

Gel filtration

Gel filtration was performed on a Fractogel TSK HW-55 column (2.4 cm $\times$ 90 cm) (Merck) at 4°C . PBS was used as elution buffer. The flow rate was set at 1.5 ml/min. Fractions of 5 ml were collected and the absorbance at 280 nm was measured. Subsequently the fractions were evaporated to dryness by lyophilization. The residues were dissolved in 1 ml of Tris/sucrose buffer and the protein concentration was calculated. $[^3\text{H}]\text{LTB}_4$ metabolism was determined in each fraction (500 μg of protein) in the presence of $\text{Ca}^{2+}/\text{Mg}^{2+}$ (1 mM/0.5 mM) and NADPH (1 mM).

Gel electrophoresis

Gel electrophoresis was performed with the supernatant of the solubilized 100000 g pellet (12.5% gel); after gel electrophoresis, the small gel was cut into five strips, and the protein was eluted from the gel by NH_4HCO_3 buffer (15 mM), extensively dialysed in PBS and subsequently lyophilized. The protein content of each fraction was determined and adjusted to 50 $\mu\text{g}/\text{ml}$, and the conversion of $[^3\text{H}]\text{LTB}_4$ was studied as described above.

Incubation conditions

NADPH-, Ca^{2+} -, Mg^{2+} -, pH- and temperature-dependence were studied with the HL-60 cell homogenate. The homogenate of 2×10^7 cells was incubated under various conditions as described in the Results section with $[^3\text{H}]\text{LTB}_4$ (3.7 kBq) for 60 min.

The effects of thermolysin and diazonium salt were tested using 1×10^7 cells in 500 μl of PBS, or the homogenate of 2×10^7 cells in 500 μl of Tris/sucrose buffer. Cells and homogenate were preincubated with thermolysin (100 $\mu\text{g}/\text{ml}$, 60 min) or diazonium salt (4.8 mM, 10 min). Subsequently the cells were washed once in PBS and resuspended in 500 μl of the same buffer. $\text{Ca}^{2+}/\text{Mg}^{2+}$ (1 mM/0.5 mM) and $[^3\text{H}]\text{LTB}_4$ (3.7 kBq) were added and the incubation was carried out over the indicated time periods. The reaction was stopped by addition of 2 ml of chloroform/methanol (2:1, v/v).

Fig. 2. Reversed-phase h.p.l.c. analysis of unlabelled LTB₄

Conversion of LTB₄ (1 µg) by 2×10^8 HL-60 cells in the presence of Ca²⁺/Mg²⁺ (1 mM/0.5 mM) for 3 h at 37 °C. The chemical structures of LTB₄ (peak 1) and the metabolite (peak 2) are shown. Peak 1 (LTB₄) was detected at 280 nm and peak 2 (dihydro-LTB₄) was detected at 230 nm.

Analysis of LTB₄ metabolites

After centrifugation of the samples (1200 g, 10 min), the supernatants were extracted with 2×3 ml of chloroform and the combined phases were dried under a stream of nitrogen. For analysis of LTB₄ metabolites by t.l.c., the remainder was resuspended in 50 µl of chloroform and spotted onto silica thin layer plates (Kieselgel 60, 250 µm; Merck). The plates were developed with diethyl ether/water/acetic acid (100:0.5:1, by vol.) as mobile phase. Typical *R_F* values were 0.53 for LTB₄ and 0.58 for dihydro-LTB₄. Radioactivity was detected by t.l.c. scan. (Isomess Radio-Dünnschichtanalysator IM 3000; Raytest, Essen, Germany).

