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THE POLYMORPHONUCLEAR LEUKOCYTE: A CELL TUNED FOR TRANSCELLULAR BIOSYNTHESIS OF CYS-LEUKOTRIENES

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Sulfidopeptide leukotrienes (cysLT) are potent vasoactive mediators that can constrict coronary vessels and alter caliber of the microcirculation. They can be formed "*in situ*" *via* a peculiar type of cell communication termed "transcellular biosynthesis" whereby donor cells (polymorphonuclear leukocytes, PMNL) feed acceptor cells (endothelial cells, EC) the unstable epoxide intermediate leukotriene A_4 for further metabolism to cysLT.

We have investigated the relative amount of leukotriene A_4 that is synthesized by PMNL and made available for transcellular biosynthesis. This has been accomplished by measuring the relative amounts of enzymatic *vs* non-enzymatic leukotriene A_4-derived metabolites after challenge with the Ca^{2+}-ionophore A23187, using PMNL suspensions at different concentrations. Non-enzymatic leukotriene A_4-derived metabolites were used as a quantitative index of the amount of leukotriene A_4 released into the extracellular milieu.

In human, as well as in bovine PMNL, the relative amounts of non-enzymatic *vs* enzymatic leukotriene A_4-derived metabolites increased with decreasing cell concentrations. By diminishing possible cell-cell interactions *via* increased dilution, it is calculated that approx. 60% of leukotriene A_4 synthesized is released from the PMNL.

These data provide evidence that, in PMNL, transfer of leukotriene A_4 to neighbouring acceptor cells is taking place as a predominant mechanism of cell communication.

Key words: *leukotrienes, polymorphonuclear leukocytes, endothelial cells, cell-cell interaction, ischemia, coronary artery, transcellular biosynthesis.*

INTRODUCTION

The generation of leukotrienes (LT) exibits a remarkable cellular specificity; polymorphonuclear leukocytes (PMNL) contain a 5-lipoxygenase (primary enzyme) to generate the 5-hydroperoxy eicosatetraenoic acid and a leukotriene A_4 (LTA_4) hydrolase (secondary enzyme) to generate leukotriene B_4 (LTB_4).

On the other hand, eosinophils share with PMNL the same primary enzyme but possess a leukotriene C_4 synthase as a secondary enzyme to synthesize the cysteinyl leukotriene (cys-LT) leukotriene C_4 (LTC_4). Recently another process of biosynthesis of cys-LT has emerged which involves the participation of different cell types whereby PMNL (i.e. a donor cell) can synthesize the unstable intermediate LTA_4 which can be further processed by neighbouring cells (acceptor cells) into LT, B_4 or C_4. Such reaction, which requires the cooperation of different cell types, has been termed "transcellular biosynthesis" and was first demonstrated by Bunting *et al.* and Marcus *et al.* (1, 2).

Recent studies using organ systems perfused with PMNL (3—5) have indicated the pathophysiological relevance of the transcellular metabolism of LTA_4 in the rabbit heart, in particular when tight cell-cell interactions occur, such as during adhesion and diapedesis of PMNL through the microvascular endothelium. In light of these observations it was of interest to assess the relative amount of LTA_4 released from PMNL, and therefore available for transcellular biosynthesis of cys-LT, with respect to total LT_4 synthesized. The release of LTA_4 into the extracellular milieu would remove this intermediate from intracellular LTA_4 hydrolase which catalyzes conversion of LTA_4 into LTB_4. The extracellular (released) LTA_4 will react with water with a half-life lower than 30 sec (6) to yield the non enzymatic products, Δ^6-*trans*-LTB_4, Δ^6-*trans*-12-epi-LTB_4, and 5,6 dihydroxy-eicosatetraenoic acid isomers. The hypothesis that in diluted cell preparations, LTA_4 would have less chance of being reabsorbed and metabolized by vicinal PMNL, has been tested. In a previous study, Cluzel *et al.* (7) showed that the use of diluted cell suspensions provided important information concerning the amount of LTB_4 released by PMNL.

In the present paper we provide evidence that significant transcellular metabolism of LTA_4 does indeed take place in a rabbit heart perfused with purified human PMNL preparations and that LTA_4 represents the predominant 5-LO metabolite released from PMNL.

