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Leukotriene A_4, and not leukotriene B_4, is the main 5-lipoxygenase metabolite released by bovine leukocytes

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Abstract The production of leukotriene A_4 (LTA_4)-derived metabolites, analysed by RP-HPLC, was studied in purified bovine polymorphonuclear leukocyte (PMNL) preparations and in PMNL-platelet coincubations after challenge with the calcium ionophore A23187. The results obtained show that in bovine PMNL LTB_4 represents the main LTA_4 metabolite. When washed platelets were added to PMNL, LTC_4 was the main enzymatic metabolite observed, indicating a substantial transfer of PMNL-derived LTA_4 to platelets. The synthesis of LTC_4 was accompanied by a significant decrease in LTB_4, suggesting that a quota of the LTB_4 synthesized in PMNL preparations is the result of transcellular metabolism of released LTA_4 by neighbouring PMNL. Reduction of PMNL-PMNL interactions through dilution of cell incubates allowed us to estimate that most of the leukotriene A_4 synthesized by PMNL is indeed released from the cell. LTA_4, and not LTB_4, represents the main 5-lipoxygenase metabolite released by bovine PMNL.

Key words: Transcellular metabolism; Leukotriene A_4; Leukotriene C_4 synthase; (Bovine polymorphonuclear leukocyte); (Bovine platelet)

1. Introduction

Arachidonic acid oxidation catalyzed by 5-lipoxygenase (5-LO; EC 1.13.11.34) activity leads to the formation of potent, biologically active molecules such as leukotriene B_4 (LTB_4), and cysteinyl leukotrienes, namely leukotriene C_4 (LTC_4), leukotriene D_4 (LTD_4), and leukotriene E_4 (LTE_4)[1,2]. While a great deal of evidence from basic biochemical studies has focused on cells that possess 5-LO enzyme, it is now clear that the formation of leukotrienes is not strictly limited to those cells that have these primary oxidative enzymes. Recent experiments have shown that the chemically reactive intermediate leukotriene A_4 (LTA_4)[3] can be transferred from one cell to another. Conversion of LTA_4 to LTB_4 and LTC_4 has been shown in cells that do not possess 5-LO activity such as red blood cells [4], platelets [5], endothelial cells [6,7] and smooth muscle cells [8].

Recent studies using organ systems perfused with PMNL [9–11] have indicated the pathophysiological relevance of the transcellular metabolism of LTA_4, in particular when tight cell-cell interactions occur, such as during adhesion and diapedesis of PMNL through the microvascular endothelium.

Polymorphonuclear leukocytes (PMNL) possess large amounts of 5-LO, the enzyme catalyzing the sequential conversion of arachidonic acid to 5-hydroperoxyeicosatetraenoic acid (5-HpETE) and LTA_4 [12]. Upon cell activation, non-enzymatic LTA_4 breakdown products, such as Δ^6-*trans*-LTB_4 isomers and 5,6-diHETE isomers [13–15] are found in cell incubation media, suggesting the extracellular release of LTA_4.

Although a few studies have carefully addressed the transcellular metabolism of LTA_4 in PMNL-platelet coincubations [5,16], as well as the amounts of LTA_4 released upon calcium ionophore A23187 challenge of human PMNL [17], no quantitative data are available that would relate the amounts of LTA_4 released from PMNL alone to those of LTC_4 synthesized by transcellular metabolism in PMNL-platelet coincubations.

In the present paper we studied the transcellular metabolism of LTA_4 in bovine blood cells, using purified polymorphonuclear leukocytes, washed platelets and coincubations of PMNL platelets. The effect of cell concentration on the quantitative profile of enzymatic (namely LTB_4) versus non-enzymatic LTA_4 metabolites produced after challenge with the calcium ionophore A23187 was also studied.

The results provide evidence that transcellular metabolism of LTA_4 takes place in bovine PMNL preparations as well as in PMNL-platelet coincubations, and that intact LTA_4 represents the main 5-LO metabolite released by bovine PMNL.

