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Preparation of high specific activity tritium-labelled leukotriene B_4 suitable for radioligand binding assay

Stanislav I. Schramm,[a]* Igor Yu. Nagaev,[a] Alan Sabirsh,[b] Valeriy P. Shevchenko,[a] Anastasiya S. Arkhipova,[a] Jesper Z. Haeggström,[b] and Nikolay F. Myasoedov[a]

We describe a method of preparation of high specific activity tritium-labelled leukotriene (LT) B_4 from [5,6,8,9,11,12, 14,15-^{3}H] arachidonic acid (AA; 6.66 TBq/mmol) utilizing a LTB_4-synthesizing enzyme system from rat basophilic leukemia (RBL-1) cells. It was shown that both cyclooxygenase inhibitor indomethacin and adenosine 5′-triphosphate induced [^{3}H] AA transformation to [^{3}H] LTB_4. In optimized conditions up to 15% of total radioactivity of the incubation mixture was present in [^{3}H] LTB_4. A separation of [^{3}H] LTB_4 from other labelled $C_{20:4}$ products was achieved by a three-step reverse phase-high-performance liquid chromatography in methanol- and acetonitrile-based solvent systems. [^{3}H] LTB_4 was confirmed to be identical to the naturally occurring LTB_4 by a radioligand binding assay using a culture of HF1 cells that express a BLT_1 receptor.

Keywords: [^{3}H] leukotriene B_4; enzymatic synthesis; rat basophilic leukemia (RBL-1) cells; BLT_1 receptor; radioligand binding assay

Introduction

Leukotriene (LT) B_4 (5S,12R-dihydroxy-6,8,10,14-ZEEZ-eicosatetraenoic acid) is a potent mediator of inflammation, and it is primarily synthesized by polymorphonuclear cells (PMNs).[1] It promotes the adhesion of leukocytes to the vascular endothelium and plays an important role in attracting leukocytes to inflammatory sites.[2]

At present there are on-going studies to find new inhibitors of LTB_4 synthesizing enzymes and antagonists of LTB_4 receptors as potential drugs.[3] A radiolabelled LTB_4 with high specific activity is an essential tool for this work. The simplest method of preparation of such substances is a selective hydrogenation of 14, 15-acetylene precursors with gaseous tritium.[4] However, in practice, the specific radioactivity of LTs prepared by this way does not exceed 1.5 GBq/mol. Another method is to employ an enzymatic transformation of tritium-labelled arachidonic acid (AA) to produce the desired substances.[5,6] This approach results in products with considerably higher specific radioactivity. Moreover, a number of other labelled compounds can be made from [^{3}H] AA using the same enzyme.[5] An enzymatic method for the preparation of [^{3}H] LTB_4 has not been described in the literature to date.

This paper reports a synthesis of high specific radioactivity tritium-labelled LTB_4 from [$^3H_8$] AA using an enzymatic system from cultured rat basophilic leukemia (RBL-1) cells as well as receptor-binding properties of the compound.

The synthesis of LTB_4 from AA in RBL-1 cells comprises three consecutive enzymatic reactions catalyzed by two enzymes: a 5-lipoxygenase (5-LO) and a LTA_4-hydrolase (Scheme 1).[7,8,9] LTB_4 is not the only product of AA metabolism in RBL-1 cells. Exogenous AA is also converted to the products of cyclooxygenase and 12-lipoxygenase oxygenation, and in the presence of reduced glutathione to the peptide LTs LTC_4, LTD_4, and LTE_4.[8,9] LTA_4 (5,6-oxido-7,9,11,14-ZZEE-eicosatetraenoic acid) is a common precursor for all LTs. In the absence of L-glutathione, LTA_4 is converted enzymatically to LTB_4 or non-enzymatically to dihydroxy derivatives of eicosatetraenoic acid (diHETEs): 5,6-diHETEs, 5S,12S- and 5S,12R-dihydroxy-6,8,10,14-EEEZ-eicosatetraenoic acid (the two latter compounds are the 6-*trans* isomers of LTB_4)[9].