MATERIAL AND METHODS

Chemicals and reagents

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

Isolated perfused heart preparation

Albino rabbits weighing between 2.5 and 3.0 kg were utilized. Hearts were isolated and perfused retrogradely at 37°C through the aorta as previously described (8). The rate of perfusion was maintained at 20 ml min^{-1} with a roller pump (Gilson Minipulse 2, Biolabo, Milano). A latex balloon was inserted into the left ventricular cavity for measurement of left ventricular pressure (LVP) and dP/dt, recorded with a Hewlett Packard carrier amplifier (mod. 8805B) and recorder (mod. 7754A). The balloon was slowly filled with saline until end-diastolic pressure stabilized between 8 and 12 mm Hg. All hearts were equilibrated for 30 minutes at a flow rate of 20 ml min^{-1} to allow extensive rinsing of the vascular bed; the hearts were then perfused in a recirculating system at the same flow rate of 20 ml min^{-1} with a total volume of 50 ml. Coronary perfusion pressure (CPP) and left ventricular end-diastolic pressure (LVEDP) were monitored continuously.

Leukocyte-perfused hearts

Isolated rabbit hearts were perfused with human neutrophils isolated from blood (40 ml) withdrawn from healthy donors that had not taken medications for at least one week and purified using a discontinuous Percoll density gradient (42% and 51%, v/v, in PPP) as described previously (9).

Immediately prior to heart perfusion, PMNL were supplied with Ca^{2+} (2 mM) and Mg^{2+} (0.5 mM), and subsequently diluted to 5 ml in Tyrode's solution for infusion into the recirculating medium of the isolated rabbit hearts.

Isolated hearts were allowed to equilibrate under recirculating flow for 15 minutes and the PMNL suspension was added at a flow rate of 0.6 ml/min, in order to avoid mechanical obstruction of coronary vasculature, and left to equilibrate for additional 5 minutes before challenge with A-23187 (0.5 μM) 30 minutes later, the entire heart reservoir (approx. 45 ml) was withdrawn for storage under argon athmosphere at −20°C until HPLC analysis.

RP-HPLC analysis

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 %l of HPLC mobile phase A (methanol/acetonitrile/water/acetic acid 10/10/80/0.02, v/v/v/v, pH 5.5 with ammonium hydroxide) and injected into an HPLC gradient pump system (Mod. 126, Beckman Analytical, Palo Alto, CA, USA) connected to a diode-array UV detector (Mod. 168, Beckman Analytical) using a microprocessor-controlled autosampler (Jasco 851-AS, Tokio, J), with 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 solvent A to solvent B (50% methanol, 50% acetonitrile) at a flow rate of 0.5 ml/min, was used to elute a 3 × 150 mm column (RP-18 endcapped Ecocart Superspher, 4 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(S),12(S)-dihydroxy-eicosatetraenoic acid (5,12-diHETE) as well as from non-enzymatic LTA_4 metabolites.

Positive identification of enzymatic and non-enzymatic LTA_4 metabolites was obtained 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) was calculated from the HPLC data. Enzymatic-LTA_4 metabolites was used as a collective name for LTC_4, LTB_4, 20-hydroxy-LTB_4 and 20-carboxy-LTB_4; non enzymatic-LTA_4 metabolites was used as a collective name for Δ^6-trans LTB_4 isomers, 5,6-dihydroxy eicosatetraenoic acids as well as 12-O-methyl-Δ^6-trans LTB_4 isomers (10).

LTA_4-metabolites were defined as (enzymatic-LTA_4 metabolites)+(non enzymatic-LTA_4 metabolites).

Normalized data were obtained expressing as 100% the total amount of LTA_4-derived metabolites observed in a given sample.

RESULTS

Langendorff heart experiments

Infusion of exogenous LTD_4 (4 nmoles, bolus injection) into the isolated rabbit heart, resulted in a rapid, but relatively transient, increase in coronary perfusion pressure (*Fig. 1*) as a result of the direct vasoconstricting action of cysLT .