For h.p.l.c. analysis of the ³H-labelled LTB₄ metabolites, the extracted residues were dissolved in 220 µl of methanol/water (3:7, v/v) and then applied to h.p.l.c. analysis as described previously, using a reversed-phase column (Nucleosil 5C18; Macherey & Nagel, Düren, Germany; 4 mm $\times$ 250 mm (Knöller *et al.*, 1988; Schönfeld *et al.*, 1988)). A three-step gradient was used as mobile phase. Solvent A consisted of methanol/acetonitrile/water (1:1:3, by vol.), to which 0.1 g of EDTA/1 litre of water and 0.75 g of K₂HPO₄ were added; the pH was titrated with H₃PO₄ to 5.0. Solvent B was methanol/acetonitrile/water (3:3:4, by vol.) with a pH of 5.0. The flow rate was set at 0.9 ml/min at 40 °C. A linear gradient was chosen, starting at $t = 0$ min (A = 100%) to $t = 20$ min (B = 100%). At $t = 45$ min, the eluent was reset to A = 100% and equilibrated for 15 min. The technical equipment was purchased from LDC Milton Roy (Hasselroth, Germany) (h.p.l.c. gradient pump CM4000) and Spectraphysics (autosampler 8780 XR). The radioactivity was monitored using an h.p.l.c. radiomonitor (Ramona; Raytest). Prior to the entrance into the monitor (supplied with a 500 µl cell), scintillation fluid (Rotiszint 2211; Roth) was mixed with the eluent at a ratio of 0.9:1.25 (v/v). Peak identification and quantification was performed with Nelson Analytical Software (chromatography system 2600; Semrau, Sprockhövel, Germany).

Gas chromatography/mass spectrometry (g.c./m.s.)

HL-60 cells (2×10^8) were incubated in the presence of Ca²⁺/Mg²⁺ (1 mM/0.5 mM) and LTB₄ (1 µg) at 37 °C for 3 h. The

incubation was terminated by centrifugation at 1200 g and further prepared as described by Powell & Gravelle (1989). The eluted eicosanoids in the methyl formate fraction were dried under a stream of nitrogen and resolved in methanol/water (3:7, v/v) and then purified by reversed-phase h.p.l.c. The solvent system consisted of methanol/acetonitrile/water (20:27:53, v/v), to which 2.4 g of K₂HPO₄ and 0.25 g of EDTA were added, adjusted to pH 5.0 with phosphoric acid. The solvent flow was set to 1 ml/min. Detection of the metabolites was carried out with a u.v. detector purchased from LDC Milton Roy (HPLC spectromonitor SM4000). All other technical instruments were as indicated above. The purified products were methylated with diazomethane (30 min at room temperature) and transformed into their trimethylsilyl ether derivatives by incubation with bismethyltrisilylfluoroacetamide (60 min, 60 °C) (Harper *et al.*, 1986).

G.c./m.s. analysis was performed with an HP 5890 gas chromatograph combined with an HP 5970 mass selective detector from Hewlett Packard (Waldbronn, Germany). The compounds were separated on an Ultra-2 column (50 m $\times$ 0.2 mm) with a temperature gradient from 100 °C to 280 °C (10 °C/min). The masses were monitored in a molecular range between 50 and 600 *m/z*.

Statistics

The experiments were done in duplicate if not otherwise stated. The s.d. was below 10% of the corresponding mean value in all cases.

RESULTS

Fig. 1 shows a typical h.p.l.c. chromatogram of the metabolism of [³H]LTB₄ by HL-60 cells. One major peak and two smaller, less polar peaks were detected; this pattern was identical with that obtained with LTB₄ metabolism in human macrophages (Schlüter *et al.*, 1988; Schönfeld *et al.*, 1988).

For further analysis of the main peak, HL-60 cells were incubated with unlabelled LTB₄ in the presence of Ca²⁺/Mg²⁺ (1 mM/0.5 mM). Fig. 2 shows the h.p.l.c. profile of LTB₄ absorbing at 280 nm (peak 1) and of its metabolite 5S,12R,-10,11-dihydroeicosatri-6,8,14-enoic acid (peak 2) absorbing at 230 nm,

Fig. 3. Time-dependent conversion of ³H-LTB₄ (3.7 kBq) by 5×10^6 HL-60 cells

Cell pellet and cell supernatant were separated immediately after the incubation period. ■, LTB₄ in supernatant; □, LTB₄ in pellet; ●, dihydro-LTB₄ in supernatant; ○, dihydro-LTB₄ in pellet.

Table 1. Metabolism of LTB₄ by various subcellular fractions of HL-60 cells

The homogenate of 4×10^8 cells was centrifuged at 10000 *g* and then at 100000 *g*. LTB₄ reduction was measured in the different fractions at the various protein concentrations by incubation (60 min) with [³H]LTB₄ (3.7 kBq) in the presence of Ca²⁺/Mg²⁺ (1 mM/0.5 mM) and NADPH (1 mM).

