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Binding of Leukotriene C_4 to Rat Lung Fibroblasts and Stimulation of Collagen Synthesis in Vitro†

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Received August 3, 1987; Revised Manuscript Received December 11, 1987

ABSTRACT: Arachidonate metabolites are potent biological mediators affecting multiple cellular functions. Although prostaglandins of the E series, which are products of the cyclooxygenase pathway, have been known as inhibitors or down-regulators of fibroblast proliferation and collagen synthesis, the more recently discovered products of the 5-lipoxygenase pathway have not been as extensively investigated with regard to fibroblast function. In this study, a sulfidopeptide product of the lipoxygenase pathway, leukotriene C_4 (LTC_4), was examined for its ability to modulate rat lung fibroblast collagen synthesis and proliferation in vitro. The data revealed the ability of LTC_4 and to a lesser extent leukotriene D_4 (LTD_4) to stimulate collagen synthesis in a dose-dependent (10^{-11}-10^{-8} M) manner without affecting cellular proliferation as determined by radiolabeled thymidine incorporation; 1 nM LTC_4 caused an 85% ($p < 0.02$) increase above untreated controls in [^{3}H]proline incorporation into collagenous protein in the media, which was blocked by the putative leukotriene receptor antagonist FPL55712 (10 μM) and inhibited by cycloheximide and actinomycin D. This LTC_4 stimulatory effect was slightly more specific for collagen synthesis vs noncollagenous protein synthesis but was not accompanied with any change in the collagen type composition. Binding of [^{3}H]LTC_4 to these cells was specific, reversible, and saturable, with a K_d of 1.8 ± 0.95 nM. Under equilibrium conditions, there was an estimated 2.39×10^4 receptors per cell. This binding was also inhibited by 10 μM FPL55712. Competitive binding studies show specificity of this binding for LTC_4 relative to LTD_4 and FPL55712. Furthermore, no significant conversion of LTC_4 to LTD_4 or leukotriene E_4 was noted during the binding studies. These results indicate the presence of specific LTC_4 receptors on these cells which may mediate the cellular effects on protein and collagen synthesis.

Understanding how arachidonate metabolites affect fibroblast growth and function is critical for uncovering the mechanism by which these substances initiate, maintain, and/or down-regulate the fibrogenic response in fibrotic diseases. Recent studies have demonstrated the inhibitory effects of prostaglandin E_2 (PGE_2)[1] on fibroblast growth and collagen synthesis (Baum et al., 1978; Clark et al., 1982; Elias et al., 1985), thus suggesting an important role as down-regulators, perhaps to maintain or return fibroblasts to a quiescent state. The more recently discovered 5-lipoxygenase pathway products of arachidonate metabolism have not been examined in as much detail. The products of this pathway are primarily the various HETE's and leukotrienes. These substances have diverse potent biological activities and are the products of various cell types (Samuelsson, 1983; Lewis & Austen, 1984).

† Supported by National Institutes of Health Grants HL28737, HL31237, HL31963, and HL39925 and by a grant-in-aid from the American Heart Association and its Michigan affiliate. Part of this work was performed during the tenure of Established Investigatorships (to S.H.P. and S.L.K.) of the American Heart Association.

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[1] Abbreviations: PGE_2, prostaglandin E_2; LTB_4, leukotriene B_4; LTC_4, leukotriene C_4; LTD_4, leukotriene D_4; LTE_4, leukotriene E_4; DMEM, Dulbecco's modified Eagle's medium; βAPN, β-aminopropionitrile; PBS, phosphate-buffered saline; PMSF, phenylmethanesulfonyl fluoride; TCA, trichloroacetic acid; FCS, fetal calf serum; HETE, hydroxyeicosatetraenoic acid; 5-HETE, 5-hydroxyeicosatetraenoic acid; SRS-A, slow reactive substance of anaphylaxis; EDTA, ethylenediaminetetraacetic acid trisodium salt; PITC, phenyl isothiocyanate; PTH, phenylthiohydantoin; HPLC, high-performance liquid chromatography; SDS, sodium dodecyl sulfate; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

0006-2960/88/0427-2846$01.50/0

Of relevance to the regulation of the fibrogenic response is the macrophage or monocyte, which in the presence of appropriate stimuli is a major producer of LTB_4, LTC_4, and, to lesser extents, the other leukotrienes (Lewis & Austen, 1984; Rouzer et al., 1980, 1982; Williams et al., 1984). Since these cells are found in areas of scarring and wound healing, production and release of such mediators may affect the accumulation of connective tissue which typifies the fibrotic lesion.

Although a recent study has demonstrated that LTB_4 is chemotactic for fibroblasts (Mensing & Czarnetzki, 1984), the functional effects of leukotrienes in cells other than inflammatory or phagocytic and contractile cells have not been well characterized. This is primarily the result of the initial discovery that LTC_4, LTD_4, and LTE_4 are the major constituents of SRS-A, thus focusing most of the interest on their smooth muscle contractile activity and other phenomena related to anaphylaxis (Samuelsson, 1983; Lewis & Austen, 1984). These and other studies, however, have clearly documented the existence of specific receptors for these leukotrienes in a wide variety of tissues and cell types (Lewis & Austen, 1984; Krilis et al., 1983; Cheng et al., 1985; Ballerman et al., 1985; Baud et al., 1985b), presumably mediating some biologically relevant responses. In this paper, the effects of LTC_4 on isolated rat lung fibroblast proliferation and collagen synthesis were examined. Binding studies were undertaken to demonstrate the existence of receptors for this leukotriene and to determine the relevant binding parameters as a means of probing the possible biological relevance of the binding phenomena.