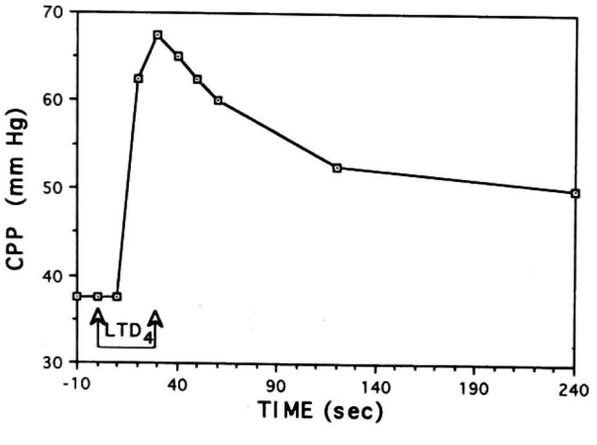

Fig. 1. Effect of a bolus injection of exogenous LTD_4 (4 nmoles) on coronary resistance to perfusion pressure (CPP) in a Langendorff rabbit heart preparation.

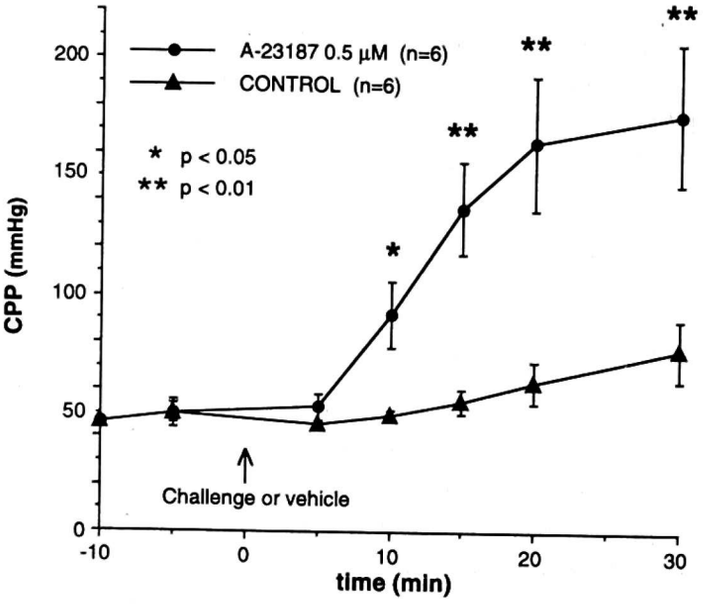

Fig. 2. Increase in coronary resistance to perfusion pressure (CPP) in isolated rabbit hearts perfused under recirculating conditions with human PMNL (1×10^5 cells/ml) and challenged with A-23187, 0.5 µM.

Challenge of the rabbit heart with A-23187 (0.5 µM), per se, did not alter myocardial contractility nor resistance to perfusion pressure. Similarly, the simple addition of 5×10^6 purified PMNL did not modify any of the functional parameters. However when the challenge with A-23187 was performed in the presence of purified PMNL a severe increase in coronary resistance to perfusion pressure took place which was slow in onset (slow reacting) and long lasting (*Fig. 2*).

The increase in coronary tone correlated significantly ($p < 0.0001$) with the concentrations of cys-LT in the recirculating buffer 30 min after challenge.

PMNL experiments

Decreasing PMNL concentration from 20×10^6 cells ml^{-1} to 1×10^6 cells ml^{-1} resulted in a significant increase in non enzymatic-LTA_4 metabolites (from 53.9 ± 1.9 to 110.4 ± 8.3 pmol/10^6 PMNL, $p < 0.01$), while the total LTA_4-derived metabolites, on a per cell basis, did not change (252.4 ± 11.3 and 272.6 ± 23.8 pmol/10^6 PMNL, at 20×10^6 and 10^6 PMNL ml^{-1} respectively). The amount of non enzymatic-LTA_4 metabolites expressed as a percent of the total LTA_4-derived metabolites, showed a progressive increase from 21.5% at 20×10^6 PMNL ml^{-1} to 41.1% at 1×10^6 PMNL ml^{-1} (*Fig. 3*).

