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Non-specific effects of leukotriene synthesis inhibitors on HeLa cell physiology

Alan Sabirsh[a,*], Jesper Bristulf[b], Ulf Karlsson[b], Christer Owman[b], Jesper. Z. Haeggström[a]

[a] *Division of Physiological Chemistry II, Department of Medical Biochemistry, Biophysics, Karolinska Institute, Sweden*
[b] *Division of Molecular Neurobiology, Department of Physiological Sciences, Lund University, Sweden*

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Abstract

We examined the effects of various leukotriene synthesis inhibitors on calcium signalling in HeLa cells, before and after transfection with BLT_1. All of the inhibitors studied were found to reduce increases in intracellular calcium concentration induced by BLT_1, but also by an ionophore or activation of various G-protein coupled receptors, regardless of BLT_1 expression. In order to explore the mechanism of these apparently general effects we examined HeLa cell expression of leukotriene receptors and biosynthetic enzymes and found that the genes for key leukotriene synthesis enzymes and all of the leukotriene receptors were not expressed. Leukotrienes are involved in the pathology of a variety of cancers, and for HeLa cells leukotrienes have been reported to be important for aspects of the carcinogenic phenotype. We find that leukotriene synthesis inhibitors have non-specific effects, so careful controls are necessary to avoid interpreting non-specific effects as evidence for leukotriene involvement.

1. Introduction

Leukotriene (LT) B_4 is classical chemoattractant and immune modulating agent. The production of LTB_4 begins with activation and translocation of cytosolic phopholipase A_2 ($cPLA_2$) to the nuclear membrane, where it liberates arachidonic acid from membrane lipids. This arachidonic acid is converted into the precursor, LTA_4, in a two-stage reaction catalysed by 5-lipoxygenase (5-LO) [1]. This enzyme also translocates to the nuclear membrane and becomes active after functional association with 5-LO activating protein (FLAP). LTA_4 is then hydrolysed to LTB_4 by LTA_4 hydrolase [2]. The activation of the biosynthetic machinery is dependent on various intracellular signalling events, but LTB_4 can also activate phospholipase A_2 and 5-LO, either directly [3,4] or via two G protein-coupled cell-surface receptors, BLT_1 or BLT_2 [5–7]. Thus LTB_4 can activate blood leukocytes expressing BLT_1 and/or BLT_2 inducing a response that involves the production of more LTB_4. Inflammatory processes may thus perpetuate themselves in an autocrine fashion. This putative mechanism of positive feedback has received considerable attention [4,8–13] because it could represent a target for drugs designed to modulate the severity of inflammation. Though a variety of drugs have been devised to interfere with leukotriene synthesis pathways, little has been reported regarding their effects on other aspects of cellular physiology.

Leukotriene synthesis and/or responses to leukotrienes have been reported to be important for the pathogenesis and pathology of a variety of cancer forms, in particular pancreatic [14,15], oesophageal [16] and

Abbreviations: LTB_4, Leukotriene B_4; BLT_1 and BLT_2, leukotriene B_4 receptors; 5-LO, 5-lipoxygenase; $cPLA_2$, cytosolic phopholipase A_2; FLAP, 5-LO activating protein; TED, 2-(-thienyl)ethyl 3, 4-dihydroxybenzylidene-cyanoacetate); OBAA, 4-(4-octadecylphenyl)-4-oxobutenoic acid; MAFP, methyl arachidonyl fluorophosphonate; MTT, Thiazolyl blue

*Corresponding author. Tel.: +46 8524 87630; fax: +46 8736 0439.
E-mail addresses: Alan.Sabirsh@ki.se (A. Sabirsh), Jesper.Haeggstrom@ki.se (J. Bristulf), Ulf.Karlsson@mphy.lu.se (U. Karlsson), Christer.Owman@mphy.lu.se (C. Owman), Jesper.Haeggstrom@mbb.ki.se (J.Z. Haeggström).

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prostatic [17] cancers. The activity of 5-LO and/or the production of LTB_4 have also been reported to be important for HeLa cell physiology (and thus cervical carcinoma), including such functions as cell spreading and adhesion [18–20], actin polymerisation [21], and cytotoxic cell death [22].

Our aim with the present work is to convey two related concepts. First, leukotriene synthesis inhibitors have general effects on HeLa cells that are unrelated to their target action. This is important given the attention that is currently being focused on these drugs. Secondly, in an attempt to suggest a mechanism for these general effects, we have examined the expression of LT synthesis enzymes as well as LT receptors. We find that LT synthesis inhibitors can affect HeLa cell calcium homeostasis even when the essential LT biosynthesis enzyme 5-LO and the LTB_4 receptor BLT_1 are not expressed. In light of the present data, the role of LTB_4 in HeLa cell physiology therefore needs to be re-examined. In the absence of experiments demonstrating the expression and preferably knockdown of relevant proteins, studies using lipoxygenase inhibitors as evidence of leukotriene involvement in carcinogenic mechanisms or other aspects of cell physiology should be interpreted with caution.

2. Materials and methods

2.1. Chemicals and reagents

All reagents were purchased from Sigma-Aldrich (Sigma-Aldrich Sweden AB, Stockholm, Sweden), unless otherwise stated. All tissue culture media and reagents were from Life Technologies (Täby, Sweden). 2-(-thienyl)ethyl 3,4-dihydroxybenzylidene-cyanoacetate (TED), and 4-(4-octadecylphenyl)-4-oxobutenoic acid (OBAA) were from Tocris (Bristol, UK). MK-886 was from Biomol (Plymouth Meeting, PA, USA), and methyl arachidonyl fluorophosphonate (MAFP) came from Cayman Chemical (Ann Arbor, MI, USA).