Materials and Methods

Materials. LTC_4, LTD_4, and LTE_4 were the generous gifts of Dr. J. Rokach (Merck Frosst Laboratories, Quebec, Canada). 5-HETE was generously provided by Drs. John Pike and Robert Gorman (Upjohn Co., Kalamazoo, MI). [14,15-^{3}H]LTC_4 (20–60 Ci/mmol), L-[2,3,4,5-^{3}H]proline (>100 Ci/mmol), and [*methyl*-^{3}H]thymidine (6.7 Ci/mmol) were obtained from New England Nuclear (Boston, MA). DMEM, FCS, and penicillin–streptomycin–fungizone (antibiotics) stock solution were from Grand Island Biological Co. (Grand Island, NY). Bacterial collagenase (CLSPA grade) was from Worthington Corp. (Freehold, NJ) and further purified by gel filtration prior to use (Phan et al., 1985). Trypsin (1:250) for isolating and passaging cells for tissue culture was from Difco Laboratories (Detroit, MI). 4-(Dimethylamino)benzaldehyde was from BDH Chemicals (Poole, England). PITC, glacial acetic acid, pyridine, trimethylamine, and constant-boiling 6 N HCl were all HPLC grade and obtained from Pierce Chemical Co., Rockford, IL. HPLC-grade acetonitrile, methanol, and ammonium acetate were from Fisher Scientific Co. (Livonia, MI). All other reagents were of reagent grade or better, unless otherwise specified.

Lung Fibroblast Isolation. Fibroblasts were isolated from male Fisher 344 rats weighing 150–200 g (Charles River, Portage, MI), exactly as reported previously (Phan et al., 1985). Cells between fifth and fifteenth passages were used for this study. Cells were maintained in DMEM plus 1% antibiotics and 10% FCS and passaged by using 0.25% trypsin with a 1:3 split ratio.

Cell Proliferation Assay. This was done essentially as previously described (Phan et al., 1985). Briefly, 5×10^3 cells were plated in each well of a 96-well microtiter plate in DMEM containing 10% FCS. After 24 h, the media were removed and replaced with fresh DMEM containing 10% FCS and with or without LTC_4. After 18–24 h of incubation, the cells were pulsed with 1 μCi of [^{3}H]thymidine per well for 6 h. After being washed, the cells were then transferred to cellulose acetate filters, lysed, washed, and counted for radioactivity as previously described (Phan et al., 1985). Data were expressed as dpm per well.

Doubling time for these cells was also determined to assess the effect of LTC_4 on cell growth. This was done as previously described by actual cell counting using a hemocytometer (Phan et al., 1985).

Collagen Synthesis. This was measured essentially as described previously (Phan et al., 1985). Briefly, confluent cell monolayers in 35-mm diameter dishes or 75 mm^2 flasks were washed twice with serum-free DMEM prior to use. The media were then replaced with fresh serum-free DMEM containing 50 μg/mL sodium ascorbate, with or without LTC_4, and incubated overnight (16–18 h). The cells were then pulsed for 6 h with 10 μCi/mL [^{3}H]proline in the presence of 0.2 mM cold or nonradiolabeled proline, 80 μg/mL βAPN, and fresh 50 μg/mL sodium ascorbate. The same system was also assayed in the presence of FPL55712, cycloheximide (0.1 mg/mL), or actinomycin D (1 μg/mL) added with the LTC_4 to examine their ability to inhibit the LTC_4 effect. After the pulse, the media were removed and pooled with one wash of the cell layer. The cell layer is gently scraped into PBS and an aliquot taken for cell count. Both media and cell layer were then analyzed for incorporation of radioactivity into collagenase-sensitive (collagen synthesis) and collagenase-insensitive (noncollagenous protein synthesis) proteins as described previously (Phan et al., 1985). Data were expressed as dpm incorporated per 10^6 cells. Under these conditions, >75% of collagenous radioactivity was located in the media fraction.

Collagen synthesis was also measured as the total amount of hydroxyproline production. Both media and cell layer were analyzed for hydroxyproline content in two ways. For these experiments, cells grown in 75 mm^2 flasks were used, and the data were based on the pooling of products from three flasks to provide adequate amounts of hydroxyproline for analysis. Both samples (media plus cell layer) were exhaustively dialyzed against 0.1 M acetic acid after treatment with 0.1 mM PMSF, 5 mM *N*-ethylmaleimide, and 5 mM EDTA. After taking an aliquot for SDS–polyacrylamide gel electrophoretic analysis, the samples were dried with a Speed Vac (Savant Instruments, Inc., Hicksville, NY). They were then resuspended in constant-boiling 6 N HCl and hydrolyzed under vacuum at 110 °C for 16 h. These were then redried in the Speed Vac, resuspended in glass-distillized–deionized water, and redried to remove all acid completely. An aliquot was then taken for the colorimetric assay using 4-(dimethylamino)benzaldehyde as described previously (Phan et al., 1985).

The remainder of the samples was then analyzed for hydroxyproline according to the method of Clark and Hildebrandt (1984), except amino acids were derivatized with PITC instead of dansyl chloride. The samples were derivatized with PITC to form their respective PTH-amino acids, according to the method of Heinrikson and Meredith (1984). Briefly, samples were suspended in 100 μL of coupling buffer (acetonitrile/pyridine/trimethylamine/H_2O, 10:5:2:3) and redried in the Speed Vac. After resuspensions in coupling buffer, PITC (0.5% final concentration) was added and allowed to react for 15 min at room temperature. The samples were then dried in the Speed Vac, resuspended in methanol, and analyzed by reverse-phase HPLC using a Varian Vista 5560 chromatography system (Varian Instruments, Palo Alto, CA). A C_{18}, 3-μm particle size column (4.6 mm × 15 cm) (LC18, Supelco, Bellefonte, PA) was used for this analysis. The effluent was detected for the absorbance at 269 nm as well as for radio-

activity using an on-line detector (Model HS, Radiomatic Instruments, Tampa, FL). The chromatography conditions were essentially as described previously (Heinrikson & Meredith, 1984) except for a few modifications. Solvent A was composed of 0.05 M ammonium acetate, pH 6.8; solvent B was composed of 0.1 M ammonium acetate, pH 6.8, in 50% methanol (in H_2O); solvent C is 100% methanol. The program started with a linear gradient of 87% A/13% B to 82% A/18% B from 0 to 20 min. This was followed by a linear gradient of 70% A/25% B/5% C to 65% A/5% B/30% C from 20.01 to 50 min. Elution was done at 50 °C and a flow rate of 1.0 mL/min. Under these conditions, PTH-hydroxyproline and PTH-proline eluted at 34.5 and 46.8 min, respectively, with base-line separation from all other PTH-amino acid peaks. The peaks were integrated, and the amount of PTH-amino acid was quantitated by using an internal standard (diethyl phthalate). The specific radioactivities of hydroxyproline in the samples were then expressed as dpm per nanomole and used to calculate total hydroxyproline in the media. This was done by dividing the total dpm of PTH-hydroxyproline by the specific radioactivity of the PTH-hydroxyproline. Data were expressed as nanograms of hydroxyproline per 10^6 cells.

