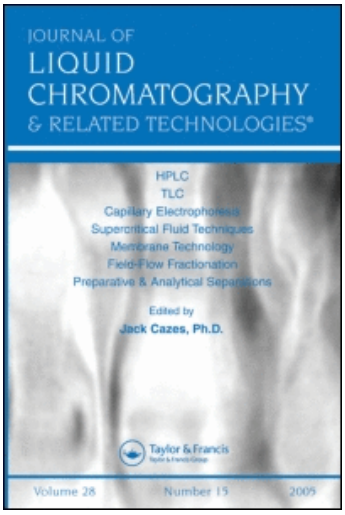

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HPLC ANALYSIS OF [^{3}H]-ARACHIDONIC ACID METABOLITES PRODUCED BY SMOOTH MUSCLE AND ENDOTHELIAL CELLS IN RESPONSE TO LEUKOTRIENE D_4

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ABSTRACT

Clonally derived murine and rat smooth muscle cells and bovine endothelial cells were incubated with [^{3}H]-arachidonic acid for 16 hours (10 μCi/ml). The cells were then rinsed twice with saline and then treated with leukotriene D_4 (LTD_4) (1 μM) for 10 min. The supernatants from these cells were acidified with phosphoric acid (0.1% vol:vol), and then extracted with two volumes of ethylacetate. The radioactivity in the organic phase was analyzed by HPLC using an on-line radioactivity detector equipped with a solid scintillating flow cell. This procedure allowed us to monitor the arachidonic acid metabolites produced in response to LTD_4. From data obtained by this method we conclude that LTD_4 increases both lipoxygenase and cyclooxygenase product formation in these cells and that this method may be useful in obtaining qualitative information concerning eicosanoid metabolism in other biological systems.

INTRODUCTION

Leukotriene D_4(LTD_4) is produced in response to inflammatory and anaphylactic stimuli (1). LTD_4 induces a number of different

responses including vasoconstriction and plasma exudation in the hamster cheek pouch microvasculature (2) and dilatation of the renal artery in the dog (3). Other investigators have reported that LTD_4 can increase prostanoid production *in vivo* and *in vitro* and that some of the physiological effects of LTD_4 may be mediated by these prostanoids (4,5).

To observe the effects of LTD_4 on arachidonic acid metabolism, it is necessary to monitor the synthesis of a large number of arachidonic acid metabolites. Therefore, we have developed a rapid HPLC technique to analyze supernatants obtained from cells which have been pre-incubated with radio-labeled arachidonic acid. In this communication, we describe this procedure and report that LTD_4 increases the biosynthesis of a number of eicosanoids including both lipoxygenase and cyclooxygenase products in smooth muscle and endothelial cells.

MATERIALS AND METHODS

Materials

Radioactive eicosanoid standards, 6 keto-$PGF_{1\alpha}$, TxB_2, PGE_2, PGD_2, LTB_4, 5HETE, 15HETE, 12HETE, LTB_4 and arachidonic acid (all tritium labeled) were obtained from New England Nuclear (Boston, MA). Non-radioactive standards 6 keto-$PGF_{1\alpha}$, TxB_2, PGE_2, PGD_2, PGF_2, PGA and PGB_2 were obtained from Sigma (St. Louis, MO). Non-radioactive 5,15 diHETE, 8,15 diHETE, 14,15 diHETE and 5,12 diHETE were obtained from BioMol Inc. (Philadelphia, PA). All solvents were of HPLC grade and were obtained from Fisher Scientific (Philadelphia, PA). LTD_4 was prepared by total synthesis and generously provided by Dr. John Gleason (Smith Kline and French Laboratories, Philadelphia, PA).

Cells and Cell Culture

Cells used in these experiments include the BC3H$_1$ murine angioma (6), the A$_7$r5 rat aortic smooth muscle cell line (7) and the CPAE bovine aortic endothelial cell line (8), and were obtained from the American Type Culture Collection (Rockville, MD). All cells were grown in Dulbecco's Modified Eagle Medium (GIBCO, Grand Island, NY) supplemented with 20% fetal calf serum, antibiotic free. Cells were labeled by adding concentrated [^{3}H]-arachidonic acid ([^{3}H]-AA) (10 μCi/ml) to the culture for 16 hours. The [^{3}H]-AA was concentrated under a stream of N_2 so that the final concentration of ethanol in the culture media was 0.1 %. The cells were then rinsed twice with Puck's Saline F (GIBCO, Grand Island, NY) and then treated with LTD_4, (1 μM) for 10 min. The saline was removed and eicosanoids were extracted.