2.2. Cell culture and transfection

Cultures of HeLa S3 cells (obtained from ATCC cat. no. CCl-2) transfected with a reporter construct (HF1) and BLT_1 (HF1pBLT1) or an empty plasmid (HF1pSham) were constructed and maintained according to Kotarsky et al. [23].

Human peripheral blood mononuclear cells (PBMC) were obtained from buffy-coats of healthy blood donors by density gradient centrifugation over Ficoll-Paque (Pharmacia, Uppsala, Sweden). The cells were washed three times in PBS and resuspended in RPMI 1640 (Life Technologies). Monocytes were further purified by allowing them to adhere to gelatine-coated plastic dishes for 45 min at 37 °C in a humidified 5% CO_2 incubator. The cells were washed five times with pre-warmed RPMI to remove loosely adhering cells. The remaining monocytes (>90% pure as assessed by morphology and FACS analysis) were detached and resuspended in cold RPMI containing 10% endotoxin-free FBS (Hyclone, Logan, UT, USA) at a final concentration of 10^6 cells/ml. Cell viability assessed by trypan blue exclusion was >95%. All reagents contained less than 1.0 pg/ml endotoxin as assessed using the limulus amebocyte lysate (LAL) test.

Neutrophils were isolated from the blood of healthy donors using Lymphoprep (Nycomed, Roskilde, Denmark), after dextran sedimentation, and contaminating erythrocytes were lysed using hypotonic shock. This procedure produced solutions containing cells that were >99% viable (as assessed using trypan blue exclusion) and >90% neutrophils (the balance consisting of eosinophils, mononuclear cells and various other cell types). The cells were washed and resuspended in RPMI to a concentration of 10^6 cells/ml and added to six-well cell culture plates in 1 ml/well. After a brief gentle centrifugation to sediment the cells, the plates were incubated for 30 min at 37 °C before use.

2.3. Preparation of crude cell fractions

Cells were added to ice-cold Tris–HCl buffer containing proteinase inhibitors (50 mM Tris base, 5 mM $MgCl_2$, 1 mM EGTA, 0.2 mM leupeptin, 0.5 mM benzamidine, 1.0 mM phenylmethylsulfonyl fluoride, soybean trypsin inhibitor 20 mg/l, pH 7.5) before they were homogenised on ice using a Teflon pestle. The cell homogenates were centrifuged at 1000*g* for 10 min at 4 °C. The resulting pellet was defined as the "nuclear" fraction. The supernatant was decanted and centrifuged once more at 100,000*g* for 30 min at 4 °C. The resulting pellet was defined as the "crude membrane" fraction, and the remaining supernatant as the "cytosolic" fraction. The protein concentration in each fraction was determined (BCA protein assay, Pierce, Rockford, IL, USA).

2.4. Microarray studies of HeLa gene expression

Cultures of HF1pBLT1 that were 80% confluent were exposed to 100 nM LTB_4 or buffer for 1 h. Total RNA was then isolated using Trizol (Invitrogen, Sweden) according to the manufacturers instructions. Affymetrix (High Wycombe, UK) microarray (U133) chips were then used to analyse gene expression following the preparation and fragmentation of cRNA according to the chip manufacturers recommendations, and standard procedures used by the Swegene Microarray Facility at Lund University, Sweden. The results were analysed using Affymetrix Microarray Suite.

2.5. Quantitative PCR analysis

cDNA was prepared from total RNA obtained from duplicate wells containing 10^6 HF1pSham cells, 10^6 HF1pBLT1 cells, or 10^5 monocytes. The cells were lysed and homogenised using QIAshredder columns (QIAgen, Valencia, CA, USA). mRNA was isolated using MG-OL particles (magnetic particles with covalently bound oligo dT, Sera-mag) according to the manufacturers instructions and was then reverse transcribed using Superscript II (Invitrogen).

To examine if 5-LO expression could be affected by LTB_4 exposure, duplicate cultures of HF1pBLT1 and HF1pSham cells were exposed to 100 nM LTB_4 or a control solution for either 1 or 16 h. cDNA isolated from monocytes was used as a positive control for 5-LO expression.

Primers (5-LO forward: GCTGCAGGACTTCGT-GAACG, and 5-LO backward: GGGATCCAGGAG-CACCAGTC) that were specific for human 5-LO and positioned across an intron, were used in polymerase chain reactions (PCR) to quantify 5-LO gene expression (Lightcycler, Roche Diagnostics Scandinavia AB, Bromma, Sweden) by comparison with known amounts of 5-LO PCR product. To facilitate quantitative comparisons between monocytes and HeLa cells, the expression of β-actin (β-actin forward primer: GGATGCAGAAGGA-GATCACTG, β-actin backward primer: CATCTGCTG-GAAGGTGGACA) was also quantified for each sample.

2.6. Western blot analysis

Various concentrations of purified 5-LO protein and aliquots (50 μg/well) of membrane proteins from various cell fractions were subjected to SDS/PAGE (4–15%, Bio-Rad, Hercules, CA, USA). Bands of protein were electroblotted onto transfer membranes (Fluorotrans, Pall Biosupport, Portsmouth, UK). The resulting membranes were soaked for 3 h in NaCl/Tris containing 3% (w/v) non-fat dry milk, rinsed once, and subsequently incubated for over night at 4 °C with a polyclonal antiserum (dilution 1:500). After washing (3×30 min at 25 °C), the membranes were incubated at 25 °C with a goat anti-(rabbit IgG) Ig coupled to horseradish-peroxidase (dilution 1:2000) in NaCl/Tris containing 2.0% non-fat dry milk. Immunoreactive bands were visualised following washing using the ECL detection method (ECL PLUS kit, Amersham Bioscience, Uppsala, Sweden) according to the manufacturer's instructions.