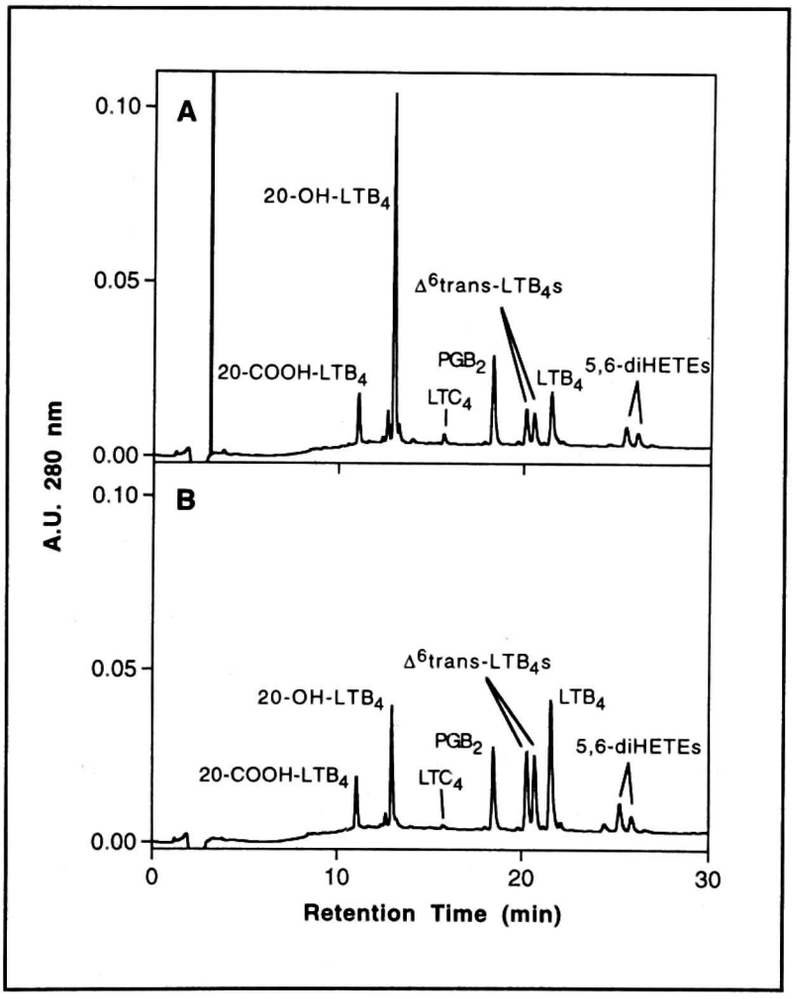

Fig. 3. Effect of cell dilution on the relative amounts of enzymatic *vs* non-enzymatic LTA_4 metabolites. Panel A, 20×10^6 PMNL ml^{-1}; Panel B, 1×10^6 PMNL ml^{-1}

The correlation between the concentration of PMNL ml^{-1} and the ratio of (enzymatic-LTA_4 metabolites)/(non enzymatic-LTA_4 metabolites) best fitted a square polynomial correlation ($r^2 = 0.72$, $p < 0.0001$) (*Tab. 1*), indicating a possible saturation of enzymatic metabolism of LTA_4 in concentrated PMNL preparations. Increasing concentrations of synthetic LTA_4 added to PMNL (20×10^6 cells ml^{-1}), resulted in preferential non enzymatic metabolism

at concentrations of LTA_4 higher than 1 μM. In fact, non enzymatic-LTA_4 metabolites represented 18.3% at 1 μM, 41.7% at 3 μM and 62.6% at 10 μM. Detectable amounts of 5-keto-(7E,9E,11Z,14Z)-eicosatetraenoic acid, identified by RP-HPLC retention time and on-line UV spectral analysis, were observed when synthetic LTA_4 was used at concentrations higher than 1 μM.

Table 1. The ratio of enzymatic- and non enzymatic-LTA_4 metabolites and cells concentration in human PMNL preparations.

PMNL concentration ($x10^6$ cells)	Enzymatic/Non enzymatic Ratio ± SEM
1	1.46 ± 0.0834
2	1.81 ± 0.138
5	2.26 ± 0.1574
10	2.76 ± 0.1411
20	3.69 ± 0.3025

Different concentrations of human PMNL were challenged with 5 μM A-23187 for 10 min at 37°C.