Fraction	Protein (μg)...	Dihydro-LTB ₄ (%)			
		500	250	100	25
10000 <i>g</i> pellet		18	8	0	0
10000 <i>g</i> supernatant		17	8	0	0
100000 <i>g</i> pellet		28	14	7	0
100000 <i>g</i> supernatant		4	0	0	0

due to the loss of one distal double bond of the conjugated triene. Peak 2 was collected and a u.v. spectrum was taken, showing a maximum at 230 nm. Derivatization for g.c.-m.s. analysis revealed evidence that the double bond at position C-10-C-11 is reduced (results not shown). Comparable data were shown by Powell (1987).

Fig. 3 demonstrates the potent LTB₄ reductase activity of HL-60 cells. Cells (5×10^6) were incubated with [³H]LTB₄ (3.7 kBq) for the indicated time period in the presence of Ca²⁺/Mg²⁺ (1 mM/0.5 mM). Within 30 min, LTB₄ was almost completely metabolized. To analyse whether the metabolites are released into the supernatant or remain cell-bound, the incubation was stopped at various time points by addition of 1 vol. of ice-cold buffer and subsequent centrifugation (1200 *g*, 10 min) (Fig. 3). The intact cells as well as the supernatant were then analysed by h.p.l.c. Separation of the cells from the cell supernatant after the

incubation period with [³H]LTB₄ revealed evidence that during the process of metabolism neither LTB₄ nor dihydro-LTB₄ were found in substantial amounts within the cell, but were almost exclusively observed in the cell supernatant (Fig. 3).

Treatment of HL-60 cells with DMSO (1.3%) induces differentiation into granulocyte-like cells (Collins *et al.*, 1978; Leftwich & Hall, 1990). We therefore analysed whether HL-60 cells treated with DMSO for 5–9 days metabolize LTB₄ by ω -oxidation, as is the case in peripheral granulocytes. Although an increase in PMA-induced NBT reduction and an increased ratio of cytoplasmic versus nuclear NBT were observed by light microscopy, no ω -oxidation products of [³H]LTB₄ were observed. Differentiated as well as undifferentiated HL-60 cells convert LTB₄ exclusively by reduction into dihydro-LTB₄ (mean of $n = 2$; results not shown).

In subsequent studies we analysed several biochemical properties of the reductase enzyme. To characterize the reductase more precisely, the cell homogenate of HL-60 cells was used as a source of the enzyme. The effects of NADPH, Mg²⁺, Ca²⁺, pH and temperature on reductase activity were studied using the unpurified homogenate. NADPH was required at a concentration of 0.5–1.0 mM to allow a sufficient turnover of [³H]LTB₄. Maximum enzyme activity was observed at pH 7.5 and 37 °C. Concentrations of 1 mM-Ca²⁺ and -Mg²⁺ resulted in maximal conversion, although substantial metabolism occurred in the presence of EDTA (1.2 mM, no Ca²⁺ and Mg²⁺ added) (mean of $n = 3$; results not shown).

Further experiments were directed towards the localization of the enzyme within the cell. For this purpose the influence of the diazonium salt of sulphanilic acid and the protease thermolysin from *Bacillus thermoproteolyticus* on LTB₄ conversion were tested in the presence of cells or cell homogenate. Both reagents are known to interact with ecto-enzymes within plasma membranes which face the surrounding medium and do not penetrate into the cell. Under the conditions used, pretreatment with thermolysin (100 μg/ml; preincubation time 60 min) or diazonium salt (4.8 mM; preincubation time 10 min) did not lead to a leakage of the cytoplasm marker lactate dehydrogenase into the medium. Diazonium salt completely blocked the enzymic activity in intact cells and the cell homogenate, whereas thermolysin suppressed LTB₄ conversion only in the cell homogenate (mean of $n = 3$; results not shown). These data suggested that the reductase enzyme was not affected by thermolysin in intact cells as was shown for ecto-enzymes.

We then studied whether the enzyme can be released from the cells after stimulation with the Ca²⁺ ionophore (7.5 μM) or PMA (100 ng). After incubation for 30 min and subsequent centrifugation, the cells and the supernatant (in the presence of 1 mM-NADPH) were incubated separately with [³H]LTB₄ (3.7 kBq) for 45 min. Metabolism within the cell pellet was around 75%, whereas no enzymic activity was released into the supernatant (mean of $n = 3$; results not shown).