2. Materials and methods

2.1. Chemicals and reagents

All chemicals used were reagent grade and obtained from commercial sources. Eicosanoids were purchased from Cayman Chemical Co. (Ann Arbor, MI, USA). HPLC-grade solvents were obtained from Merck (Darmstadt, Germany). Ficoll was from Pharmacia (Uppsala, Sweden). Type I 'plus' water was obtained using a MilliQ Plus water purifier (Millipore, Molsheim, France), fed with double-distilled water.

2.2. Preparation of bovine platelets and polymorphonuclear leukocytes

Bovine polymorphonuclear leukocytes and platelets were prepared according to Hansbrough et al. [18] and Henricks et al. [19], with modifications. Briefly, blood (7 vols.) was collected at time of slaughter in 1 vol. ACD (citric acid·H_2O, 41 mM; Na^+ citrate·$2H_2O$, 100 mM; glucose, 136 mM) and carefully mixed. After centrifugation for 20 min at room temperature and $380 \times g$, platelet-rich plasma (PRP) was removed and reacidified with 1:10 (v/v) ACD. The residual blood was further centrifuged for 10 min at room temperature and $1300 \times g$, and the pellet subjected to erythrocyte lysis by gentle resuspension in 1 vol. of a 0.2% (w/v) NaCl solution and further dilution with 1 vol.

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Abbreviations: LTA_4, leukotriene A_4; LTB_4, leukotriene B_4; LTC_4, leukotriene C_4; PMNL, polymorphonuclear leukocytes; 5-LO, 5-lipoxygenase; $PBS^=$, PBS without Ca^{2+} and Mg^{2+}; PRP, platelet-rich plasma; Δ^6-*trans*-LTB_4 isomers, 5(*S*),12(*R*)-dihydroxy-6,8,10-*trans*-14-*cis*-eicosatetraenoic acid and 5(*S*),12(*S*)-dihydroxy-6,8,10-*trans*-14-*cis*-eicosatetraenoic acid; 5,12-diHETE, 5(*S*),12(*S*)-dihydroxy-6,10-*trans*-8,14-*cis*-eicosatetraenoic acid; 5,6-diHETE isomers, 5(*S*),6(*S*)-dihydroxy-7,9-*trans*-11,14-*cis*-eicosatetraenoic acid and 5(*S*),6(*R*)-dihydroxy-7,9-*trans*-11,14-*cis*-eicosatetraenoic acid

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of a balancing solution (1.6% NaCl, w/v; 0.2% sucrose, w/v) at +4°C. Mononuclear cells were then separated by centrifugation (30 min at room temperature and 400×g) on Ficoll cushions (density 1.077 g ml^{-1}) and the pellet, containing neutrophils (75–95%) and eosinophils (5–25%), was washed twice in PBS without Ca^{2+} and Mg^{2+} ($PBS^{=}$), resuspended at a final concentration of approx. 30×10^6 cells ml^{-1} and kept on ice until use.

PRP was centrifuged for 15 min at room temperature and 1000×g, and platelets resuspended with 5 ml of washing buffer (citric acid·H_2O, 36 mM; glucose·H_2O, 5 mM; KCl, 5 mM; $CaCl_2$, 2 mM; $MgCl_2\cdot6H_2O$, 1 mM; NaCl, 103 mM; pH 6.5) containing 0.4% (w/v) BSA and prostaglandin E_1 (final concentration 100 nM), according to Patscheke [20]. Platelets were further centrifuged for 15 min at room temperature and 400×g, and finally resuspended in $PBS^{=}$.

Platelets and PMNL were counted using a modified Neubauer chamber and viability, as assessed by trypan blue dye exclusion, amounted to ≥95%.