HPLC analysis of [^{3}H] Arachidonic acid metabolites

The saline removed from the cells was acidified using phosphoric acid (0.01% final concentration) and eicosanoids were extracted using 2 volumes of ethylacetate. This procedure resulted in the recovery of greater than 95% of the total radioactivity found in the saline (data not shown). The volume of the organic phase was reduced to 200 μl under a stream of N_2.

Samples were analyzed using a Waters Address HPLC system as follows. The arachidonic acid metabolites were separated by reverse phase chromatography using a Waters Nova Pak 4μ C-18 column in a Waters Z module. A gradient of acidified water (0.005% phosphoric acid) and acetonitrile was used. The initial 4 min after the sample injection was isocratic (24% acetonitrile). During the next 8 min a final

concentration of 28% acetonitrile was achieved using a convex gradient curve 5. During the next 13 min the acetonitrile concentration was increased to 45% using a concave gradient curve 8. The acetonitrile concentration was then increased to 100% over the next 15 min using a convex gradient curve 5. An equilibration time of 10 min was used between samples and a constant flow rate of 4 ml/min was used throughout. The radioactivity was monitered using an on line Ramona D radioactivity detection system (INUS, Philadelphia, PA) equipped with a yttrium packed flow cell. The flow cell volume was 400 µl and an integration time of 5 seconds was used. The counting efficiency of the flow cell was approximately 1%. The volume of the flow cell was critical in obtaining optimal sensitivity and resolution. If the flow cell volume was increased to 1 ml, baseline resolution of a number of metabolites was not obtained. It was also found that if the flow cell volume was reduced to 250 µl, a 2-4 fold decrease in sensitivity was observed (data not shown). The data were collected, stored and analyzed using the software obtained from INUS.

In the course of these experiments we found that contamination, possibly radioactive phospholipids, accumulated on the yttrium flow cell and that it was necessary to remove this contamination after every 20-30 injections. Contamination was removed by bypassing the column and pumping nitric acid (20% vol to vol) through the flow cell (2 ml/min for 10 min). This procedure did not reduce the efficacy of the flow cell.

RESULTS

The simple extraction procedure used resulted in excellent recovery of the various eicosanoids standards (Table 1). The chromatographic conditions described in the Materials and Methods provided baseline

Table 1

RETENTION TIMES AND EFFICIENCY OF RECOVERY OF STANDARDS

	RT (min.)	Percent Recovery*		RT (min.)	Percent Recovery*
6Keto $PGF_{1\alpha}$	6.54*	70	5,15 diHETE	28.54**	ND
TxB_2	14.00*	85	LTB_4	29.75*	>95
PGF_2	16.24**	ND	HHT	29.95***	>95
PGE_2	16.68*	90	14,15 diHETE	31.04**	ND
PGD_4	19.20*	>95	5 HETE	32.05***	>95
PGA_2	26.20**	ND	15 HETE	32.65***	>95
PGB_2	26.48**	ND	12 HETE	32.90***	>95
5-12 diHETE	28.18**	ND	AA	37.60*	>95
8-15 diHETE	28.31**	ND			