2.7. Intracellular calcium concentration assays

Confluent cultures of monoclonal HF1pBLTR or HF1pSham cells were loaded with FURA-2AM and analysed according to Sabirsh et al. [24].

For experiments designed to investigate the effects of UV light on LTB_4-induced calcium release, we used Fluo-3AM as a probe for intra-cellular calcium levels. The cells were exposed to LTB_4 as above, illuminated with 485 nm light, and the light emitted at 520 nm was quantified. The cells were treated between each observation in one of four different ways: (1) exposure to 485 nm visible light; (2) exposure to full spectrum light, including UV wavelengths; (3) no exposure to light; and (4) exposure to full spectrum light, but not LTB_4. The experimental light exposures were of the same duration as those used for observation: 10 short flashes/4 s cycle from a xenon arch lamp. The illumination time including both observation and experimental exposures was about 1 s per 4 s cycle, or about 30 s in total. The light exposure level at the surface of the cell monolayer was measured using a calibrated PIN-diode.

2.8. Luciferase reporter assays

Receptor activation-induced changes in luciferase reporter activity were assayed according to Kotarsky et al. [23].

2.9. Radioligand binding assays

Radioligand binding assays were performed using opaque white 96-well filter plates with FC glass fibre filters (model MAFC-NOB, Multiscreen Assay System, Millipore, Bedford, MA, USA). Binding buffer containing 0.5 nM ^{3}H-LTB_4 with or without 1.0 μM of unlabelled LTB_4 was added to re-homogenised HF1pSham or HF1pBLT1 crude membrane preparations containing 1.0 μg of membrane protein. The membrane-ligand solution was incubated for 1 h at room temperature before the reaction was terminated by rapid filtration. The filters were washed, dried and 25 μl of Microscint-O (Packard) was then added to each well and ^{3}H-LTB_4 binding was evaluated using a TopCount NXT scintillation counter (Packard).

2.10. LTB_4 production assays

HF1pSham and HF1pBLT1 cells were seeded into six-well plates and grown until 80–90% confluent. Neutrophils were isolated and seeded into six-well plates as described above. The cells were stimulated and LTB_4 was extracted from the cells according to Peppelenbosch et al. [21]. Briefly, the cells were rinsed with PBS, and then thapsigargin (T), ionomycin (I), LTB_4 (L) or control PBS solutions (C) were added to the cells before they were incubated for 10 min at 37 °C. The medium was then removed and replaced with 1 ml methanol and the cells were incubated for 5 min at room temperature. The methanol solutions were then centrifuged to remove particles before the samples were dried under nitrogen.

The dry product was then redissolved in 10 μl methanol and diluted in PBS (or EIA assay buffer for EIA experiments).

The LTB_4 content in each sample was analysed using a LTB_4 EIA kit (Amersham Bioscience) according to the manufacturer's instructions.

LTB_4 content was also analysed using a luciferase reporter system (see above) as a bioassay. The samples from above, or known LTB_4 concentrations, were added to the cells, and following 16 h of incubation at 37 °C, luciferase activity was measured.

2.11. MTT toxicity assay

Thiazolyl blue (MTT) was purchased from Sigma-Aldrich and the assay was performed according to the manufacturer's instructions. All substances to be tested were added to cell cultures 1.5 h prior to a 1 h incubation in the presence of MTT at 37 °C.

3. Results

3.1. Leukotriene synthesis inhibitors affect BLT_1-induced calcium release

HeLa cells do not express BLT_1 or BLT_2, and using northern blots, microarray data, flow cytometric measurements and radioligand binding experiments no endogenous LTB_4 receptor expression by these cells has ever been detected (see Fig. 1 and work by this lab and others [24–26]). Sham transfected HeLa HF1 cells did not respond to LTB_4 following exposure to concentrations as high as 10 μM. Similarly, HeLa HF1 cells were also unresponsive to LTC_4, LTD_4 and LTE_4 (Fig. 1a and b).

In HeLa HF1 cells transfected with BLT_1, LTB_4-induced robust calcium release, and this was attenuated by all of the leukotriene synthesis inhibitors we tested (Fig. 1c–g). The phospholipase inhibitors, MAFP and OBAA, had an effect similar to the cellular LO inhibitor TED, and the FLAP (5-LO activating protein) inhibitor MK-886.

Given that phospholipase inhibitors reduced calcium responses to LTB_4, we reasoned that increasing the amount of available arachidonic acid could possibly increase responses to exogenous LTB_4. A 60 min incubation with 50 μM arachidonic acid prior to stimulation with LTB_4 had no effect on the response attenuation following exposure to LT synthesis inhibitors (data not shown).

3.2. Leukotriene synthesis inhibitors have general effects on calcium homeostasis

Inducing calcium release with agonists for receptors endogenously expressed by HF1pBLT1 cells revealed that leukotriene synthesis inhibitors could also reduce the calcium release induced by both the chemically related eicosanoid, dideoxy prostaglandin $F_{2\alpha}$ (dideoxy-$PGF_{2\alpha}$), and the chemically unrelated agonist, ATP (Fig. 2a). Similar results were obtained using these agonists with leukotriene synthesis inhibitors and HF1pSham cells (data not shown), indicating that activation of BLT_1 receptors was not involved in the attenuation of calcium responses.