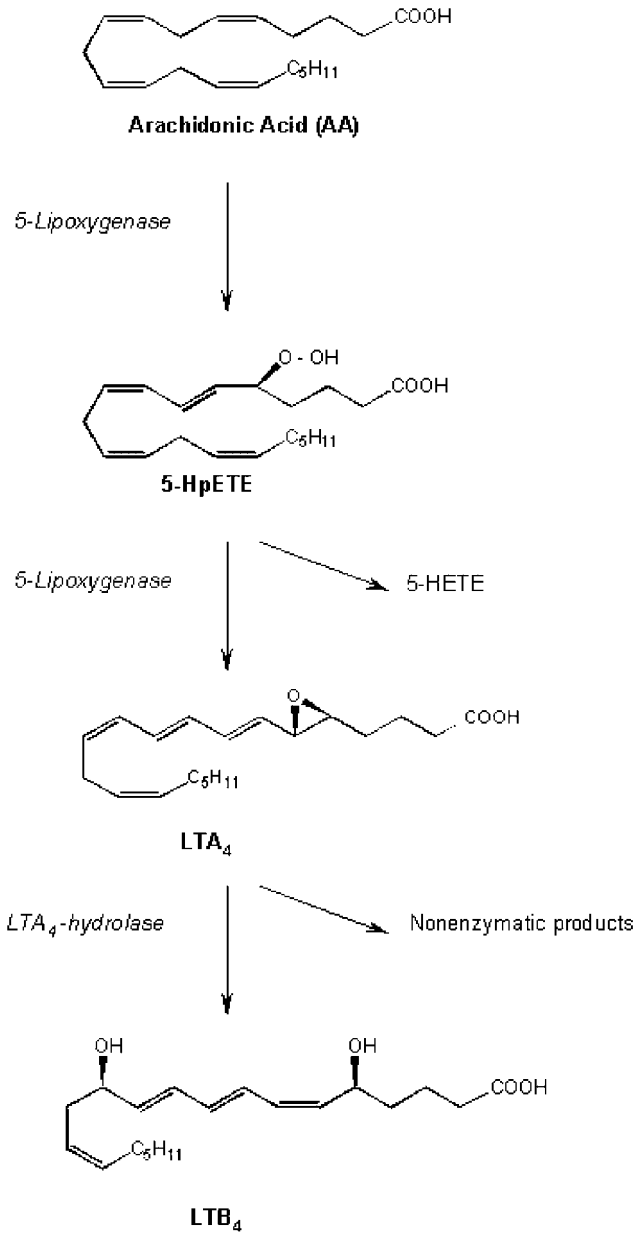
Results and discussion

In RBL-1 cells, the activities of 5-LO and LTA_4-hydrolase have been found in 10 000*g* supernatant fraction derived from cellular homogenates.[9,10] Therefore, we utilized this cellular fraction for the preparation of tritium-labelled LTB_4.

[a]*Department of Chemistry of Physiologically Active Compounds, Institute of Molecular Genetics of the Russian Academy of Sciences, Kurchatov Sq. 2, Moscow 123182, Russia*

[b]*Department of Medical Biochemistry and Biophysics, Karolinska Institutet, S-171 77 Stockholm, Sweden*

**Correspondence to: Stanislav I. Schramm, Department of Chemistry, Institute of Molecular Genetics, Kurchatov Sq. 2, Moscow 123182, Russia. E-mail: shram@img.ras.ru*



Scheme 1

Only small amounts of 5S-hydroxy-eicosatetraenoic acid (5-HETE) were detected when enzyme preparations from RBL-1 cells were incubated with labelled AA under standard conditions (Table 1). But when cyclooxygenase inhibitor indomethacin was added to the standard reaction mixture, the total amount of 5-LO products notably increased (Table 1). The high-performance liquid chromatography (HPLC) analysis showed that LTB_4, 6-*trans* isomers of LTB_4, 5,6-diHETEs, and 5-HETE are the main radioactive products detected (Figure 1). LTB_4 content was higher when incubations were carried out at 30°C.