Analysis of Collagen Products. After pulsing with [^{3}H]-proline, media and cell layer were prepared as described above and analyzed by SDS–polyacrylamide gel electrophoresis. After dialysis against 0.1 M acetic acid and drying with the Speed Vac, the samples were dissolved in 10 mM Tris-HCl, pH 6.8, containing 2% SDS, 10 mM EDTA, and 10% glycerol. After being heated for 2 min in a boiling water bath, the samples were cooled, reduced with 5% mercaptoethanol, and subjected to electrophoresis as previously described (Phan et al., 1985). The gels were treated with scintillant, dried, and exposed to X-ray film (Phan et al., 1985). After development, the autoradiograms were scanned with an LKB Ultroscan densitometer, and the peaks were quantitated by electronic integration.

LTC_4 Binding Studies. Confluent cells in 35-mm diameter dishes were used for these experiments. Upon reaching confluence, the media were removed and replaced with 1.0 mL of serum-free DMEM supplemented with 10 mM serine borate and chilled on a bed of ice. [^{3}H]LTC_4 was then added at final concentrations as indicated in the figures and tables. Nonspecific binding was determined as dpm bound in the presence of 1 μM cold LTC_4 and maximally was <30% of total binding. When the effect of FPL55712 was tested, 10 μM was added prior to addition of [^{3}H]LTC_4. For kinetic studies, the incubation was continued at 0–4 °C for the times indicated in Figure 4. For all other equilibrium binding assays, the standard incubation time was 60 min. To demonstrate reversibility of binding, 4.0 μM cold LTC_4 was added after 60 min of incubation (see Figure 4) and the residual specific binding determined. All incubations were stopped by rapid removal of media followed by two washes with ice-cold PBS. The cells were then resuspended in PBS by brief trypsinization (3 min, 0.25%), transferred to scintillation vials, and counted for radioactivity after the addition of scintillant. Binding assays were done in triplicate, except where indicated otherwise, and expressed as femtomoles of [^{3}H]LTC_4 specifically bound per 10^6 cells.

To determine the specificity of LTC_4 binding, competitive binding studies were undertaken. Conditions were as described above for equilibrium binding studies, except where indicated the [^{3}H]LTC_4 binding assay was done in the presence of the indicated concentrations of cold LTC_4, LTD_4, LTE_4, or FPL55712 (Figure 7). These assays were all done in the presence of 2.6×10^{-9} M [^{3}H]LTC_4.

Table I: Effect of LTC_4 on Cell Growth

treatment	cell growth	
	doubling time[a] (h)	dpm[b]
none	41 ± 2.1	5880 ± 480
10^{-9} M LTC_4	43 ± 1.3 (NS)	5566 ± 338 (NS)

[a] This refers to the number of hours needed to double the number of cells per flask. All data are presented as means ± SE (N = 3). NS means $p \leq 0.05$ as determined by the t test between treated vs untreated mean values. [b] This refers to the amount (dpm) of [^{3}H]thymidine incorporated per well in a 6-h pulse period.

Analysis of [^{3}H]LTC_4 Metabolism. To examine the degree of conversion of [^{3}H]LTC_4 to [^{3}H]LTD_4 and [^{3}H]LTE_4, fibroblasts were grown to confluence in 75 mm^2 flasks. The cells were then exposed to 2.6×10^{-9} M [^{3}H]LTC_4 (0–4 °C) for 55 min in the presence of 0, 10, or 50 mM serine borate. The media were then removed and the cells washed twice with 20 mL of cold PBS each time. They were then resuspended in 5 mL of 0.1% acetic acid titrated to pH 5.6 with 0.5 N NaOH (solvent A for HPLC analysis). After scraping the cells, the suspension was sonicated on a bed of ice, 3 times with 10-s bursts of 30 W. After centrifugation, the clarified samples were concentrated to 0.5 mL by vacuum centrifugation (Speed Vac); 200 μL of each sample was then injected onto a C18 reverse-phase column (Supelcosil LC-18DB, 4.6 × 150 mm) and eluted isocratically with 42% solvent A and 50% solvent B (CH_3OH). The instrumentation was as described for PTH-amino acid analysis. Peaks were identified by comigration with authentic pure standards for LTC_4, LTD_4, and LTE_4.

Glutathione S-Transferase Assay. Intact fibroblasts, fibroblast lysates, and sonicates were assayed for glutathione S-transferase activity as described previously (Habig et al., 1974; Chan et al., 1986). Intact fibroblasts were directly scraped from tissue culture dishes and suspended at 2.5×10^6 cells/mL. Lysates were prepared by directly adding assay buffer (0.05 M potassium phosphate, pH 6.5) containing 0.2% Triton X-100 to fibroblast monolayers. Sonicates were obtained by sonicating the suspension of intact fibroblasts in assay buffer for 30 s at 30 W on ice; 100-μL aliquots of these samples were then assayed for transferase activity using 1-chloro-2,4-dinitrobenzene as substrate (Chan et al., 1986). Rat liver homogenates were used as positive controls.

Statistical Analysis. This was done by using the unpaired, two-tailed Student's t test when comparing means.

Results

Effects on Cell Proliferation. Addition of 1 nM LTC_4 to rat lung fibroblasts failed to affect [^{3}H]thymidine incorporation or cellular doubling times in a significant manner (Table I). Although this assay was undertaken in the presence of 10% FCS, similar experiments in the presence of 0.4% FCS also failed to affect [^{3}H]thymidine incorporation (data not shown). These two conditions should allow for the expression of both inhibitory and stimulatory effects if either one were present as a result of the addition of 1 nM LTC_4. No significant effects were seen with incubation times as long as 24 h.