2.3. Cell incubations

PMNL (5×10^6 cells ml^{-1} for coincubations or $1–20\times10^6$ cells ml^{-1} for dilution experiments) and/or platelets (2×10^8 cells ml^{-1}) were supplied with Ca^{2+} (2 mM) and Mg^{2+} (0.5 mM) and, following pre-incubation at 37°C for 5 min, the calcium ionophore A23187 (Calbiochem, La Jolla, CA, USA; 5 μM) was added to trigger eicosanoid metabolism.

Stimulation was terminated after 10 min with 2 vols. of ice-cold methanol containing the HPLC internal standard prostaglandin B_2 (PGB_2, 25 ng) and samples stored at −20°C until RP-HPLC analysis.

2.4. Analysis of leukotriene A_4-derived metabolites

Samples were diluted with water to a final methanol concentration lower than 20% and extraction was quickly carried out using a solid phase cartridge (Supelclean LC-18, Supelco, Bellafonte, PA); the retained material was eluted using 90% aqueous MeOH. After evaporation, the dried extract was reconstituted in 600 μl of HPLC mobile phase A (methanol/acetonitrile/water/acetic acid 10:10:80:0.02, v/v, pH 5.5 with ammonium hydroxide), and injected into an HPLC gradient pump system (model 126, Beckman Analytical, Palo Alto, CA, USA) connected to a diode-array UV detector (model 168, Beckman Analytical), using a microprocessor-controlled auto sampler (Jasco 851-AS, Tokyo, Japan), with the sample kept at 4°C. UV absorbance was monitored at 280 nm, and full UV spectra (210–340 nm) acquired at a rate of 0.5 Hz.

A multilinear gradient from mobile phase A to mobile phase B (methanol/acetonitrile 50:50) at a flow rate of 1 ml min^{-1}, was used to elute a 4×250 mm column (RP-18 endcapped Lichrospher, 5 μm, Merck). Solvent B was increased to 35% over 6 min, to 65% over 25 min and to 100% over 3 min. This method allows separation of LTB_4 from 5,12-diHETE as well as from non-enzymatic LTA_4 metabolites.

Positive identification of enzymatic and non-enzymatic LTA_4 metabolites was made through UV spectral analysis of chromatographic peaks eluting at characteristic retention times. Quantitation was carried out on positively identified peaks only, using their HPLC peak areas relative to that of PGB_2 at 280 nm, and calculated from the responses of standard compounds. The ratio (enzymatic LTA_4 metabolites)/(non enzymatic LTA_4 metabolites) in PMNL preparations at different cell density, was calculated using the amount of LTB_4 divided by the amount of Δ^6-*trans*-LTB_4 isomers+5,6-diHETE isomers.

LTA_4 metabolites in PMNL preparations and PMNL-platelet coincubations were defined as [enzymatic LTA_4 metabolites (namely LTB_4+LTC_4)]+[non-enzymatic LTA_4 metabolites (namely Δ^6-*trans*-LTB_4 isomers+5,6-diHETE isomers)].

2.5. Data presentation and statistics

The results were expressed as means ± standard error of the mean (S.E.M.) of 4–5 different cell preparations.

Difference analyses between PMNL and PMNL-platelet coincubations were performed by Student's *t*-test. Comparison of enzymatic and non-enzymatic LTA_4 metabolites in different cell concentration groups were carried out by analysis of variance (ANOVA) and post hoc analysis performed with the Tukey-Kramer HSD test. ANOVA and linear regression analysis were used to examine the relationship between the cell concentration and the ratio (enzymatic LTA_4 metabolites)/(non-enzymatic LTA_4 metabolites).

The level of significance considered was $< 5\%$.