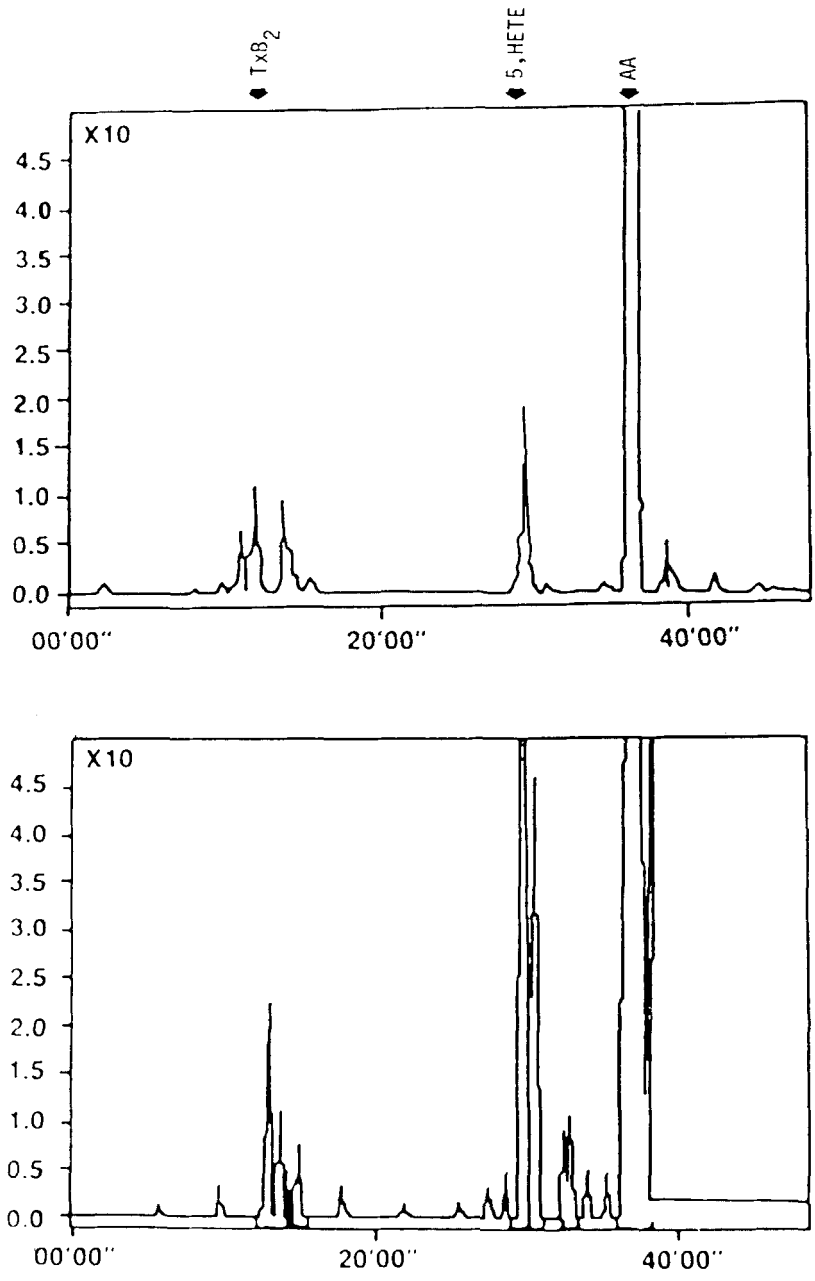
ND - Not determined

* radioactive and non radioactive standards
** non radioactive standards
*** radioactive standards

Standard solutions of non-radioactive eicosanoids were dissolved in methanol (1 mg/ml) and 20 µg were chromatographed as described in Materials and Methods and the absorbance was monitored at 214 nm. Where possible, radioactive standards (10^5 cpm) were added to the non-radioactive standards. Several of the compounds shown above were available in only the radioactive or in the non-radioactive form as noted in the Table. Recovery of the radioactive standards from saline, using the method described in Materials and Methods, is also shown.

separation of all of the standards. The nonradioactive standards as monitored by UV absorbance (214 NM) cochromatographed with the radiolabeled standards. The retention times of these standards are shown in Table 1.

In these experiments three different cell lines were used, each of which responded differently to LTD_4 treatment. The first cell line examined was the $BC3H_1$ smooth muscle cell line. Chromatograms of both control (Fig. 1A) and LTD_4 treated (Fig. 1B) are shown. The major prostanoids produced TxB_2 and PGE_2 are noted for their spasmogenic properties. In addition these cells also produced 5 Hete. The biosyn-



Figures 1A and B. HPLC analysis of [^{3}H]-arachidonic acid metabolites produced by BC3H$_1$ cells. Cells (~5x10^6) were incubated with [^{3}H]-arachidonic acid (10 μCi/ml) for 16 hr then removed and incubated in saline (A) or saline containing LTD_4 (1 μM) for 10 min (B). The supernatant was then extracted and analyzed. The chromatograms are representative examples of 6 obtained.

thesis of all of these eicosanoids, as well as the release of non-metabolized arachidonic acid, was increased as a result of LTD_4 treatment.