Effects on Collagen Synthesis. The data in Table II revealed that LTC_4 significantly stimulated collagen synthesis by isolated rat lung fibroblasts as assessed by three different methods of measuring collagen production. Measurements of [^{3}H]proline incorporation into collagenase-digestible proteins revealed an averge 85% increase over untreated control cells in the media, i.e., secreted procollagens. In the cell layer, the

Table II: Effects of LTC_4 and FPL55712 on Fibroblast Collagen Synthesis

sample	treatment	collagen synthesis[a] dpm/10^6 cells[b]	ng of Hyp/10^6 cells[c]	ng of Hyp/10^6 cells[d]
media	none	69416 ± 5631	140 ± 11.8	209 ± 7.1
	1 nM LTC_4	128322 ± 12374 ($p < 0.02$)	245 ± 19.7 ($p < 0.02$)	291 ± 11.2 ($p < 0.005$)
	10 μM FPL55712, 1 nM LTC_4	73522 ± 3988 (NS)	151 ± 11.8 (NS)	219 ± 10.7 (NS)
cell layer	none	71495 ± 5622	ND[e]	105 ± 1.0
	1 nM LTC_4	97534 ± 1893 ($p < 0.02$)	ND	152 ± 8.7 ($p < 0.01$)
	10 μM FPL55712, 1 nM LTC_4	76213 ± 5767 (NS)	ND	109 ± 3.9 (NS)

[a] Data were presented as means ± SE, $N = 3$. Numbers in parentheses refer to p values of Student's t tests comparing each treatment vs its respective control (untreated) values (NS means $p \geq 0.05$). [c] Calculated by dividing the total dpm in PTH-hydroxyproline by the specific radioactivity of PTH-hydroxyproline in the sample. [d] Refers to results of the standard colorimetric assay for hydroxyproline using 4-(dimethylamino)-benzaldehyde. [e] ND, not determined.

Table III: Effects of Cycloheximide and Actinomycin D on Protein Synthesis

treatment[b]	protein synthesis[a] (dpm/10^5 cells)	treatment[b]	protein synthesis[a] (dpm/10^5 cells)
none	7989	LTC_4	12234
actinomycin D	3846	acinomycin D + LTC_4	4021
cycloheximide	3869	cycloheximide + LTC_4	3784

[a] Data represents the means of duplicate determinations of [^{3}H]proline incorporation into nondialyzable proteins in the media after a 16-h pulse. [b] Actinomycin D, cycloheximide, and LTC_4 were added at final concentrations of 1 μg/mL, 0.1 mg/mL, and 1 nM, respectively.

increase was less dramatic (mean of 36% increase) but was statistically significant ($p < 0.02$). A similar increase (an average of 75% above control) in total hydroxyproline content of the media was noted when the assay was based on the determination of hydroxyproline specific radioactivity (Clark & Hildebran, 1984). This method of measuring collagen synthesis is not influenced by changes in intra- and extracellular proline pool sizes, or intracellular prolyl-tRNA pool sizes (Clark & Hildebran, 1984), and thus is a reliable indicator of actual effects on collagen synthesis. A less sensitive assay using the standard colorimetric method with 4-(dimethylamino)benzaldehyde showed similar effects (Table II), although the increase (an average of 39% above control, $p < 0.005$) was significantly less compared to results using the preceding methods. These stimulatory effects were virtually totally abolished by 10 μM FPL55712, a putative leukotriene receptor antagonist (Table II).

This stimulatory effect was also inhibited by cycloheximide and actinomycin D (Table III). Although these agents alone inhibited protein synthesis (by approximately 40–50%), the addition of LTC_4 failed to significantly alter [^{3}H]proline incorporation in the presence of either inhibitor. These data would suggest that protein synthesis and transcription of collagen genes are required for the observed LTC_4-induced increase in synthetic rates.

This LTC_4-induced increase in collagen synthesis was not due to increased production of a new collagen type nor was a change in the collagen type composition noted, as assessed by SDS–polyacrylamide gel electrophoresis (Figure 1). These cells also show normal processing of procollagen in the LTC_4-treated cells.

To examine the dose dependence of this phenomenon, the same experiment was done in the presence of varying doses of LTC_4, and for comparison, the effects of LTD_4 and 5-HETE were also studied. The results are shown in Figure 2 and show a dose-dependent stimulation, peaking at approximately between 1 and 10 nM. These curves gave approximate ED_{50}'s of 5×10^{-11}, 7×10^{-11}, and 4×10^{-10} M for LTC_4, LTD_4, and 5-HETE, respectively. Furthermore, maximal stimulation by

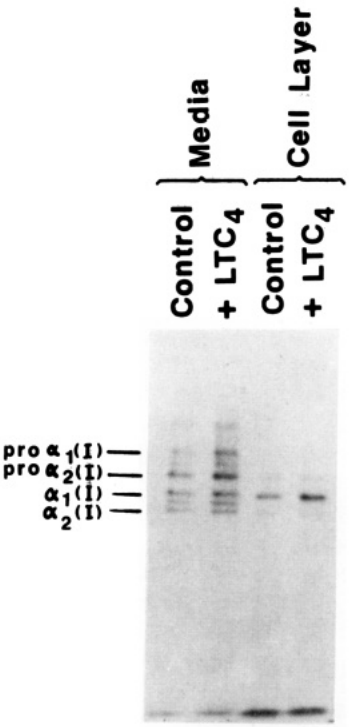

FIGURE 1: SDS gel electrophoretic analysis of collagen products. Cells were exposed to [^{3}H]proline in the presence (+LTC_4) or absence (control) of LTC_4. The cell layer and media were then analyzed for radiolabeled products by gel electrophoresis as described under Materials and Methods. The mobilities of type I procollagen and collagen α chains are indicated. The other bands, with the exception of bands migrating slower than proα_1(I), correspond to the various processing products of procollagen I. These bands were sensitive to digestion by purified bacterial collagenase.

LTC_4 was significantly greater than that by LTD_4 and 5-HETE. Thus, this stimulation of collagen synthesis showed a relative specificity for LTC_4 on a per unit dose basis. The stimulatory effects again are greater for secreted (media) than for cell-associated procollagens.

Although noncollagenous protein synthesis, as defined by collagenase-insensitive radioactivity, was also increased (data not shown), the ratio of collagen vs non-collagen products in the media was significantly increased by LTC_4 treatment. Thus, in Figure 3, this ratio was increased from 36.4% ± 0.32% in controls to 44.1% ± 1.39% in LTC_4-treated cultures ($p < 0.01$). This slightly greater specificity for collagen vs non-collagen synthesis was also seen with LTD_4 (41.3% ± 1.45%, $p < 0.05$) but was not found to be statistically significant for 5-HETE (Figure 3).