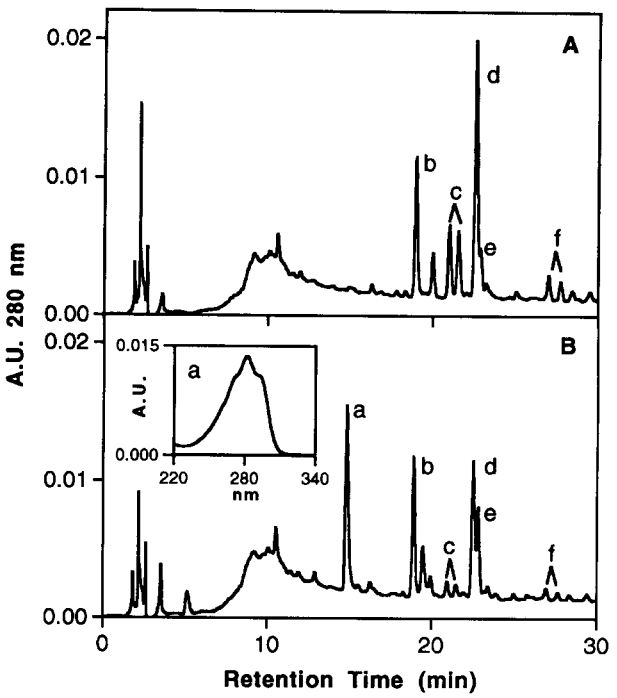

Fig. 1. LTA_4 metabolites synthesized by bovine PMNL and PMNL-platelet coincubations. UV absorbance profile at 280 nm from the RP-HPLC of bovine PMNL (5×10^6 cells ml^{-1}) (A) and PMNL-platelet coincubations (ratio 1:40) (B) after challenge with the Ca^{2+} ionophore A23187 (5 μM, 10 min, 37°C). Peaks are labeled as follows: a, LTC_4; b, PGB_2 (HPLC internal standard); c, Δ^6-*trans*-LTB_4 isomers; d, LTB_4; e, 5,12-diHETE; f, 5,6-diHETE isomers. Inset: UV spectrum of peak a, obtained on-line with the diode-array UV detector.

3. Results

3.1. Bovine PMNL-platelet coincubations

Challenge of PMNL preparation (5×10^6 cells ml^{-1}) with the calcium ionophore A23187 (5 μM, 10 min at 37°C), resulted in the production of LTB_4 (21.9 ± 3.1 pmol/10^6 cells) and of non-enzymatic LTA_4 metabolites (Δ^6-*trans*-LTB_4 isomers+5,6-diHETE isomers, 17.4 ± 2.6 pmol/10^6 cells) while neither LTC_4 nor ω-oxidized LTB_4 metabolites [21] were detectable. No LTA_4 metabolites were observed in the absence of calcium ionophore A23187 challenge.

Addition of washed, homologous platelets in a ratio of 40:1 with PMNL resulted in the appearance of substantial amounts of LTC_4 (18.1 ± 4.4 pmol/10^6 cells), representing the main LTA_4-derived metabolite (Fig. 1). A statistically significant decrease in both LTB_4 and non-enzymatic LTA_4 metabolites was also observed, while the total amount of LTA_4 metabolites remained unchanged (Fig. 2).

5,12-diHETE, a metabolite arising from the sequential action of the 5- and 12-lipoxygenase on arachidonic acid [22], was detected in PMNL incubations (4.9 ± 2.2 pmol/10^6 cells), according to the presence of 12-lipoxygenase in bovine leukocytes [18]. Addition of homologous platelets caused a significant increase in this double oxygenation metabolite (22.7 ± 5.7 pmol/10^6 PMNL), consistent with the presence of the platelet 12-lipoxygenase.