We further attempted to purify the enzyme by subcellular fractionation, gel filtration and gel electrophoresis. Subcellular fractionation (10000 *g* and 100000 *g*) of the cell homogenate revealed evidence that the highest specific activity of the enzyme was located within the membrane fraction (100000 *g* pellet; Table 1); in this fraction lactate dehydrogenase was not detectable, whereas the highest activity of acid phosphatase as a typical marker for membrane fractions was observed (results not shown). A sample of 100 μg of protein from the 100000 *g* pellet converted LTB₄ into dihydro-LTB₄, whereas less than 5% of the LTB₄ was metabolized by 500 μg of protein derived from the 100000 *g* supernatant. Moreover, almost 80% of the enzymic activity could be solubilized (0.3% Triton X-100, 40 min on ice) from the 100000 *g* pellet.

Fig. 4. Purification of LTB₄ reductase by gel filtration

Chromatography was performed on a TSK HW-55s column with the solubilized 100 000 g supernatant of a HL-60 cell homogenate; samples of 5 ml/fraction were used. The curve shows absorption at 280 nm, and the bars show conversion of [³H]LTB₄ by the respective fraction (500 μg of protein). Standards: 1, ovalbumin (43 kDa); 2, α-chymotrypsin (25 kDa); 3, α-ribonuclease (13.7 kDa).

Fig. 5. SDS/PAGE of solubilized HL-60 membranes

Lane A, standard mixture, consisting of phosphorylase (97.4 kDa), bovine serum albumin (66.2 kDa), ovalbumin (45 kDa), carbonic anhydrase (31 kDa), soybean trypsin inhibitor (21.5 kDa) and lysozyme (14.4 kDa) (Bio-Rad, München, Germany); Lane B, fraction containing the highest enzymic activity; lane C, crude homogenate; [³H]LTB₄ conversion was measured in each fraction (50 μg of protein/sample; 37 °C, 60 min).

Further purification of the enzyme was performed by gel filtration on a HW 55 column (Fig. 4). The homogenate from 4 × 10⁸ cells was treated with Triton X-100 (40 min on ice) to solubilize the enzyme. After incubation, the homogenate was centrifuged at 100 000 g for 60 min and the supernatant was separated on the column. The greatest enzyme activity was eluted with the exclusion volume. To exclude the possibility that the high molecular mass was due to the formation of oligomers or membrane-bound fragments, SDS/PAGE of the solubilized membranes was performed. Fig. 5 shows a typical pattern of the separated proteins obtained from the 100 000 g supernatant of the solubilized membranes. Parallel lanes of this gel were cut into five strips, the protein was eluted and the conversion of LTB₄ in each fraction was tested as described in the Materials and Methods section. The highest enzymic activity had an apparent molecular mass of 40 kDa.

DISCUSSION

Our data clearly demonstrate that LTB₄ is metabolized differently within the myeloid lineage. In contrast to granulocytes, the promyelocytic HL-60 cells exclusively metabolize LTB₄ into non-polar products. This was originally noted by Benjamin *et al.* (1985), who described the existence of a non-polar metabolite in differentiated as well as undifferentiated HL-60 cells. In this study, we demonstrate that HL-60 cells rapidly convert LTB₄ into its metabolite dihydro-LTB₄ and two further unknown metabolites. U.v. spectroscopic data and g.c.-m.s. analysis led to the identification of dihydro-LTB₄ and indicated that the molecule has a molecular mass 2 Da greater than that of LTB₄, which suggests the hydrogenation of one double bond by the action of a LTB₄ reductase. As reported previously, the metabolite lacks the u.v. absorption at 280 nm which is typical of the triene structure of the original LTB₄. The pattern of molecule fragments obtained by g.c.-m.s. revealed evidence that the double bond at position C-10-C-11 is reduced. Comparable data were reported by Powell & Gravelle (1989), who described the metabolism of LTB₄ into dihydro- and dihydro-oxo products by pig leucocytes. Moreover, after reduction of LTB₄, an epimerization of the 10,11-dihydro-LTB₄ into 10,11-dihydro-12-epi-LTB₄ might occur (Wainwright *et al.*, 1990). Whether the two additional metabolites are intermediates of the complex reaction remains to be clarified.