Binding Studies. Incubation of fibroblasts with 2.6 nM [^{3}H]LTC_4 resulted in specific binding which approached equilibrium at times greater than 60 min, at 0–4 °C (Figure 4). At this concentration of [^{3}H]LTC_4, specific binding was

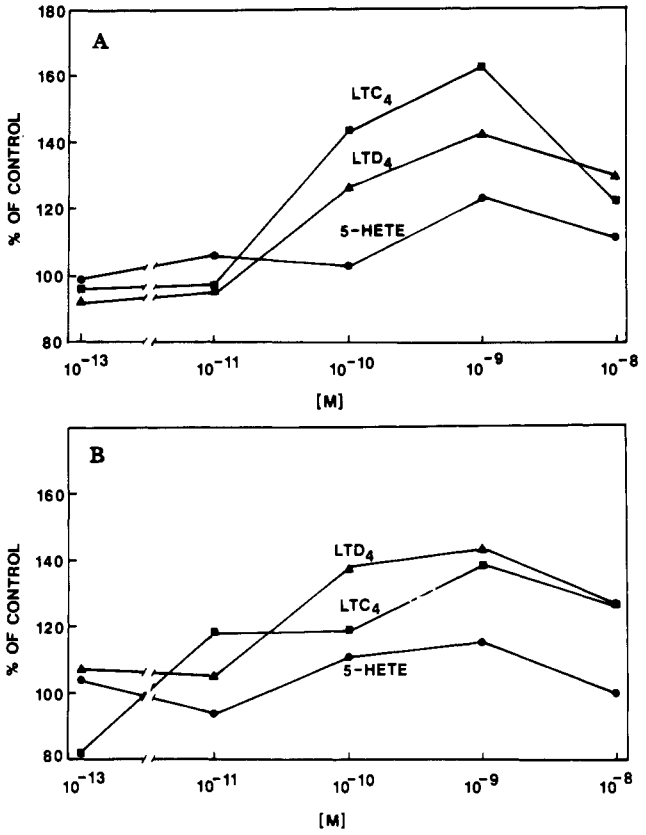

FIGURE 2: Effects of lipoxygenase pathway products on collagen synthesis. Confluent fibroblasts were incubated in the presence of the indicated concentrations of leukotrienes or 5-HETE. Data represented the means of triplicate determinations expressed as dpm of [^{3}H]proline incorporated to collagenase-sensitive proteins and normalized to the untreated control mean values (100%) (mean ± SE, N = 3) of 37275 ● 943 dpm/10^6 cells for the media (A) and 10064 ± 324 dpm/10^6 cells for the cell layer (B).

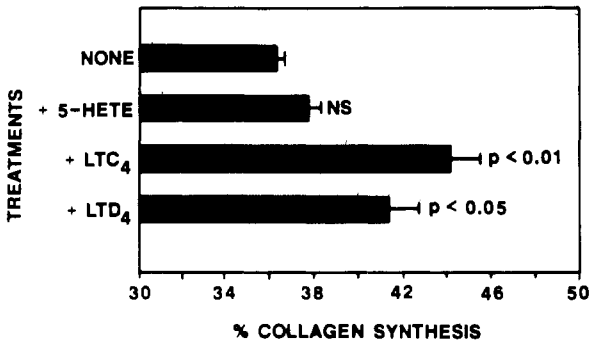

FIGURE 3: Effects on percent collagen synthesis. Percent collagen synthesis was calculated by the formula $\%C = 100[C/(C + 5.04N)]$ where $\%C$ = percent collagen synthesis, C = dpm of collagenous product (as defined by sensitivity to collagenase), and N = dpm of noncollagenous product (as defined by insensitivity to collagenase). All data were derived from analysis of the media, and the incubation conditions were as described in the legend to Figure 2. All assays were done in triplicate, and results were expressed as means ± SE, and the p values were the results of Student's t test between each experimental mean vs the untreated control mean value.

75% ± 3.9% (SE, N = 3) of total binding. Addition of 4.0 μM cold LTC_4 after reaching equilibrium (60 min) resulted in loss of specifically bound [^{3}H]LTC_4 to <5% of the original in 30 min (Figure 4). This binding was markedly decreased in the presence of 10 μM FPL55712 (Table IV) and was saturable, as indicated by the data in Figure 5. The dose–response curve started to taper off at doses >4.0 nM (Figure 5). Scatchard analysis of the binding data using the LIGAND program (Munson & Rodbard, 1980) revealed a K_d of 1.8 ± 0.95 (SD, N = 3) nM and an estimated (2.39 ± 0.17) × 10^4 (SD, N = 3) receptors per cell.

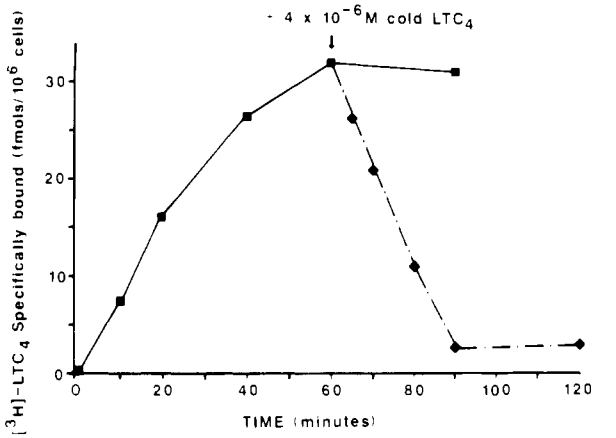

FIGURE 4: Kinetics of LTC_4 binding. Confluent cells were incubated, for the times indicated on the abscissa, with 2.6 nM [^{3}H]LTC_4. After being washed, the cells were transferred and counted for radioactivity. After subtraction of nonspecific binding (done in the presence of 1 μM LTC_4), the dpm bound was converted to femtomoles by division with the specific radioactivity of the [^{3}H]LTC_4. Data were expressed as means of duplicate determinations. To examine for reversibility, 4 μM LTC_4 was added after 60 min of binding and at the indicated times were examined for residual specifically bound radioactivity (dashed lines).