The effects of the leukotriene synthesis inhibitors were unrelated to cellular toxicity, at least for the duration of these experiments (90 min at most). An (MTT) assay for the activity of mitochondrial dehydrogenases indicated that the incubation times and concentrations used were not toxic (Fig. 2b). Incubation with toxic concentrations (>10%) of ethanol, for example, inhibited agonist-induced calcium release, whereas sub-toxic concentrations did not (data not shown).

It has been shown using macrophages primed with lipopolysaccharide (LPS) that exposure to UV light can result in the activation of cellular phospholipases [27]. Since UV wavelengths are widely used as part of the method for measuring intracellular calcium concentrations with FURA-2, we investigated whether or not this illumination had any effect on calcium responses to LTB_4 stimulation. Balsinde et al. [27] exposed cells to 366 nm UV light with an energy output of 9.6 $mJ/s\,cm^2$ for a total of 4 s. For comparison, the lamp we used had a constant output of 9.3 mJ/s. The cells were illuminated with broad-spectrum light (including UV wavelengths down to at least 340 nm) for a total of about 15 s, over a surface area equivalent to one-well in a 96-well cell culture plate (38.5 mm^2, or 0.38 cm^2). Our experiments involving FURA-2 imaging of intracellular calcium exposed the cells to 24.5 $mJ/s\,cm^2$ for 15 s, including 2.5 s of exposure before LTB_4 was injected into the wells. (The energy exposure measured at either 340 or 380 nm was found to be approximately 8.7 mW.) Using a calcium-sensitive dye that is excited using visible light (Fluo-3), we found that exposing the cells to broad-spectrum UV light did not have any significant effect on the magnitude of the $[Ca^{2+}]_i$ change following exposure to LTB_4 (data not shown). Therefore, the use of UV light and FURA-2 does not affect HeLa cell physiology in this context.

3.3. HeLa cells do not express 5-LO

Microarray data indicated that while HeLa HF1pBLT1 cells express a number of phospholipases, they do not express any LOs (Table 1). HeLa cell expression of 5-LO was unaffected by BLT_1 expression or incubation with LTB_4. To validate the microarray data we focused on 5-LO and assessed 5-LO gene expression using quantitative PCR. In contrast to monocytes, HeLa cells were found to have extremely