Decreasing the concentration of PMNL caused a marked increase of LTB_4 with respect to 20-OH- and 20-COOH-LTB_4, in agreement with previous data and suggesting that ω-oxidation of LTB_4 is mainly carried out after reuptake of released LTB_4.

DISCUSSION

The generation of leukotrienes exhibits a remarkable cellular specificity; however, in complex organ systems the cellular environment can influence markedly the final biosynthetic profile and create conditions whereby production of LT differs from that expected from individual cell types.

As shown repeatedly, activated PMNL generate not only LTB_4, but cooperate with endothelial cells, platelets, mast cells to generate large amounts of LTC_4 (11—13). LTC_4 itself activates endothelial cells to facilitate PMNL adhesion, e.g. by P-selectin expression (14).

The results obtained in our "*in vitro*" experiments, employing the spontaneously beating rabbit Langendorff heart preparation, indicate clearly that the addition of human PMNL to the A-23187-perfused rabbit heart triggers a massive increase in the coronary perfusion pressure (CPP) (and of the left-ventricular end-diastolic pressure, data not shown). This response is cys-LT dependent (5) and is fully ablated by pretreatment with the leukotriene

synthesis inhibitor BAY X1005 (15), which is a selective and bioavailable 5-lipoxygenase inhibitor (16—17). Similar results have been obtained by Grimminger and co-workers (4), using a different experimental model, who showed that perfusion and activation of PMNL in the isolated lung of the rabbit resulted in the production of increased amounts of cysteinyl leukotrienes with respect to activation of PMNL alone.

The existence of a temporal sequence between the a) increased adhesion of leukocytes to the vascular endothelium and b) the enhanced permeability of the arterial wall strongly supports the notion that conditions of tight adhesion between neutrophils and endothelial cells may create an ideal microenvironment for transcellular biosynthesis of cys-LT, leading to a very efficient transfer of LTA_4 from one cell to the other. Recenty, Brady and Serhan reported on the transcellular synthesis of cys-LT in PMNL-glomerular EC coincubations (18). In this model, cys-LT formation was significantly blunted in the presence of monoclonal antibodies directed against CD11/CD18 integrins and L-selectin, providing the grounds for the hypothesis that adhesion molecule-mediated cell-cell interactions facilitate cys-LT biosynthesis. In addition, recent data obtained in our laboratory point to a possible role of NO as an endogenous modulator of PMNL adhesion and of transcellular synthesis of cys-LT (19). Pretreatment of the isolated rabbit heart with the NO-synthase inhibitor L-NMMA, followed by perfusion of PMNL and challenge, caused a very rapid adhesion of PMNL associated with synthesis of large amounts of cys-LT and dramatic increase in CPP. Restoration of NO synthesis with L-Arginine pretreatment, significantly decreased the challenge-induced adhesion of perfusing PMNL. None of these pharmacological interventions had any direct effect on LTA_4 formation, but rather emphasized the importance of PMNL-EC adhesion toward transcellular synthesis of cys-LT.

In light of these observations it was of interest to assess the relative amount of LTA_4 released from PMNL, and therefore available for transcellular biosynthesis of cysteinyl leukotrienes, with respect to total LTA_4 synthesized.

The results of the dilution experiments indicate that a significant fraction of LTA_4 is released before being metabolised to LTB_4, and is therefore made available for transcellular biosynthesis to cysteinyl leukotrienes by proximal cells. Recent results obtained by Sala *et al.* (20) indicate that in human PMNL preparations approx. 60% of the LTA_4 synthesized through activation of 5-LO by A-23187 is actually released into the extracellular milieu. Using a different experimental approach Palmentier (21) has come to the same conclusion suggesting that the fraction of LTA_4 secreted by PMNL could represent as much as 80% of the total. This is at variance with what is generally accepted (i.e. PMNL synthesize and release $LTBA_4$) and suggest that PMNL may represent cells specifically tuned for transcellular synthesis rather than for the synthesis of a chemotactic factor (LTB_4).

Finally, the finding that LTA_4 represents the main 5-LO-derived metabolite released by PMNL, indicates that this unstable reactive intermediate could be considered as a lipid mediator itself, not as much for its intrinsic biological activity, as for its ability to promote the production of bioactive compounds in cells other than those in which it is synthesized.

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