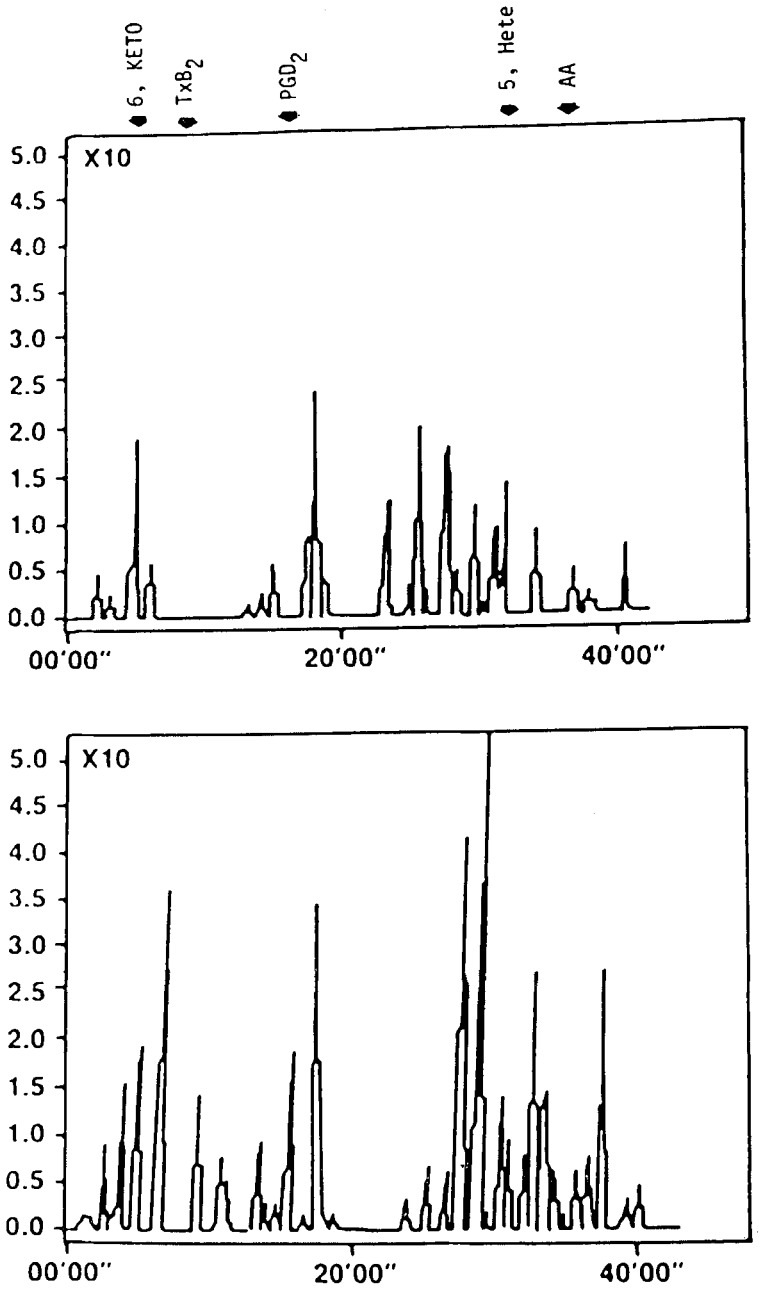
The second cell line studied was the CPAE endothelial cell line. Shown are chromatogram of both control (Fig. 1A) and LTD_4 treated (Fig. 1B) cell supernatents. These cells produced a greater variety of eicosanoids than did the $BC3H_1$ cells. Most notably, these cells synthesized large amounts of 6 Keto-$PGF_{1\alpha}$ and PGD_2, and made relatively smaller amounts of TxB_2 and PGE_2. The CPAE cells also produced both 5 Hete as well as 5-12 and 5-15 di-Hete.

The third cell line examined, the A_7r5 smooth muscle cell line, produced the fewest number of arachidonic acid metabolites. In both control (Fig. 3A) and LTD_4 treated (Fig. 3B) supernatant, the major prostanoid was PGD_2, the major lipooxygenase metabolite produced by these cells was 5 Hete. LTD_4 treatment resulted in an increase in both of these metabolites.