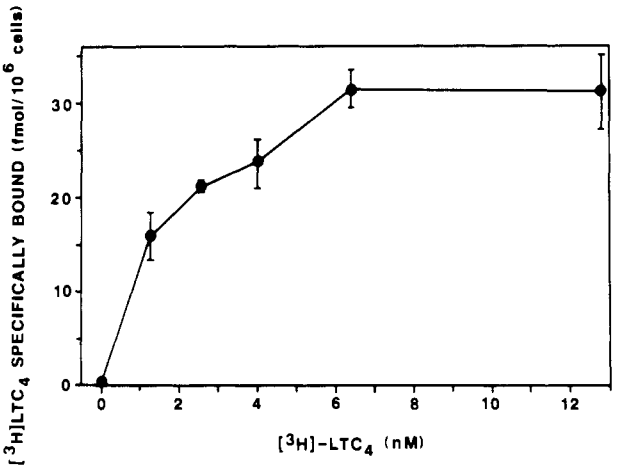

FIGURE 5: Dose–response of LTC_4 binding. Confluent cells were incubated for 60 min in the presence of the indicated final concentrations of [^{3}H]LTC_4. The amount of specific binding was then determined and expressed as mean ± SE, N = 3.

Table IV: Effect of FPL55712 on LTC_4 Binding

treatment	specific binding (fmol/10^6 cells)[a]	
	$+10^{-10}$ M LTC_4	$+10^{-9}$ M LTC_4
none	4.1 ± 0.92	17.5 ± 0.83
10^{-5} M FPL55712	1.3 ± 0.31	5.9 ± 1.08

[a] Data are presented as means ± SE (N = 3). Nonspecific binding for these series of experiments is 24.3%. Concentrations of LTC_4 refer to total used in the binding assays.

To confirm that the LTC_4 binding sites are specific for this leukotriene, competitive binding studies were undertaken. [^{3}H]LTC_4 binding was gradually inhibited by increasing doses of cold LTC_4 (Figure 6). Similar inhibition curves were obtained with LTD_4 and FPL55712, but these curves were shifted to the right by approximately 1 logarithmic unit. LTE_4 was totally ineffective in displacing or inhibiting [^{3}H]LTC_4 binding at doses <10^{-4} M. These results demonstrate the specificity of these binding sites for LTC_4.

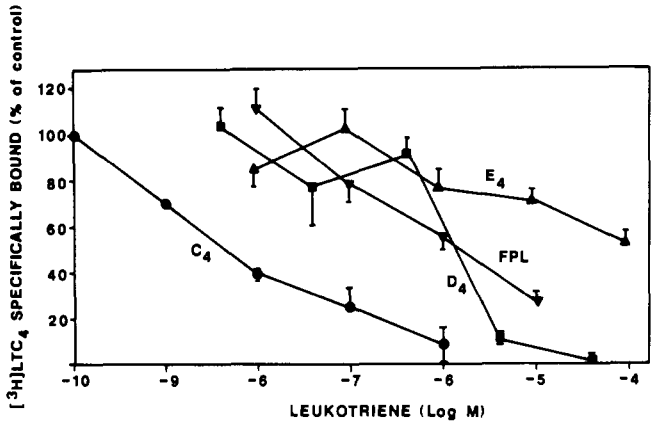

FIGURE 6: Competitive binding analysis. Specific binding of $[^3H]LTC_4$ was determined in the presence of the indicated concentrations of unlabeled LTC_4, LTD_4, LTE_4, or FPL55712. Data were expressed as percent of specific binding in the absence of added unlabeled competing ligand.

These data do not rule out the possibility that part of the observed cell-bound radioactivity represents $[^3H]LTD_4$ binding to LTD_4-specific receptors after hydrolysis of the $[^3H]LTC_4$ ligand. To discriminate between these possibilities, cell-bound radioactivity was analyzed by reverse-phase HPLC to directly quantitate, if any, $[^3H]LTC_4$ conversion to $[^3H]LTD_4$ and $[^3H]LTE_4$ by cellular γ-glutamyltranspeptidase. The original ligand ($[^3H]LTC_4$) obtained from the manufacturer showed <1% contamination by $[^3H]LTD_4$ or $[^3H]LTE_4$ (Figure 7). The major contaminant was a peak with a retention time of 8.7 min (Figure 7B), while LTC_4, LTD_4, and LTE_4 had retention times of 12.2, 22.1, and 29.6 min, respectively (Figure 7A). When binding assays were done in the absence of serine borate, less than 5% of the total radioactivity was associated with LTD_4, while LTC_4 represented 75% of the total (Figure 7C). An unidentified peak representing approximately 10% of the total radioactivity was also present with a retention time of 13.4 min. LTE_4 was present in trace amounts (<0.1%) under these conditions. In the presence of 10 or 40 mM serine borate (Figure 7D,E, respectively), >85% of the radioactivity was associated with LTC_4, with none or trace amounts associated with LTD_4 or LTE_4. Thus, even in the absence of serine borate, conversion of LTC_4 to LTD_4 and LTE_4 was minimal under the conditions of the binding assay. These results confirm that the $[^3H]LTC_4$ binding sites are specific but does not rule out the existence of specific and separate receptors for LTD_4 and/or LTE_4. They also do not rule out the existence of a class-specific receptor which could bind all three peptidoleukotrienes, albeit with differing affinities.

Specific, saturable, and reversible binding of LTC_4 to rat liver glutathione *S*-transferases has been reported (Sun et al., 1986). To demonstrate that this LTC_4 binding phenomenon in fibroblasts is not due to such enzymes, intact cells, lysates, and sonicates were assayed for this activity. No detectable activity was found in either whole cells, lysates, or sonicates, although rat liver homogenates exhibited high activity as has been reported (Sun et al., 1986). Samples equivalent to cell concentrations as high as 2.5×10^6 cells/mL failed to show any activity; 0.2% Triton X-100 used to lyse the cells did not inhibit the positive control. Thus, it is unlikely that LTC_4 binding to fibroblasts is due to such enzymes.

DISCUSSION

It is becoming clear that products of the lipoxygenase pathway of arachidonate metabolism have a wide variety of biological activities beyond those related to anaphylaxis.