3.2. Bovine PMNL dilution experiments

Challenge of bovine PMNL preparations (20×10^6 cells

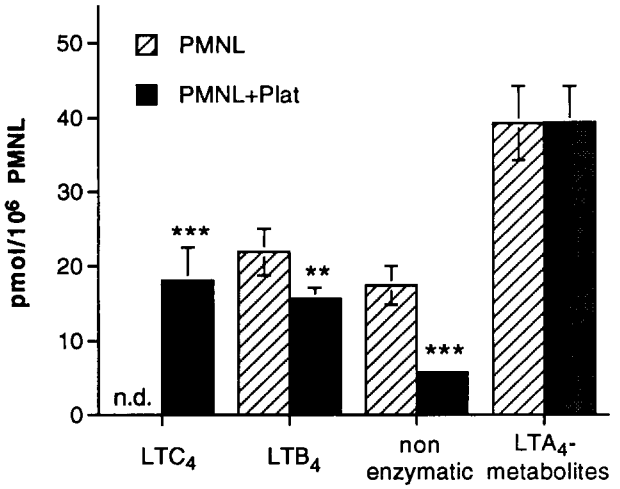

Fig. 2. LTA_4 metabolites synthesized by bovine PMNL and PMNL-platelet coincubations. Amounts of LTA_4 metabolites (expressed as pmol/10^6 cells) synthesized by bovine PMNL (5×10^6 cells ml^{-1}) (cross-hatched bars) and PMNL-platelet coincubations (ratio 1:40) (solid bars) after challenge with the Ca^{2+} ionophore A23187 (5 μM, 10 min, 37°C). Values are means ± S.E.M. of 5 different PMNL preparations. n.d., not detectable. Differences between the groups, assessed by Student's *t*-test, are indicated: $^{**}p<0.01$, $^{***}p<0.001$ from the PMNL group.

ml^{-1}) with the calcium ionophore A23187 (5 μM, 10 min, 37°C) resulted in the production of LTB_4 (17.3 ± 0.5 pmol/10^6 cells) and of non-enzymatic LTA_4 metabolites.

Decreasing cell concentration from 20×10^6 to 10^6 cells ml^{-1} resulted in a significant increase in non-enzymatic LTA_4 metabolites (from 6.6 ± 1.1 to 24.9 ± 2.2 pmol/10^6 cells, $p<0.001$ by Tukey-Kramer HSD test).

The normalized percentile composition of enzymatic and non-enzymatic LTA_4 metabolites over the total LTA_4-derived metabolites showed a progressive increase of non-enzymatic LTA_4 metabolites representing 27% at 20×10^6 cells ml^{-1} and 59.6% at 1×10^6 cells ml^{-1} (Fig. 3).

A statistically significant linear correlation ($r=0.783$, $p<0.001$) between the concentration of PMNL ml^{-1} and the ratio (enzymatic LTA_4 metabolites)/(non-enzymatic LTA_4 metabolites) was observed (Fig. 4).

4. Discussion

The importance of transcellular metabolism of leukotriene A_4 with regard to the final profile of 5-lipoxygenase metabolites observed in biological systems has recently been supported by an increasing body of evidence. The cellular environment may strongly influence the qualitative as well as quantitative profile of LTA_4-derived metabolites generated upon activation of polymorphonuclear leukocytes. Endothelial cells, smooth muscle cells and platelets have been shown to possess LTC_4-synthase (EC 2.5.1.37) and are able to synthesize LTC_4 using exogenous LTA_4 as well as PMNL-derived LTA_4. This process may lead to a pronounced shift from the production of a chemotactic lipid mediator, such as LTB_4, to cysteinyl leukotrienes, which cause oedema and vasoconstriction [23,24]. The overall potential for LTA_4 transfer from PMNL (donor cell) to acceptor cells [25], has usually been quantitated using the amounts of non-enzymatic LTA_4 metabolites in purified PMNL incubations [9]. Nevertheless, given the capacity of PMNL to take up actively LTA_4 from the extracellular milieu and convert it to LTB_4 [26], this estimate could represent an undervaluation of the amount of LTA_4 indeed leaving the cell for the extracellular environment. In a previous work by Fiore and Serhan [17], increased amounts of LTA_4-derived products were shown in human PMNL after A23187 challenge, by the trapping of LTA_4 outside of PMNL with liposomes. These observations suggested that the amount of LTA_4 released by human PMNL could indeed be greater than that estimated using non-enzymatic LTA_4 metabolites in purified PMNL preparation.