Since it is known that adenosine-5′-triphosphate (ATP) stimulates the 5-LO,[7,11] we also investigated the effect of this compound on the LTB_4 synthesis. Table 1 shows that ATP is able to promote the synthesis of LTB_4 even in the absence of indomethacin but ATP had a larger effect when given together with a cyclooxygenase inhibitor. The yield of LTB_4 in this case reaches 15% of the total radioactivity. This is two or three times as much as in the procedures described elsewhere.

An analysis of kinetics of the LTB_4 synthesis showed that the metabolism of AA and the production of 5-HETE, LTB_4, and 6-*trans*-LTB_4 isomers occurred concurrently (Figure 2). This fact suggests that the conversion of AA to 5S-hydroperoxy-eicosatetraenoic acid (5-HpETE) is the rate-limiting step of LTB_4 synthesis. Once synthesized 5-HpETE is then rapidly converted to either LTA_4 (enzymatically) or 5-HETE (enzymatically or non-enzymatically). The high content of non-enzymatic products of 5-HpETE and LTA_4 suggests that the LTA-synthase and LTA-hydrolase activities were insufficient in the enzyme preparation used. Similar results have been reported early by Jakschik and Kuo.[10]

HPLC analysis data on $C_{20:4}$ products derived from exogenous AA in polymorphonuclear leukocytes, RBL-1 cells, and lung homogenate have been well described in literature.[12,13] According to these publications, LTB_4 and its two 6-*trans* isomers can easily be separated using reverse phase (RP)-HPLC in methanol-based solvent system, resulting in a chromatographic triple peak that is typical for this reaction. In our experiments, an enzymatically prepared tritium-labelled LTB_4 and an unlabelled standard behaved in the same way in all variants of RP-HPLC used.

It has been demonstrated that in RBL-1 cell homogenates AA can undergo double oxygenation by 5- and 12-lipooxygenases.[10] One of the main products of these reactions is a 5S,12S-dihydroxy-6,8,10,14-EZEZ-eicosatetraenoic acid (5,12-diHETE). During HPLC analysis in the methanol-based system, this compound co-eluted with LTB_4. To reveal the 5,12-diHETE content in LTB_4 fraction obtained after the first HPLC, we analyzed it once more using an acetonitrile-based system (Figure 3). This analysis showed that the content of 5,12-diHETE in LTB_4 fraction did not exceed 15% of the total radioactivity.

An HPLC analysis of the radioactive AA products derived from the reaction mixture revealed a number of unidentified products amounting to 50% of the total radioactivity. The larger portion of these products consists of polar substances with shorter retention times (1.5–5.0 min) when using RP-HPLC and a methanol-based solvent system. The proportion of these products increased when a cellular homogenate was used instead of 10 000*g* supernatants and decreased when the incubation temperature was reduced from 37 to 30°C. This agrees well with previously published data by Jakschik *et al.*[14] showing that the proportion of unidentified polar products produced following the incubation of AA with calcium-free RBL-1 cell enzyme preparations reaches 55%. The addition of Ca^{2+} reduced the proportion of these products notably. The authors believe that these polar products may be a mixture of different phospholipids as well as unmetabolized radiolabelled AA.

A scaled-up synthesis of [^{3}H] LTB_4 was carried out under the optimal conditions determined above. [5,6,8,9,11,12,14,15-^{3}H] AA (6.66 TBq/mmol, 2.59 GBq) was used as a parent substance. The content of labelled LTB_4 in extracts after incubation was about 15% of the total radioactivity. The first two steps of purification (RP-HPLC in methanol-based solvent system) isolated LTB_4 and its two 6-*trans* isomers. And a third RP-HPLC (in an acetonitrile-based solvent system) was used to separate LTB_4 from 5,12-diHETE. One hundred and thirty MBq of LTB_4 with a radiochemical purity of 97% and specific radioactivity of 6.29 TBq/mmol was obtained after the final purification. The