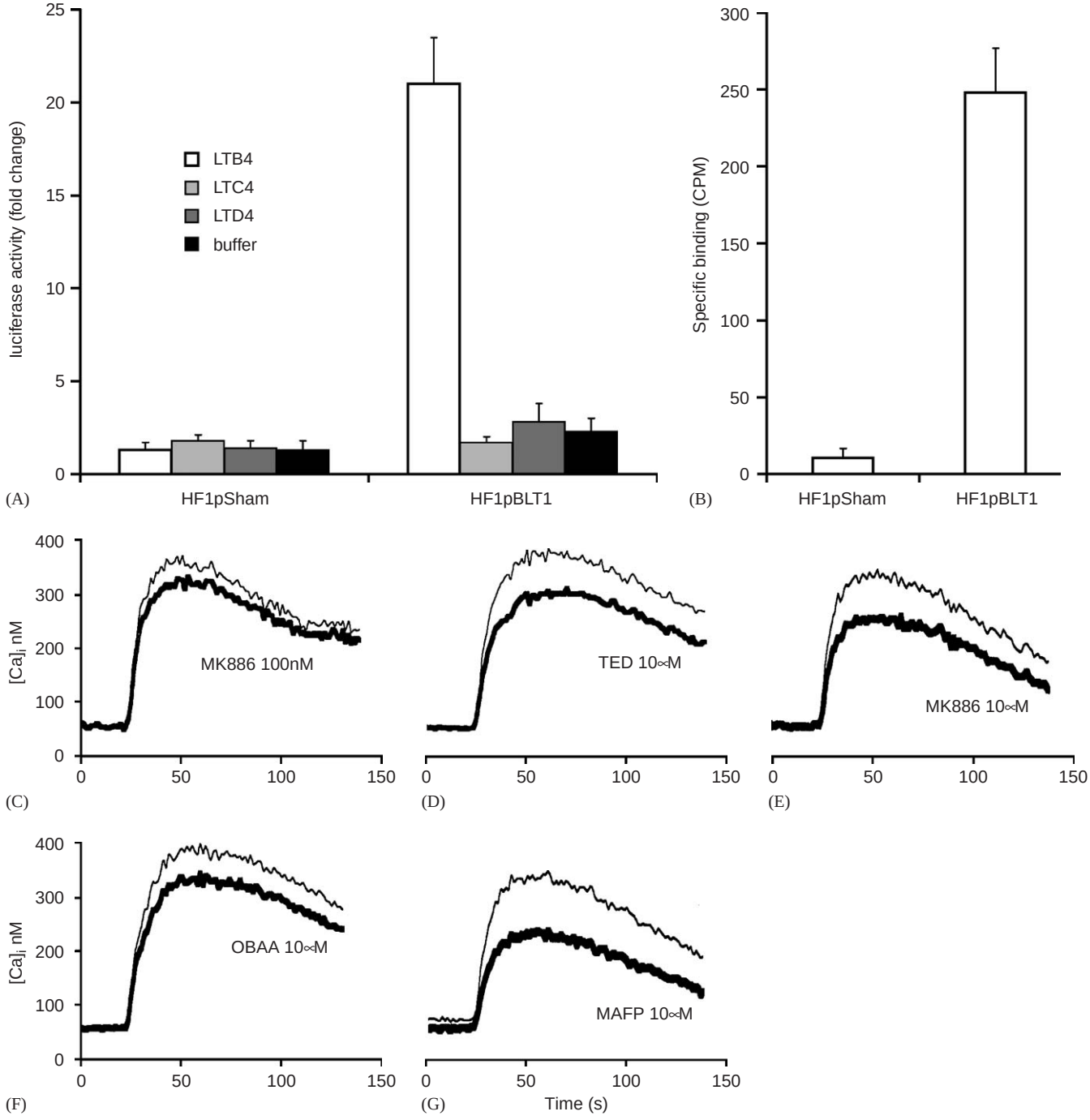

Fig. 1. HeLa cells express BLT_1 only when transfected and, leukotriene biosynthesis inhibitors can attenuate BLT_1 induced increases in intracellular calcium concentration. (A) HeLa HF1 cells were transfected with BLT_1 (HF1pBLT1) or an empty vector (HF1pSham) and exposed to 100 nM LTB_4, LTC_4, LTD_4 or buffer control solutions. Only BLT_1 transfected cells responded, and they responded only to LTB_4. (B) Crude cell membrane fractions from BLT_1 transfected cells or sham transfected cells were incubated with 0.5 nM 3H-LTB_4 with (total binding) or without 1 μM LTB_4 (non-specific binding) in order to measure specific binding. Crude cell membrane fractions from BLT_1 transfected cells bind tritiated LTB_4 specifically, whereas sham transfected cells do not. The effects of 5-LO inhibitors on BLT_1-induced calcium release is shown in (C–E). Control wells, receiving only buffer control solutions and LTB_4, are shown as thin lines. Thick lines represent wells receiving LTB_4 following a 60 min pre-incubation with 100 nM MK886 (C), 10 μM TED (D) or 10 μM MK-866 (E). Similarly, the effects of PLA_2 inhibitors on BLT_1-induced calcium release is shown in (F) and (G). The 10 μM of OBAA (F) or 10 μM of MAFP (G) were incubated with HF1pBLT1 cells for 60 min prior to LTB_4 exposure.

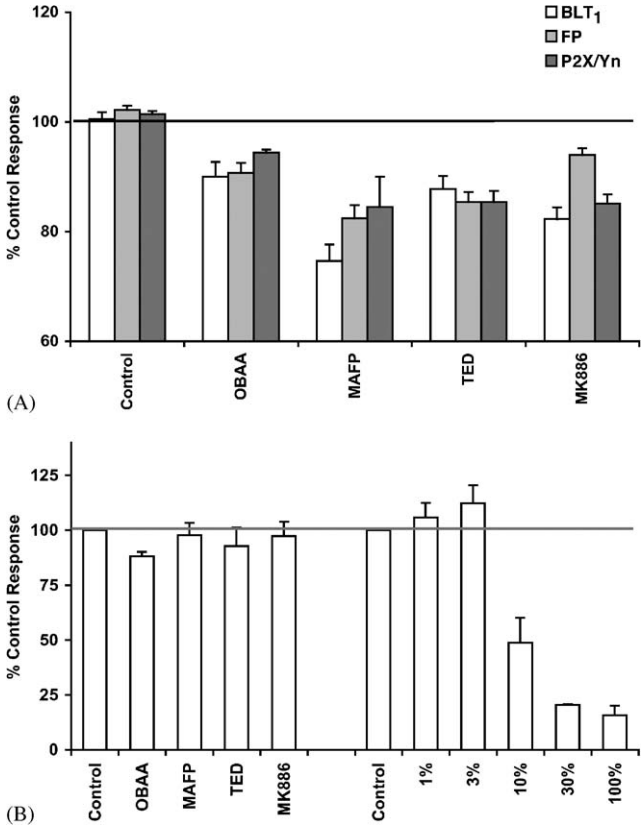

Fig. 2. The non-specific effects of leukotriene synthesis inhibitors. (A) The effects of various drugs on intracellular calcium concentrations following the activation of the LTB_4 receptor BLT_1 or, the endogenously expressed dideoxy-$PGF_{2\alpha}$ receptor, FP, and P2Xn/Yn purino receptors (activated with ATP). Calcium release was measured in HF1pBLT1 cells as described in Methods, and the curves were integrated, averaged and are shown as the mean of 16 wells (four experiments in quadruplicate) ± 95% CI. Similar results were obtained with sham transfected HF1 cells, except that no response to LTB_4 exposure was observed. (B) The toxicity of various drugs measured over 90 min using MTT. The results shown are from two experiments in triplicate using HF1pBLT1 cells and are presented as mean absorbance ± SD. To the far right on the *x*-axis are various alcohol solutions presented as v/v. Sham transfected HF1 cells produced similar results.

low levels 5-LO mRNA (Table 2). This is reflected at the protein level as well. While western blots of isolated leukocytes revealed 5-LO expression, no expression was detected in HeLa cells (Fig. 3). This is the first work to examine both mRNA and protein expression of 5-LO in HeLa cells.