At present, the relationship between the expression of LTB₄ metabolism and cell differentiation within the myeloid lineage has to be elucidated. A high activity is found in human alveolar macrophages, whereas peripheral human monocytes have only low activity, which increases during culture for 24–72 h (Fauler *et al.*, 1989). Similarly, LTB₄ metabolism is expressed only in differentiated monocytic U937 cells (induced by DMSO) (Schönfeld *et al.*, 1990). In contrast, myeloid HL-60 cells which can be differentiated into either monocytes or granulocyte-like cells express a highly active LTB₄ reductase in the undifferentiated state. Differentiation of HL-60 cells with DMSO failed to induce the typical LTB₄ ω-oxidation found in mature granulocytes. It is suggested that DMSO-treated HL-60 cells mainly differentiate into myelocytes and only to a very limited degree into mature

granulocytes, as described previously (Collins *et al.*, 1979; Trinchieri *et al.*, 1987). Preliminary results obtained with myeloid cells from patients suffering from chronic myeloid leukaemia indicate that the precursor cells also express high LTB₄ reductase activity (results not shown), suggesting that the reductase may have an important function within the bone marrow. Additionally, other lipoxygenase products may serve as substrates of the LTB₄ reductase. For example, Murphy *et al.* (1988) reported the conversion of 12*R*-hydroxyeicosatetraenoic acid (HETE) into a highly active 12*R*-dihydro-HETE, which caused chemotaxis of neutrophils, neovascularization and vasodilation. Thus the enzyme may have additional functions in the regulation of the biological activity of various lipid mediators.

Studies characterizing the LTB₄ reductase more precisely suggested that the enzyme is localized within the membrane fraction. Gel electrophoresis indicates a molecular mass of about 40 kDa, and the highest specific activity is found within the membrane fraction. The conversion of LTB₄ by fractions of a higher molecular mass shown by gel filtration could be explained by the formation of oligomers or binding of the enzyme to membrane fractions. The enzyme is possibly located on the interior side of the cellular membrane, since it is not attacked by the protease thermolysin from *Bacillus thermoproteolyticus*. Moreover, if the incubation of the cells with [³H]LTB₄ is stopped at various time points and the supernatants and cell pellets are separated, the radioactivity is almost exclusively observed in the supernatant. This is in contrast with the metabolism observed in granulocytes via ω -oxidation, which lead to a substantial accumulation of 20-carboxy-LTB₄, and to a lesser extent of 20-hydroxy-LTB₄, in the cells (Brom & König, 1989). Comparable with the ω -oxidation of LTB₄, NADPH (serving as a proton donor) is required for adequate turnover of LTB₄ into dihydro-LTB₄ in the cell homogenate. Incubation with potent cell activators such as PMA and Ca²⁺ ionophore failed to release detectable reductase activity into the cell supernatant. Whether the reductase may be released under certain conditions into biological fluids such as serum, urine or bronchoalveolar lavage has to be analysed; however no activity is detected in human or mouse sera (S. Kasimir, unpublished work).

Metabolism of LTB₄ into ω -oxidation products by human neutrophil granulocytes is thought to be dependent on the expression of specific binding sites which may support the transport of LTB₄ into the cell (Brom & König, 1989). It has to be questioned whether specific receptors are involved in the metabolism of LTB₄ into dihydro-LTB₄. Benjamin *et al.* (1985) reported that HL-60 cells do not express receptors for LTB₄ on the cell surface. On may therefore suggest that the conversion into dihydro-LTB₄ does not require the presence of LTB₄-binding sites.

Our data suggest that the reduction of LTB₄ to form dihydro-LTB₄ plays an important role in the regulation of the biological activity of this lipid mediator. The precise mechanisms of differentiation of the myeloid lineage require investigation. Moreover, studies are required to elucidate the role of the LTB₄ reductase in immunoregulatory and physiological conditions.