Bovine PMNL represent a very useful model for the study of LTA_4 transcellular metabolism for different reasons:

(i) Bovine PMNL do not synthesize LTC_4, in spite of the presence of eosinophils [18,19,27]; the observed formation of LTC_4 in PMNL-platelet coincubations is therefore totally dependent on the transfer of LTA_4 from PMNL to thrombocytes

(ii) Bovine PMNL do not show significant ω-oxidative metabolism of LTB_4; this simplifies the evaluation of PMNL enzymatic metabolites. In human PMNL ω-oxidized derivatives of LTB_4 (20-hydroxy- and 20-carboxy-LTB_4)[21] represent the predominant enzymatic metabolites and their formation is mainly carried out by neighbouring PMNL, taking up LTB_4 synthesized by other PMNL [28,29]. Diluting cell concentration would result, in the case of human PMNL, in substantial changes of the relative amounts of LTB_4 and its ω-oxidized metabolites.

The results obtained with this study show that bovine platelets, as previously shown for human platelets [5,16], are able to take up efficiently PMNL-derived LTA_4 and metabolize it to LTC_4, representing the main LTA_4-derived metabolite in PMNL-platelet coincubations. Accordingly, non-enzymatic

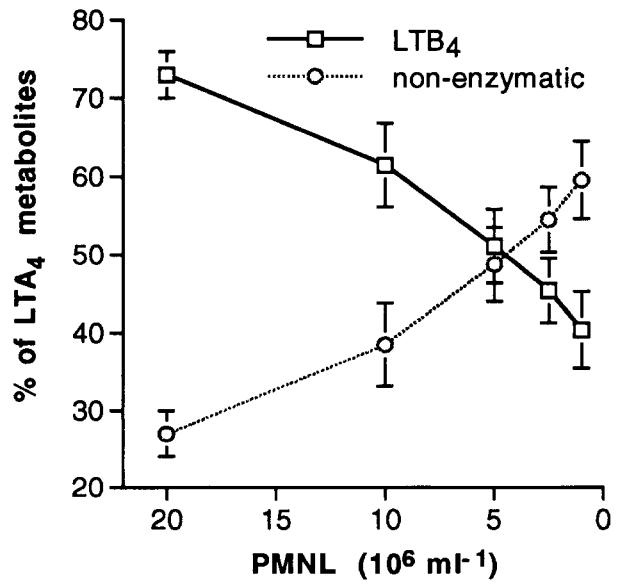

Fig. 3. Normalized percent composition of LTA_4 metabolites synthesized by bovine PMNL. Relative amounts of enzymatic and non-enzymatic LTA_4 metabolites synthesized by bovine PMNL at different concentrations, after challenge with the Ca^{2+} ionophore A23187 (5 μM, 10 min, 37°C). Data are expressed as % of total (enzymatic+non-enzymatic) LTA_4 metabolites. Values are means ± S.E.M. of 4 different PMNL preparations.