Table 1. Effect of indomethacin and ATP on the content of radioactive $C_{20:4}$ products formed following the incubation of [$^3H_8$] AA with 10 000*g* supernatant of RBL-1 cell homogenates

		Yield (%)				
Additives	*T* (°C)	LTB_4*	6-*trans*-isomers of LTB_4	5,6-diHETEs	5-HETE	AA
No additives	30	<0.5	<0.5	<0.5	5.9	34.7
	37	<0.5	<0.5	<0.5	3.8	47.1
Indomethacin – 25 μM	30	5.8	3.7	3.7	14.1	8.3
	37	1.9	6.3	5.1	14.5	10.2
ATP – 1 mM	30	2.8	12.0	4.4	12.4	17.6
Indomethacin – 25 μM	30	14.4	8.9	7.3	6.5	11.1
ATP – 1 mM						

*As it was shown in this work (see the text), the LTB_4 peak obtained after the analytical reverse phase-HPLC in methanol-based solvent system corresponds not only to the LTB_4 but also to the 5,12-diHETE. The content of latter did not exceed 15% of the total radioactivity in this fraction.

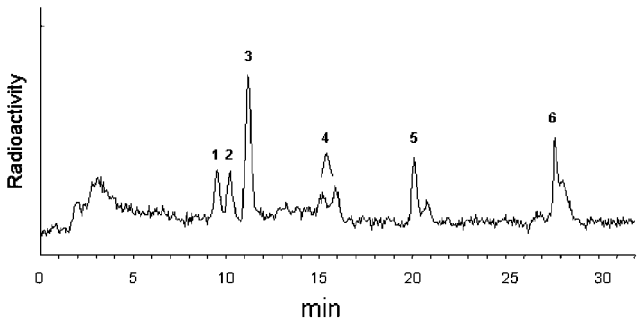


Figure 1. RP-HPLC analysis of the radioactive $C_{20:4}$ products in methanol-based solvent system. The peaks on the chromatogram are corresponded to: 1 and 2–6-trans isomers of LTB_4; 3 – LTB_4; 4–5,6-diHETEs; 5–5-HETE; 6–AA. RP-column – Kromasil 100 C18 (4 × 150 mm, 6 μm).

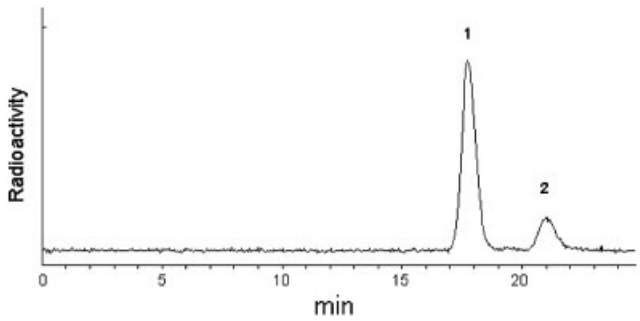


Figure 3. Separation of tritium-labelled LTB_4 and 5,12-diHETE by RP-HPLC in an acetonitrile-based solvent system. The peaks on the chromatogram correspond to: 1 – LTB_4; 2 – 5,12-diHETE. RP-column – Inertsil (4.6 × 125 mm, 5 μm).

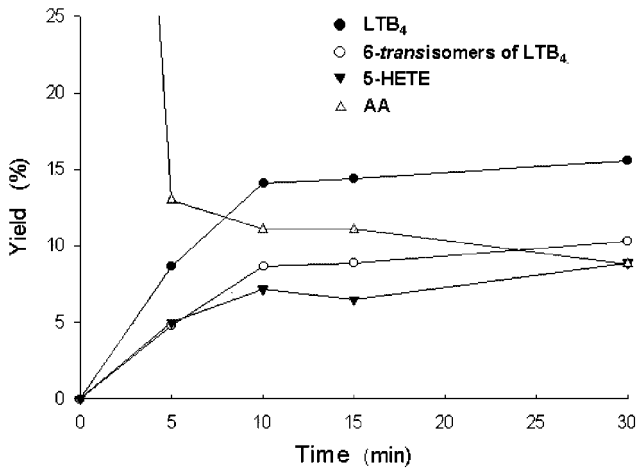


Figure 2. Kinetics of radioactive $C_{20:4}$ product formation during the incubation of [$^3H_8$] AA with 10 000*g* supernatant of RBL-1 cell homogenates.