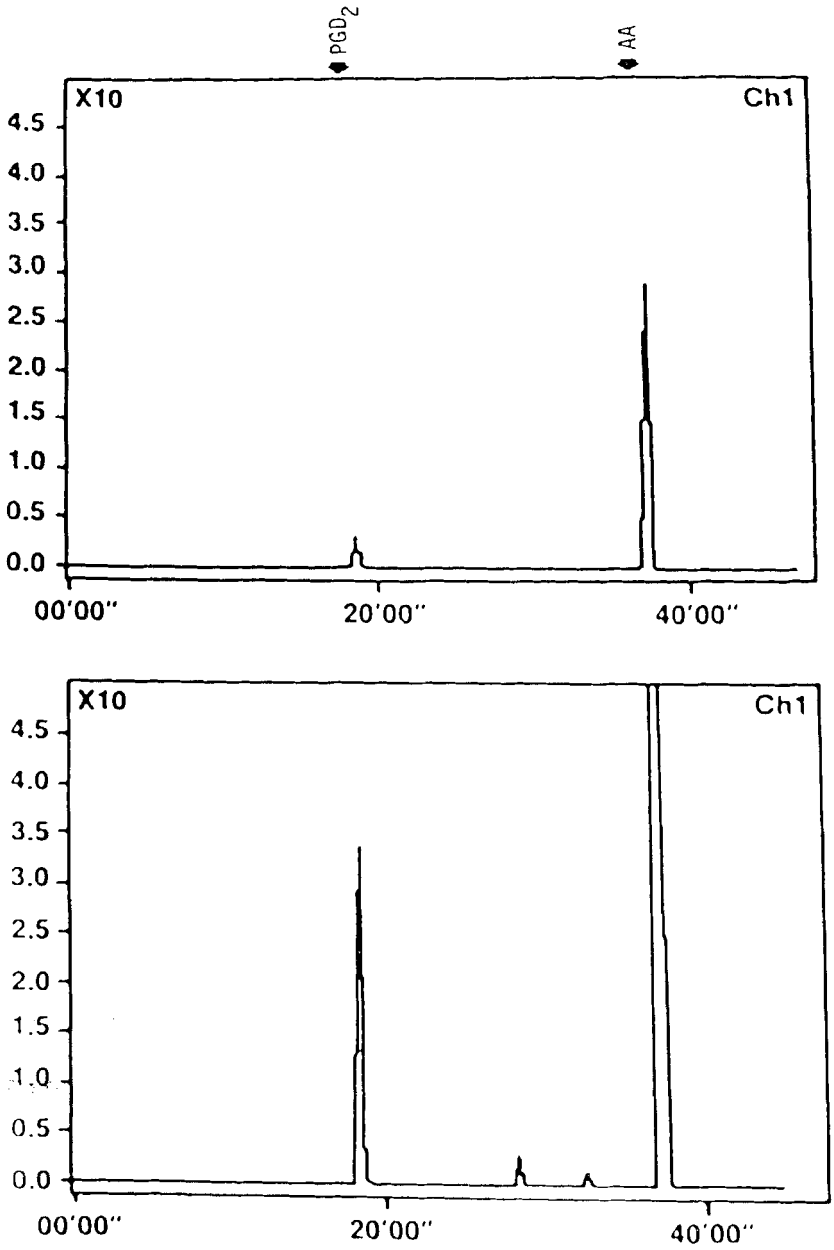
The data from 2 separate experiments was integrated and the results are shown in Table 2. We observed that LTD_4 treatment resulted in an increase in the production of both cyclooxygenase and lipooxygenase products.

DISCUSSION

LTD_4 has a number of in vivo effects including vasoconstriction, vasodilation and plasma exudation. In cell culture, LTD_4 causes $BC3H_1$ cells to contract, the A_7r5 to relax, and the CPAE cells to retract. The cells that contract produce the smooth muscle spasmogen thromboxane. The cells that relax produce the dilatory prostanoid PGD_2, while the endothelial



Figures 2A and B. HPLC analysis of [^{3}H]-arachidonic acid metabolites produced by CPAE cells. Cells (~5x10^6) were incubated with [^{3}H]-arachidonic acid (10 μCi/ml) for ~14 hr then removed and incubated in saline (A) or saline containing LTD_4 (1 μM) for 10 min (B). The supernatant was then extracted and analyzed. The chromatograms are representative examples of 6 obtained from each run of the experiment.



Figures 3A and B. HPLC analysis of [^{3}H]-arachidonic acid metabolites produced by A$_7$r5 cells. Cells (~5x10^6) were incubated with [^{3}H]-arachidonic acid (10 µCi/ml) or ~14 hr then removed and incubated in saline for 10 min (A) or saline containing LTD_4 (1 µM) for 10 min (B). The supernatant was then extracted and analyzed. The chromatograms are representative examples of 6 obtained from each run of the experiment.