3.4. HeLa cells do not produce LTB_4

A commercial EIA assay for LTB_4 was employed to analyse LTB_4 production (Fig. 4a). Neutrophils stimulated with thapsigargin produced about 15 times more LTB_4 than controls, whereas no LTB_4 production could be detected in the HF1pBLT1 extracts. Exogenous LTB_4 was added as a positive control prior to methanol extraction. Compared to neutrophil cultures, much less LTB_4 was recovered from cultures of HF1pBLT1 cells, implying that some of the LTB_4 may have been lost during extraction or due to metabolism (either enzymatic or non-enzymatic). Assuming the recovery of ionophore-induced LTB_4 is the same as for exogenous LTB_4, it would still have been possible to detect endogenous LTB_4 production by HF1pBLT1 cells. Using HF1 reporter cells the effect of BLT_1 expression was also examined. When transfected with BLT_1, HF1 cells respond to LTB_4 stimulation by producing luciferase, which in turn is used to produce luminescence that is proportional to BLT_1 activation [23]. The results illustrated in Fig. 4b show that these reporter cells are sensitive to LTB_4 concentrations as low as 0.3 nM ($P < 0.05$). The EC_{50} for LTB_4 under these conditions was 4.8 nM (3.3–7.1, 95% CI, note that the entire dose range is not shown in Fig. 3). Using this bioassay to analyse LTB_4 production we found that while neutrophils produce LTB_4 in response to stimulation with thapsigargin or ionomycin, HF1pSham and HF1pBLT1 cells do not. As a positive control exogenous LTB_4 was added to the cell culture medium before LTB_4 extraction with methanol. Only a fraction of the exogenous LTB_4 was detected, indicating that much of it remained in the culture medium or was metabolised, but the exogenous LTB_4 was clearly detectable using HF1pBLT1 cells. HF1pSham cells, which do not express BLT_1, did not respond to LTB_4 (although they are activated somewhat by the methanol extracts). Taken together, these assays show that under the conditions of these experiments HF1pBLT1 and HF1pSham cells do not produce BLT_1 agonists.

4. Discussion and conclusions

We have found that agents interfering with LTB_4 synthesis at the level of phospholipase A_2 or 5-LO do indeed reduce the calcium responses to LTB_4 observed in HeLa cells expressing BLT_1, the primary LTB_4 receptor. This is not, however, dependent on inhibition of enzymatic LTB_4 synthesis because HeLa cells do not express 5-LO, the first enzyme in the leukotriene synthesis pathway, and they are unable to produce LTB_4. Furthermore, the same effects on calcium release are observed using HeLa cells regardless of whether or not they express receptors for LTB_4 and, calcium signalling by a variety of receptors is also affected, suggesting that these inhibitors have non-specific effects on calcium homeostasis in HeLa cells.

Several studies have also reported that leukotriene synthesis inhibitors can affect HeLa cell physiology, suggesting that leukotrienes must therefore be involved.

Table 1
Microarray gene expression analysis of leukotriene biosynthesis enzymes and receptors for leukotrienes in HeLa HF1 cells

Name	Abbreviation	Accession	Signal	SEM	Present/Absent
Leukotriene A_4 hydrolase	LTA_4H	NM_000895	2030	61	P
Microsomal glutathione S-transferase 2	MGST2	NM_002413	1503	118	P
5-Lipoxygenase-activating protein	FLAP	NM_001629	190	21	A
3-Lipoxygenase	3-LO	NM_021628	179	10	A
15-Lipoxygenase, second type	15-LO	NM_001141	47	18	A
5-Lipoxygenase	5-LO	NM_000698	31	11	A
Leukotriene C_4 synthase	LTC_4S	NM_000897	27	9	A
5-Lipoxygenase	5-LO	NM_000698	25	1	A
12-Lipoxygenase	12R-LO	NM_001139	12	2	A
Gamma-glutamyltransferase-like activity 1	GGTLA1	NM_004121	9	1	A
5-Lipoxygenase-like	5-Lolike	AW003512	8	1	A
5-Lipoxygenase	5-LO	AA995910	5	1	A
15-Lipoxygenase	15-LO	NM_001140	4	0	A
Leukotriene B_4 receptor 1	BLT_1	NM_181657	102	12	A
Cysteinyl leukotriene receptor 2	$CysLT_2$	NM_020377	84	19	A
Leukotriene B_4 receptor 1	BLT_1	NM_181657	43	14	A
Cysteinyl leukotriene receptor 1	$CysLT_1$	NM_020377	33	6	A
Leukotriene B_4 receptor 2	BLT_2	NM_019839	11	4	A

Affymetrix HU-133 cDNA microarray chips were used to examine the expression of leukotriene receptors and genes involved in leukotriene biosynthesis. HeLa HF1 cells with and without BLT_1 were exposed for 1 h to 100 nM LTB_4 or a control solution prior to mRNA isolation. None of the leukotriene receptors were expressed (= absent) at the mRNA level (data for BLT_1 was obtained using sham-transfected cells only). Of the genes known to be involved in leukotriene biosynthesis only two ubiquitously expressed genes were expressed (= present) by HeLa HF1 cells. 5-LO in particular is represented three times by different probe sets, and in all cases was not found to be expressed. BLT_1 is represented by two different probe sets and was also not expressed.

Table 2
Quantitative PCR analysis of 5-LO gene expression

Cell type	Treatment	Copy number, mean ± SD
Monocytes	Control	8700 ± 800
HeLa HF1pBLT1	1 h control	Not detectable
	1 h LTB_4	5 ± 1
	16 h control	32 ± 27
	16 h LTB_4	43 ± 28
HeLa HF1pSham	1 h control	5 ± 1
	1 h LTB_4	3 ± 2
	16 h control	Not detectable
	16 h LTB_4	Not detectable

Messenger RNA was isolated from the total RNA present in approximately 100,000 monocytes or 500,000 HeLa cells. Quantitative PCR was used to determine the number of β-actin or 5-LO mRNA copies in each sample. Results from three PCR reactions performed using samples from duplicate cell populations were normalised using β-actin expression. HeLa cells with and without the leukotriene B_4 receptor, BLT_1 were exposed to either 100 nM LTB_4 or buffer and RNA was isolated after 1 and 16 h.