purified radioactive product had an UV spectrum typical of LTB_4 with an absorbance maximum at 270 nm.

Using cell membranes prepared from HF1 cells expressing BLT_1 receptors (HF1pBLT_1), the K_d and B_{max} (both mean ± SEM) for [^{3}H] LTB_4 were determined to be 1.2 ± 0.2 nM and 221 ± 20 fmol/mg membrane protein (Figure 4). There was a linear correlation between the amount of a radioligand bound and the amount of a membrane protein added. No specific [^{3}H] LTB_4 binding to sham-transfected HF1 cells was observed. Under similar conditions using membranes obtained from human PMNs, [^{3}H] LTB_4 bound to LTB_4 membrane binding sites with a K_d = 1.7 ± 0.7 nM, and the calculated B_{max} value was 19 ± 2 fmol/mg membrane protein (data not shown).

Using unlabelled LTB_4, [^{3}H] LTB_4 could also be displaced from HF1pBLT_1-membrane binding sites with K_i = 1.4 nM (95% confidence interval – from 0.9 to 2.2 nM) (Figure 5). A BLT_1-binding monoclonal antibody that has previously been shown to competitively inhibit ligand binding to BLT_1[15] could also be used to displace [^{3}H] LTB_4 with K_i = 6.8 nM (95% confidence interval – from 4.8 to 9.6 nM). No [^{3}H] LTB_4 displacement was observed when using the chemically related LTs LTC_4 and LTD_4 or a 7B1 isotype control antibody even at concentrations up to 10 μM (data not shown).

Thus, the affinity of the prepared [^{3}H] LTB_4 to BLT_1 receptor agrees well with the previously published work that uses [^{3}H] LTB_4 from other sources[16] as well as with the data obtained using fluorescence polarisation.[17] Specific binding required the presence of BLT_1 receptors and this binding could be

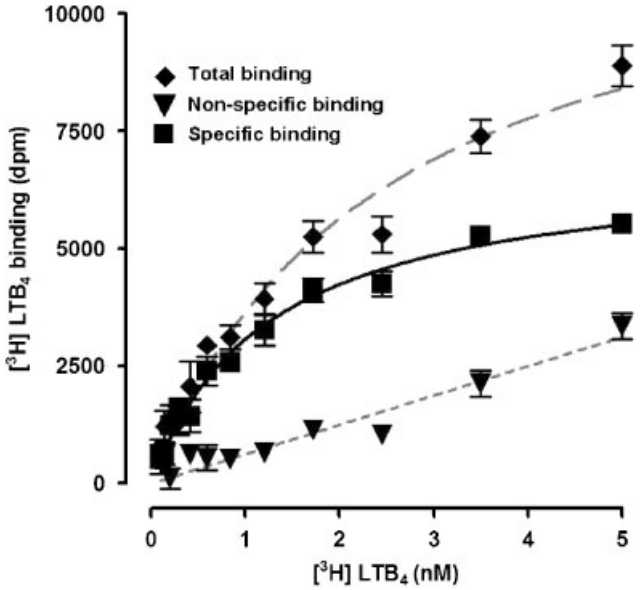


Figure 4. Saturation binding of [^{3}H] LTB_4 to membranes prepared from $HF1pBLT_1$. Data are shown as the mean value (±SEM) from three experiments using duplicate wells. The error bars are hidden by symbols in some cases.