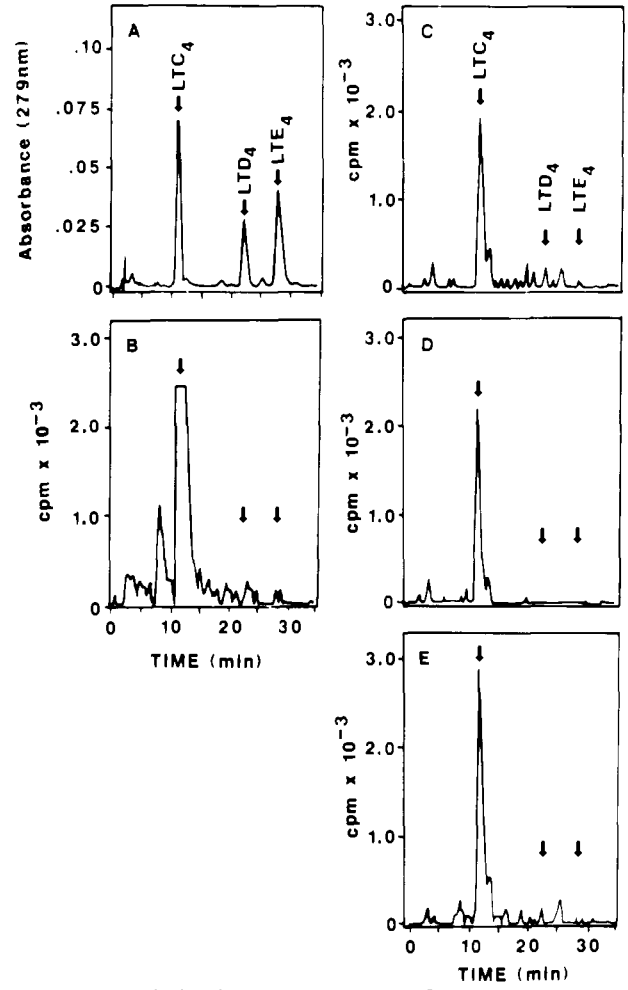

FIGURE 7: Analysis of LTC_4 conversion. Cells were exposed to $[^3H]LTC_4$, and after being washed, the cell layer was extracted and analyzed for $[^3H]LTC_4$ conversion to $[^3H]LTD_4$ and $[^3H]LTE_4$ by reverse-phase HPLC. Authentic pure standards were used to calibrate the column (A). Analysis of the $[^3H]LTC_4$ stock solution from the manufacturer is shown in (B). (C), (D), and (E) show chromatograms of samples from binding assays done in the presence of 0, 10, and 50 mM serine borate, respectively. Arrows represent the expected retention times of the indicated leukotriene.

Recent reports, for instance, have dealt with the chemotactic, adhesion, and growth-promoting activities of the leukotrienes (Mensing & Czarnetzki, 1984; Baud et al., 1985a,b). These activities are detected at concentrations which could be encountered in vivo (Lewis & Austen, 1984) and thus are potentially physiologically relevant. In this paper, the effects of LTC_4 were examined with regard to protein and collagen synthesis and cell proliferation in isolated rat lung fibroblast cultures. The data revealed that LTC_4 could stimulate fibroblast protein synthesis, with relative selectivity for collagenous proteins, in a dose-dependent manner. The effect required transcription and protein synthesis and could be blocked by the putative leukotriene receptor antagonist, FPL55712. The LTC_4 stimulatory effect was observed even when an assay method, independent of changes in intracellular proline or prolyl-tRNA pool size, was used. These data confirmed that the LTC_4 effect was actually at the level of synthesis, not precursor pool size (Clark & Hildebran, 1984). LTD_4 and 5-HETE have smaller simulatory effects. This stimulation by LTC_4 was unaccompanied by any effect on cellular growth or proliferation, in contrast to the effects on human glomerular epithelial cells in culture (Baud et al., 1985b). These phenomena were accompanied by the identification of binding sites on these cells, with properties consistent

with the existence of specific receptors for LTC_4 (Cuatrecasas & Hollenberg, 1976). This conclusion is supported by data directly demonstrating (a) specificity of the binding for LTC_4 relative to LTD_4, LTE_4, and FPL55712 (Figure 6) and (b) insignificant conversion of LTC_4 to LTD_4 and LTE_4 under the conditions of the binding assay (Figure 7). In view of the lack of significant conversion of LTC_4 to LTD_4, the inhibitory effect of FPL55712 may be due to mechanisms other than that due to its activity as a specific LTD_4 receptor antagonist (Snyder & Krell, 1984). The data, however, did show the ability of 10 μM FPL55712 to inhibit specific LTC_4 binding (Figure 6), which correlates with its ability to inhibit the LTC_4 collagen synthetic effects. This correlation supports the conclusion that the biosynthetic effect may be mediated by binding of LTC_4 to these binding sites.

Recently, Sun and co-workers (Sun et al., 1986) reported that LTC_4 can bind to rat liver homogenate glutathione *S*-transferases in a specific, saturable, and reversible manner, mimicking the behavior of empirically defined specific receptors. Binding of LTC_4 in intact fibroblasts, however, is not due to such interactions with these transferases since we could not detect such enzymatic activity in our cell preparations using 1-chloro-2,4-dinitrobenzene as substrate. Bovine aortic endothelial cells also do not contain such transferases (Chan et al., 1986). Although the lack of transferase activity using this subtrate is not conclusive proof of the total absence of such transferases in these cells, it nevertheless represents the currently accepted method for demonstrating such an activity. The available data, however, do not rule out the possibility for the existence of glutathione transferase(s) which cannot use 1-chloro-2,4-dinitrobenzene as substrate but could bind LTC_4 in a specific, saturable, and reversible manner. Thus, it is likely that the binding of LTC_4 in isolated rat lung fibroblasts is to specific LTC_4 receptors, which then stimulates collagen and protein biosynthesis via as yet undefined signal transduction pathways.

The K_d for this binding phenomenon is in the nanomolar range, which is consistent with that for other cell types, including a smooth muscle cell line and endothelial cells (Krilis et al., 1983; Chan et al., 1986). Specific receptors for LTC_4 have also been reported to be widely distributed in almost all tissues of the body (Cheng et al., 1985). The number of receptors per cell is highly variable, ranging from as low as several thousand to as high as hundreds of thousands (Lewis & Austen, 1984). The data from a smooth muscle cell line show approximately a 10-fold higher number of receptors per cell (10) than in rat lung fibroblasts as estimated in this study. It is unclear at this time whether the difference is due to differences in cell type, in animal species, or even in assay conditions. The binding parameters for fibroblasts are more similar to those for bovine aortic endothelial cells with a $K_d = 6.8 \pm 2.2$ nM and an estimated 7.2×10^4 binding sites/cell (Chan et al., 1986).