Table 2. Integration of Data Shown in Figures 1, 2 and 3

Table 2a

Cell Line	Exp. #	Treatment	6 keto $PGF_{1\alpha}$	TXB_2	PGE_2	PGD_2
$BC3H_1$	1	Control	17	61	150	0
		+ LTD_4	76	217	245	0
	2	Control	0	211	0	0
		+ LTD_4	34	1228	94	0
A,r5	1	Control	0	0	0	15
		+ LTD_4	0	0	0	83
	2	Control	17	0	0	28
		+ LTD_4	78	0	0	933
CPAE	1	Control	200	33	400	233
		+ LTD_4	766	199	866	766
	2	Control	96	128	128	480
		+ LTD_4	960	1120	640	1440

Table 2b

Cell Line	Exp. #	Treatment	5-12 diHETE	5-15 diHETE	HHT	5 HETE	AA
$BC3H_1$	1	Control	0	0	28	128	570
		+ LTD_4	0	0	933	800	1071
	2	Control	0	0	0	120	716
		+ LTD_4	0	0	0	955	4383
A,r5	1	Control	0	0	0	0	47
		+ LTD_4	61	0	0	28	476
	2	Control	0	0	0	128	825
		+ LTD_4	0	0	0	800	102320
CPAE	1	Control	533	133	0	60	133
		+ LTD_4	810	1200	0	166	567
	2	Control	128	160	0	320	441
		+ LTD_4	480	320	160	1920	16128

All values are expressed as counts per second.

The major peaks, identified by their retention times, were integrated and are shown above. These data are obtained from 2 representative experiments.

cells produce prostacyclin which mediates vascular permeability and vasodilitation. All these morphological changes are blocked by inhibition of cyclooxygenase with meclofenamic acid (9,10). It is probable that the behavior of tissue in response to LTD_4 depends upon the predominant prostanoid produced within the tissue, but the significance of the increase in prostanoid synthesis in mediating the physiological response to LTD_4 remains to be better defined.

Until recently, only radioimmunoassays provided the necessary sensitivity to quantitate the prostanoid level. Two major problems associated with this method are the speed (metabolites are assayed one at a time) and crossreaction of the antibody with other metabolites. In our work we have used HPLC and a radioactivity detector to characterize all the eicosanoids produced in the 3 different cell lines. A problem encountered in using the HPLC technique was the interexperimental variability in the number of counts observed in each peak. Because of this, it was necessary to compare controls and treated cultures on the same day in the same experiment. Repetitive assays of supernatants from the same experiment typically had little variability (<15%), however, experiments performed over several months had differences of several fold. From this we conclude that the major problems were not analytical but resulted from changes in the cells and cell culturing. These problems made statistical analysis difficult and therefore only representative experiments of control and treated cultures obtained and analyzed at the same time are shown. In spite of these difficulties we always observed that LTD_4 increased total eicosanoid production. Another disadvantage of this technique is that it is unable to quantitate the amount of each of the arachidonic acid products produced. However we are able to use this

procedure to obtain qualitative information about the identity of the eicosanoids produced and the relative amounts produced as a result of LTD_4 treatment.

Employing radioimmunoassays, several laboratories have reported that leukotrienes increase the synthesis of several arachidonic acid metabolites. LTC_4 and LTD_4 treatment of endothelial cells has been reported to increase prostacyclin (PGI_2) synthesis (9,11). In contrast, Dunham, et al (12), reported that LTD_4 induced TXA_2 synthesis in these cells. By using HPLC we are able to reconcile these observations by demonstrating that the endothelial cells produce both TxB_2 and 6 keto $PGF_{1\alpha}$ and that both of these metabolites are increased in response to LTD_4 treatment.

In the smooth muscle cell line $BC3H_1$, LTC_4 and LTD_4 have been reported to increase TXA_2 synthesis (10). Studies in our laboratory have suggested that these peptidyl leukotrienes induce phospholipase A_2 (PLA_2) activity resulting in an increase in the release of arachidonic acid (13). However, the ultimate pattern of arachidonic acid metabolism appears to be largely predetermined by genetic and epigenetic factors in the cells and is relatively unaffected by the leukotrienes.

In addition to the increase in prostanoid production in response to LTD_4 we have also observed an increase in the production of the lipoxygenase metabolites produced by these cells. This observation is consistent with the observation that LTD_4 increases phospholipase A_2 activity and thus increases the amount of arachidonic acid available for both the lipoxygenase and the cyclooxygenase pathways. Furthermore, it is conceivable that some of the lipoxygenase products may have biological activity and, in turn, mediate some of the physiological responses to LTD_4.

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