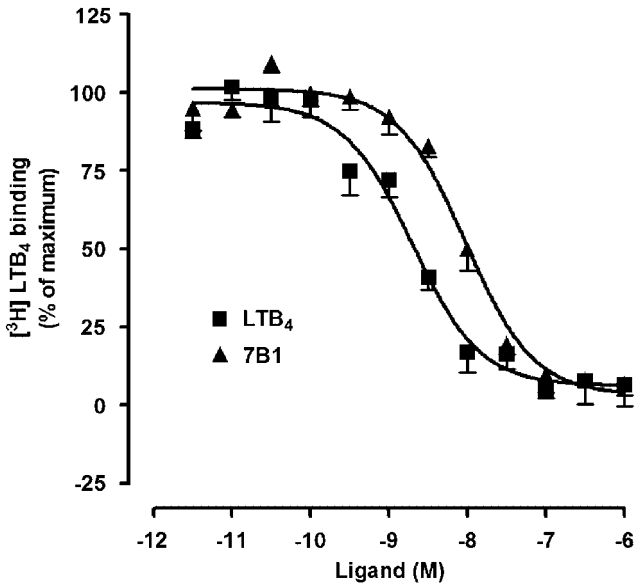


Figure 5. [^{3}H] LTB_4 (0.5 nM) displacement from specific binding sites on $HF1pBLT_1$ cell membranes by LTB_4 and BLT_1 monoclonal antibody. Data are shown as the mean value (±SEM) from three experiments using duplicate wells.

displaced in a predictable way using either unlabelled LTB_4 or an antibody that has been previously shown to interfere with LTB_4 binding to BLT_1.[15] Taken together these results demonstrate that the [^{3}H] LTB_4 prepared is useful for labelling LTB_4 receptors obtained from both recombinant and *ex vivo* cell cultures.

Experimental

General

Chemicals and reagents: Indomethacin, $Na_2 \cdot EDTA$, ATP, and 15-HETE were purchased from 'Sigma-Aldrich' (St. Louis, USA). LT B_4 was obtained from 'BIOMOL Res. Lab.' (Plymouth Meeting, USA). A BLT_1 monoclonal antibody (clone 7B1) was obtained from 'Serotec' (Hamar, Norway). Tissue culture media and reagents were from 'Biolot' (St.-Petersburg, Russia) and 'Life Technologies' (Täby, Sweden). [5,6,8,9,11,12,14,15-^{3}H] AA (6.66 TBq/mmol) was prepared as previously described.[18]

Cell cultures: Culture of RBL-1 cells was obtained from the Russian Collection of Cellular Cultures (St.-Petersburg, Russia) and cultivated as described by Jakschik *et al.*[8] Cultures of HeLa HF1 luciferase reporter cells were created and maintained according to Kotarsky *et al.*[19] HF1pSham cells and $HF1pBLT_1$ cell lines expressing wild-type untagged BLT_1 receptors were created by transfecting parent HF1 cells with plasmid DNAs using Lipofectamine PLUS ('Invitrogen', Carlsbad, USA) as described by the manufacturer, and stable receptor-expressing clones were selected using puromycin.

Human PMNs were isolated from buffy coats, following red cell sedimentation with 0.6% dextran, Lymphoprep density gradient centrifugation ('Axis Shield', Olsa, Norway) and washing.

Enzyme preparation for LTB_4 synthesis: A 10 000*g* supernatant of RBL-1 cell homogenates was prepared as described by Jaskchik *et al.*[14]