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REFERENCES

- Benjamin, C. W., Ruppel, P. L. & Gorman, R. R. (1985) *J. Biol. Chem.* **260**, 14208–14213
- Borgeat, P. & Samuelsson, B. (1979) *J. Biol. Chem.* **254**, 7865–7869
- Brom, J. & König, W. (1989) *Immunology* **68**, 479–485
- Brom, J., Schönfeld, W. & König, W. (1988) *Immunology* **64**, 509–518
- Collins, S. J., Ruscetti, F. W., Gallagher, R. E. & Gallo, R. C. (1978) *Proc. Natl. Acad. Sci. U.S.A.* **75**, 2458–2462
- Collins, S. J., Ruscetti, F. W., Gallagher, R. E. & Gallo, R. C. (1979) *J. Exp. Med.* **149**, 969–974
- de Pierre, J. W. & Karnovsky, M. L. (1974) *J. Biol. Chem.* **249**, 7111–7115
- Fauler, J., Marx, K. H., Kaever, V. & Frölich, J. C. (1989) *Prostaglandins Leukotrienes Essent. Fatty Acids* **37**, 193–196
- Hansson, G., Lindgren, J. A., Dahlen, S. E., Hedqvist, P. & Samuelsson, B. (1981) *FEBS Lett.* **130**, 107–111
- Harper, T. W., Garrity, M. J. & Murphy, R. C. (1986) *J. Biol. Chem.* **261**, 5414–5418
- Kaever, V., Martin, M., Fauler, J., Marx, K. H. & Resch, K. (1987) *Biochim. Biophys. Acta* **922**, 337–344
- Kaever, V., Damerau, B., Wessel, K. & Resch, K. (1988) *FEBS Lett.* **231**, 385–388
- Knöller, J., Schönfeld, W., Köller, M., Hensler, T. & König, W. (1988) *J. Chromatogr.* **427**, 199–208
- König, W., Schönfeld, W., Raulf, M., Köller, M., Knöller, J., Scheffer, J. & Brom, J. (1990) *Eicosanoids* **3**, 1–22
- Leftwich, J. A. & Hall, R. E. (1990) *J. Leukocyte Biol.* **47**, 87–96
- Lewis, R. A. & Austen, F. (1984) *J. Clin. Invest.* **73**, 889–897
- Lewis, R. A. & Austen, K. F. (1988) in *Inflammation: Basic Principles and Clinical Correlates* (Collins, J. I., Goldstein, J. M. & Snyderman, R., eds.), pp. 121–128, Raven Press, New York
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
- Murphy, R. C., Falck, J. R., Lumin, S., Yadagiri, P., Zirrolli, J. A., Balazy, M., Masferrer, J. L., Abraham, N. G. & Schwartzman, M. L. (1988) *J. Biol. Chem.* **263**, 17197–17202
- Parker, C. W. (1987) *Annu. Rev. Immunol.* **5**, 63–84
- Parker, C. W. (1984) in *Fundamental Immunology. Mediators: Release and Function* (Paul, W. E., ed.), pp. 697–705, Raven Press, New York
- Powell, W. S. (1987) *Biochem. Biophys. Res. Commun.* **45**, 991–998
- Powell, W. S. & Gravelle, F. (1989) *J. Biol. Chem.* **264**, 5364–5369
- Powell, W. S. & Gravelle, F. (1990) *J. Biol. Chem.* **265**, 9131–9139
- Rola-Pleszczynski, M. (1989) *J. Lipid Mediators* **1**, 149–157
- Rola-Pleszczynski, M., Bouvrette, L., Gingras, D. & Girard, M. (1987) *J. Immunol.* **139**, 513–517
- Scheffer, J., König, W., Hacker, J. & Goebel, W. (1985) *Infect. Immun.* **50**, 271–278
- Schlüter, B., Schönfeld, W. & König, W. (1988) *Scand. J. Immunol.* **27**, 451–459
- Schönfeld, W., Schlüter, B. & König, W. (1988) *Immunology* **65**, 529–536
- Schönfeld, W., Knöller, J., Kasimir, S., Brom, J. & König, W. (1990) *FASEB J.* **26**, 2101 (abstr.)
- Shak, S. & Goldstein, I. M. (1985) *J. Clin. Invest.* **76**, 1218–1228
- Soberman, R. J., Harper, T. W., Murphy, R. C. & Austen, K. F. (1985) *Proc. Natl. Acad. Sci. U.S.A.* **82**, 2292–2295
- Trinchieri, G., Rosen, M. & Perussia, B. (1987) *Blood* **69**, 1218–1224
- Wainwright, S., Falck, J. R., Yadagiri, P. & Powell, W. S. (1990) *Biochemistry* **29**, 1180–1185