The data from this study, in their totality, would support the conclusion that LTC_4 is capable of stimulating protein and collagen synthesis and that such stimulation may be mediated by binding to specific LTC_4 receptors on these fibroblasts. Although the actual receptor and its absolute specificity remain to be determined, this effect represents yet another potentially important biological function of peptidoleukotrienes which has not been previously reported. The concentrations (0.1–1 nM) at which LTC_4 stimulates collagen synthesis and the binding parameters (K_d = 1.8 nM) are both consistent with the possibility that these observations may be biologically relevant. This is especially likely in sites of inflammation and injury, wherein activated phagocytes are likely to be encountered. Activated phagocytes have been reported to release large amounts of leukotrienes in vitro, including LTC_4 (Lewis & Austen, 1984; Rouzer et al., 1980, 1982; Williams et al., 1984; Rankin et al., 1984). Adjacent fibroblasts would respond to LTB_4 and would thus be recruited to these sites chemotactically (Mensing & Czarnetzki, 1984), while LTC_4 and possibly LTD_4 would result in increased collagen (and other proteins) production by these cells, thus promoting wound healing or scar formation. These effects would complement and augment the other leukotriene effects on macrophage mediator production (Dinarello et al., 1984; Rola-Pleszczynski & Lemaire, 1985; Kunkel et al., 1986). The presence of these activated phagocytes in fibrotic lesions (Phan & Kunkel, 1986) and the ability of the lipoxygenase inhibitor nordihydroguaiaretic acid to inhibit pulmonary fibrosis (Phan & Kunkel, 1986) provide additional support for the fibrogenic activity of leukotrienes.

ACKNOWLEDGMENTS

We thank Jeny Brown and Kathleen Atkins for excellent secretarial assistance.

Registry No. LTC_4, 72025-60-6; LTD_4, 73836-78-9.

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X-ray Diffraction Study of the Polymorphic Behavior of N-Methylated Dioleoylphosphatidylethanolamine†

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Received February 27, 1987; Revised Manuscript Received November 6, 1987

ABSTRACT: The polymorphic phase behavior of aqueous dispersions of dioleoylphosphatidylethanolamine (DOPE) and its N-methylated analogues, DOPE-Me, DOPE-Me_2, and DOPC, has been investigated by X-ray diffraction. In the fully hydrated lamellar (L_α) phase at 2 °C, the major structural difference is a large increase in the interlamellar water width from DOPE to DOPE-Me, with minor increases with successive methylation. Consistent with earlier reports, inverted hexagonal (H_{II}) phases are observed upon heating at 5–10 °C in DOPE and at 65–75 °C in DOPE-Me and are not observed to at least 85 °C in DOPE-Me_2 or DOPC. In DOPE, the L_α–H_{II} transition is facile and is characterized by a relatively narrow temperature range of coexistence of L_α and H_{II} domains, each with long-range order. DOPE-Me exhibits complex nonequilibrium behavior below the occurrence of the H_{II} phase: Upon heating, the L_α lattice spontaneously disorders on a time scale of days; on cooling from the H_{II} phase, the disorder rises on a time scale of minutes. It is shown that, in copious water, the disordered state transforms very slowly into phases with cubic symmetry. This process is assisted by the generation of small amounts of lipid degradation products. The relative magnitudes of the monolayer spontaneous radius of curvature, R_0 [Kirk, G. L., Gruner, S. M., & Stein, D. L. (1984) *Biochemistry 23*, 1093; Gruner, S. M. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 3665], are inferred from the H_{II} lattice spacings vs temperature and are shown to increase with increasing methylation. The relative magnitudes of R_0 are categorized as small for DOPE, intermediate for DOPE-Me, and large for DOPC. It is suggested, and examples are used to illustrate, that small R_0 lipid systems exhibit facile, low-temperature L_α–H_{II} transitions, intermediate R_0 systems exhibit complex nonequilibrium transition behavior and are likely to form cubic phases, and large R_0 systems are stable as L_α phases. The relationship between the cubic phases and minimal periodic surfaces is discussed. It is suggested that minimal periodic surfaces represent geometries in which near constant, intermediate R_0 values can be obtained concomitantly with monolayers of near constant thickness, thereby leading to equilibrium cubic phases. Thus, the relative magnitude of the spontaneous radius of curvature may be used to predict mesomorphic behavior. The geometry of the equilibrium phases that occur may be largely understood on the basis of a competition between a spontaneous tendency for the monolayers to curl to a radius, R_0, and the need to pack similar hydrocarbon chains at near constant density and at a uniform mean length.

An outstanding problem of membrane biology is to understand the roles of the numerous lipid species typically found in biomembranes (Raetz, 1982). The realization that large fractions of these lipid species do not individually form bilayers under physiological conditions [see Cullis et al. (1985) for a review] has focused much attention on the mesomorphic behavior of lipid liquid crystals (Gruner et al., 1985). A number of questions immediately arise: How do the so-called "nonbilayer" lipids affect the physical properties of the biological bilayers into which they are incorporated? What is the biological significance of these physical effects? What molecular characteristics determine the mesomorphic phase behavior of pure and mixed lipid systems? Are there readily measurable quantities that serve as predictors of classes of mesomorphic behavior?

One approach toward answering these questions is to attempt to understand the microscopic interactions present in lipid layers. Although an understanding of the molecular interactions is ultimately desirable, it is in practice, limited by the number and complexity of interatomic forces present in lipid–water dispersions. Moreover, the observation that chemically diverse lipids often exhibit similar mesomorphic behavior leads one to suspect that many complicated micro-

† This work was supported by the NIH (Grant GM32614), the DOE (Contract DE-FG02-87ER60522-A000), and the Medical Research Council (MRC) of Canada. P.R.C. is an MRC scientist. G.L.K. was additionally supported by the John B. Putman Foundation and M.W.T. by a Liposome Co. Fellowship.
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