Enzyme incubations and analysis of $C_{20:4}$ products derived from AA

Standard procedures for measuring the enzymatic conversion of AA to LTB_4 under different incubation conditions were as follows. Aliquots (200 µl) of 10 000*g* supernatant were placed in 1.5 ml plastic microcentrifuge tubes and mixed with 250 µl of reagent containing indomethacin (12.5 nmol), $Cà_2(NO)_3$ (1 µmol), and ATP (0.5 µmol). After pre-incubation (3 min at 30°C), tritium-labelled AA (37 MBq, 5.6 nmol) in 50 µl of a 50 mM sodium-phosphate buffer pH 7.4, containing $Na_2 \cdot EDTA$ (1 mM), and gelatine (0.1%) were added to the reaction tubes and the samples were incubated for 15 min at 30°C at continuous stirring. To stop the reaction, 1 ml of cooled methanol containing 0.1% acetic acid was added to the reaction mixture and the samples were incubated for 5 min in an ice bath. The resulting solutions were centrifuged and radioactive products were extracted with 3 ml of chloroform. The organic phase was collected and evaporated to dryness under nitrogen atmosphere. The residue was dissolved in 100 µl of methanol, and $C_{20:4}$ products were analyzed by HPLC using a Kromasil 100 C18 (4 × 150 mm, 6 µm) RP column. The column was eluted with methanol/water mobile phase containing 0.1% acetic acid at a flow rate of 1 ml/min. The percentage of methanol in eluent was increased linearly from 70 to 100% over 30 min. Labelled $C_{20:4}$ products were monitored in eluate with a flow radioactivity detector.

Preparation of tritium-labelled LTB_4

An amount of 2.59 GBq of [5,6,8,9,11,12,14,15-^{3}H] AA in methanol was placed into a round-bottom flask. After the solvent was removed, 10 ml of H_2O and 3 ml of 50 mM sodium-phosphate buffer, pH 7.4, containing 1 mM $Na_2 \cdot EDTA$ and 0.1% gelatine, were added to the flask. The solution was thoroughly mixed and pre-incubated for 5 min at 30°C. Then water solutions of ATP (10 mM, 350 µl), $Ca(NO_3)_2$ (100 mM, 700 µl), indomethacin (10 mM, 88 µl), and 17 ml of 10 000*g* supernatant of RBL-1 cell

homogenates were added. The resulting reaction mixture was incubated for 15 min at 30°C with constant stirring. The reaction was stopped by adding two volumes of cooled methanol. Undissolved components were removed by centrifugation, and the reaction products were extracted using two volumes of chloroform.

The chloroform–methanol solution was evaporated under vacuum. The remaining dry substance was dissolved in 1 ml of starting solvent for the first HPLC, and the sample was injected into the preparative Kromasil 100 C18 (8 × 150 mm, 7 μm) RP column. The column was eluted using a methanol/water mobile phase containing 0.1% acetic acid at a flow rate of 2 ml/min. The percentage of methanol in the eluent was increased linearly from 70 to 100% over 30 min. The fractions with [^{3}H] LTB_4 were combined before undergoing the second HPLC purification on an analytical Kromasil 100 C18 (4 × 150 mm, 6 μm) RP column as defined above. To separate tritium-labelled LTB_4 from 5,12-diHETE, the final purification of [^{3}H] LTB_4 was performed using an Inertsil (4.6 × 125 mm, 5 μm) RP column, which was eluted with acetonitrile/water mobile phase containing 0.1% acetic acid at a flow rate of 1 ml/min. A 30 min linear gradient of acetonitrile (70–100%) was used.

Radioligand binding assays

Membranes from the sham-transfected HF1 cells, the HF1pBLT$_1$ cell line, and from purified human PMNs were prepared as previously described.[16] Radioligand binding assays were performed as follows. Radiolabelled LTB_4 was added to 1.0 μg resuspended cell membranes to a final concentration of 0.5 nM, and incubated for 1 h in opaque white 96-well filter plates MAFC-NOB with FC glass fiber filters ('Millipore', Bedford, USA). Unlabelled LTB_4 was added as necessary to determine the non-specific binding. The reaction was terminated by rapid filtration and the filters were then washed, dried, and 25 μl of Microscint-O ('Packard Instrument Co.', Meriden, USA) was added to each well. Measurement of radioactivity in plates was evaluated by using a Micro Beta scintillation counter ('EG&G Wallac', Turku, Finland). Statistical analysis was performed using Prism, by GraphPad Software.

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