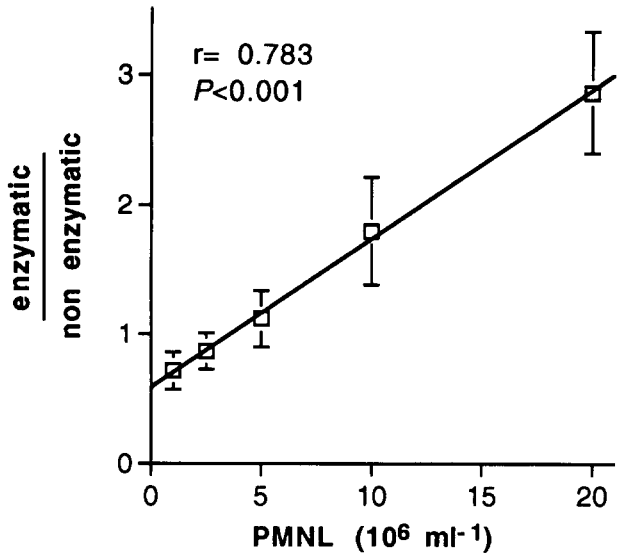

Fig. 4. Correlation between the ratio enzymatic/non-enzymatic LTA_4 metabolites and cell concentration in bovine PMNL preparations. Relationship between the ratio (enzymatic LTA_4 metabolites)/(non-enzymatic LTA_4 metabolites) and the concentration of bovine PMNL challenged with the Ca^{2+} ionophore A23187 (5 μM, 10 min, 37°C). Values are means ± S.E.M. of 4 different PMNL preparations. $r = 0.783$, $p < 0.0001$

LTA_4 metabolites (namely Δ^6-*trans*-LTB_4 isomers and 5,6-diHETE isomers) decreased by 70% compared to PMNL alone, indicating that the presence of platelets prevented the non-enzymatic decay of LTA_4 released by PMNL. Addition of platelets to PMNL preparations also resulted in a statistically significant decrease of LTB_4. It is important to note that the decrease in non-enzymatic LTA_4 metabolites, together with the decrease in LTB_4, quantitatively accounted for the amounts of LTC_4 detected. The decrease of LTB_4 observed, suggested that over 25% of LTB_4 synthesized in PMNL preparations at a concentration of 5×10^6 PMNL ml^{-1} arises from the release of intact LTA_4 and further metabolism to LTB_4 by neighbouring PMNL. In order to test this hypothesis, reduction of cell concentration, resulting in increased average distance between cells, has been used as a tool to limit the amount of further enzymatic metabolism of released LTA_4 by neighbouring PMNL. Decrease of cell concentration in PMNL preparations challenged with the calcium ionophore A23187 resulted in increased amounts of non-enzymatic LTA_4 metabolites with respect to enzymatic LTA_4 metabolites (LTB_4). The decrease of the ratio LTB_4/(non-enzymatic LTA_4 metabolites) linearly correlated with the decreased number of PMNL per ml of incubation medium, indicative of a diminished contribution of neighbouring PMNL to the final amounts of LTB_4 observed.

Albumin is known to stabilize LTA_4, increasing its half-life in buffered solution at pH 7.4 from less than 10 s to several minutes [28,17]. Nevertheless, in the experiments on PMNL dilution, we were took advantage of the very short half-life of intact LTA_4 to trap the LTA_4 released from the cell, by non-enzymatic hydrolysis to Δ^6-*trans*-LTB_4 and 5,6-diHETE isomers. Therefore, albumin was not used throughout these experiments because it was anticipated that increasing the half-life of the LTA_4 released would result in a greater chance of being taken up again and enzymatically metabolized by surrounding cells.

In PMNL-platelet coincubation, a very efficient transfer of PMNL-derived LTA_4 to platelets in the absence of albumin was observed, supporting the hypothesis that cell-cell contact may play a pivotal role in the efficient transfer of LTA_4 resulting in the formation of cysteinyl leukotrienes. In fact, inhibition of LTC_4 formation arising from the interaction of human PMNL and glomerular endothelial cells, by antibodies against CD18 and L-selectin, has recently been reported [30]. It has previously been shown that challenge of PMNL while perfusing isolated lung or heart of the rabbit resulted in a shift from LTB_4, observed after challenge of isolated PMNL, to cysteinyl leukotrienes, representing the main 5-LO product observed in the perfusion media [9–11]. The data obtained in this work support the hypothesis that a diversion from LTA_4 hydrolase to LTC_4 synthase metabolites could indeed take place when PMNL are activated in the context of tight interactions with cells (i.e. platelets, endothelial cells, smooth muscle cells) possessing the LTC_4 synthase enzyme.

In addition, the results obtained clearly show that intact LTA_4, and not LTB_4, represents the main leukotriene released upon activation by bovine PMNL. Further studies are in progress to test whether this observation may be also relevant for human PMNL.

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