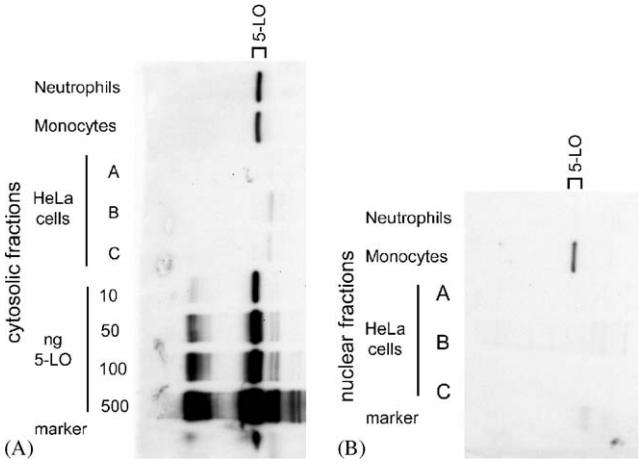

Fig. 3. 5-LO expression in leukocytes compared with HeLa cells. (A) Various concentrations of purified 5-LO in the same western blot as 50 μg of protein from cytosolic fractions isolated from three different HeLa cell cultures (A–C), neutrophils and monocytes. (B) A parallel blot showing 50 μg of proteins from nuclear fractions, isolated from the same cells as in (A).

The survival of HeLa cells exposed to tumour necrosis factor-α (TNF-α) and cyclohexamide was found by Chang et al. [22] to be enhanced by two non-selective LO inhibitors. They proposed that superoxide anions were generated from LO products, which reduced cell survival. Chun and Jacobson [18] used a variety of PLA_2 and LO inhibitors in an extensive study of HeLa cell spreading on gelatin substrates. They found that inhibiting leukotriene synthesis pathways could attenuate cell spreading and, that this could be partly reversed by adding arachidonic acid. Similar results have been reported by Auer and Jacobson [20]. Actin polymerisation in HeLa cells provoked by exposure to epidermal growth factor (EGF) has also been shown by

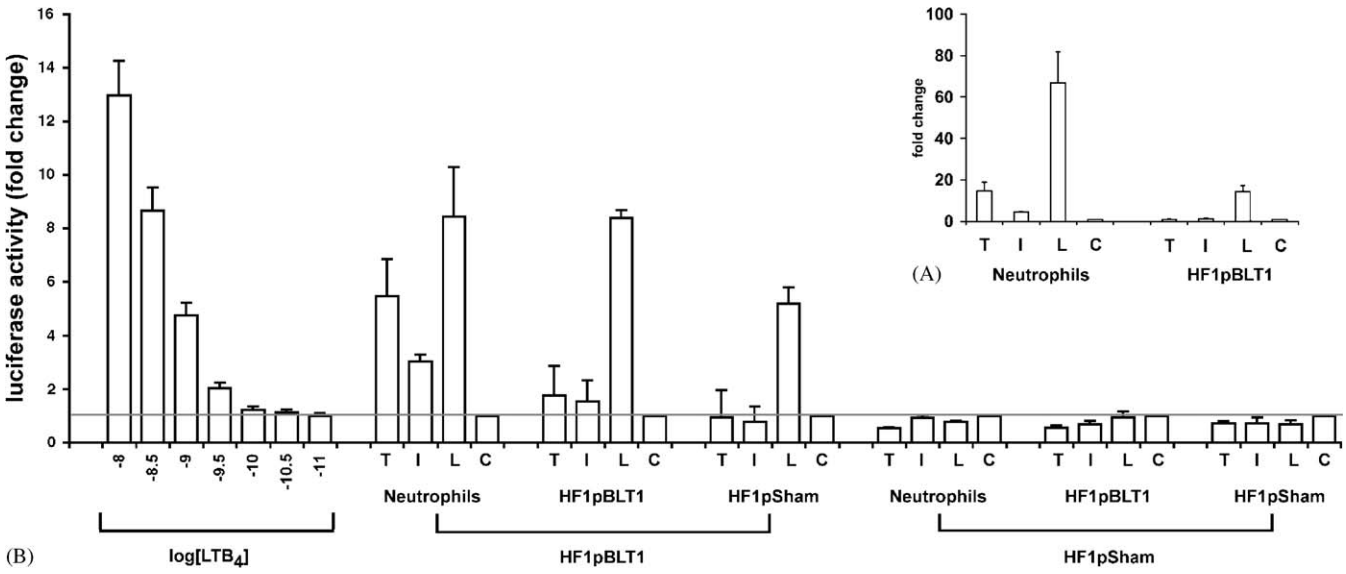

Fig. 4. Exposure to thapsigargin or ionomycin does not result in measurable LTB_4 production by HeLa cells. (A, inset) HF1pBLT1 cells or neutrophils were exposed to 1 μM Thapsigargin (T), 10 μM Ionomycin (I), 100 nM LTB_4 (L) or PBS control (C) for 10 min at 37 °C. The medium was removed, and LTB_4 was extracted using methanol according to Peppelenbosch et al. [21]. The resulting extracts were then added to an EIA assay specific for LTB_4. HF1pBLT1 cells did not produce LTB_4 in response to ionophores (T, I), but added LTB_4 (L) could be detected. The assay results are expressed as mean fold change (treatment response, divided by control) ± SEM, $n = 6$. (B) To examine the possible effect of BLT_1 expression on LTB_4 production, we also used a two-step bioassay. In the first step, cultures of neutrophils, HF1pSham, and HF1pBLT1 cells were treated as in (A). In the second step the extracts were added to HF1pBLT1 or HF1pSham cells, and luciferase production was analysed after 16 h. Known LTB_4 concentrations were used to produce a standard curve (far left). When measured using this bioassay system, the ionophore treatment did not result in measurable LTB_4 production by HF1 cells, regardless of BLT_1 expression. Exogenous LTB_4 (L) could, however, be detected, but only if the culture in the second step expressed BLT_1 (middle group, HF1pBLT1). Luciferase assay results are expressed as mean fold change (treatment response divided by control) ± SEM, from three separate experiments.

Peppelenbosch et al. [21] to be abolished by pre-treatment with LO inhibitors or the FLAP inhibitor MK886. Peppelenbosch et al. also reported that HeLa cells could produce peptidyl leukotrienes following exposure to EGF. None of these reports present data demonstrating the expression of 5-LO (or any of the other LOs), despite the fact that they are predated by a study stating that HeLa cells do not express 5-LO mRNA [28]. This latter work has recently been confirmed [29] and it has been shown that the lack of 5-LO mRNA expression is likely due to gene promoter methylation. None of the above reports have examined both 5-LO mRNA and protein expression however, so the possibility existed that 5-LO protein was expressed without a detectable amount of mRNA message. It is clear from our results that HeLa cells do not produce LTB_4, and that they lack both 5-LO mRNA and, the 5-LO enzyme protein necessary to do so. We were unable to detect the production of any BLT_1 agonists by HeLa HF1pSham or HF1pBLT1 cells, even though neutrophils could produce LTB_4 (and related metabolites) under similar conditions. HeLa cells might produce leukotrienes non-enzymatically, but this production was below the limits of detection. Microarray analysis of gene expression in HF1pBLT1 cells indicated that these cells do not express 5-LO, and although we could sometimes detect very low 5-LO expression using quantitative PCR, this was only possible using large numbers of cells, and many reaction cycles to increase sensitivity. Even so, the quantities observed were at the limit of detection and probably of little physiological relevance. In complete agreement with the present data, several publicly accessible microarray data sets contain HeLa cells as controls and report similar results (e.g. NCBI GEO data set GDS885).

Several of the previously published reports contain results obtained using HeLa S3 cells [18–20], whereas others have not defined the lineage of the HeLa cells used [21,22]. It is possible that our HeLa cells (HF1pSham and HF1pBLT1 derived from HeLa S3) have different patterns of gene expression from those used in previous studies, and this may explain the differences in leukotriene production. In all cases except Chang et al. the exposure times for the various treatments used were also quite short. Unfortunately, shorter experiments are optimal for observing more acute responses that could be a result of the altered calcium homeostasis caused by leukotriene synthesis inhibitors.

When we used agonists for surface receptors that are endogenously expressed by HeLa cells, we observed that all of the leukotriene synthesis inhibitors tested had

similar effects on calcium release, regardless of whether or not the cells expressed BLT_1. Thus, a putative BLT_1-LTB_4 ligand-receptor feedback loop was not part of this response. This also suggests that other newly formed (but currently unknown) mediators that might activate BLT_1 are not involved. In addition, under the conditions used in this study we did not observe cellular toxicity, in line with a previous report [18]. Our results are also in agreement with previous reports that MK886 has a number of effects unrelated to leukotriene synthesis inhibition, ranging from calcium mobilisation to apoptosis [30–33].

We have found that leukotriene synthesis inhibitors have non-specific effects on HeLa cell calcium homeostasis. Changes in cell physiology following exposure to these inhibitors could therefore be misinterpreted as being related to the inhibition of leukotriene (or eicosanoid) production. Our experiments do not reveal the mechanisms of these non-specific effects because additional experiments, with other leukotriene synthesis inhibitors or analogs of LTB_4, and modulators of calcium signalling, would be necessary to further characterise exactly how calcium homeostasis is affected. It is clear, however, that the LTB_4 receptor, BLT_1, is not involved and nor are any mediators that could act via this receptor. It is entirely possible that calcium homeostasis in leukocytes or other cancer cells are also affected in a similar fashion. This is important given the attention that is currently being focused on leukotriene synthesis inhibitors and the general importance of calcium homeostasis for every cell in the human body.

We find that the commonly used cervical carcinoma cell line HeLa cannot produce leukotrienes because the essential enzyme 5-LO is not expressed. A number of reports [18–22] regarding the importance of leukotriene production (and leukotriene B_4 in particular) for various aspects of HeLa cell physiology, including some of the carcinogenic traits, should therefore be re-evaluated in light of this finding. While many leukotriene inhibitors probably do affect aspects of HeLa cell physiology and perhaps cervical carcinogenesis, the mechanism appears to be more complicated than is widely appreciated. Studies relying solely on the activity of leukotriene synthesis inhibitors as evidence of leukotriene involvement in carcinogenesis should therefore be interpreted with caution.

Acknowledgements

AS designed the study, drafted the manuscript, and performed all experiments. JB assisted with cDNA preparation and QPCR. UK prepared cDNA from monocytes. CO and JZH supervised the study and assisted with preparation of the manuscript. The authors declare no conflicts of interest.

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