Release of Leukotriene A₄ Versus Leukotriene B₄ from Human Polymorphonuclear Leukocytes

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The reactive intermediate formed by 5-lipoxygenase metabolism of arachidonic acid, leukotriene A₂, is known to be released from cells and subsequently taken up by other cells for biochemical processing. The objective of this study was to determine the relative amount of leukotriene A₂ synthesized by human polymorphonuclear leukocytes (PMNL) that is available for transcellular biosynthetic processes. This was accomplished by diluting cell suspensions and measuring the relative amounts of enzymatic versus nonenzymatic leukotriene A₂-derived metabolites after challenge with the Ca²⁺ ionophore A23187. Nonenzymatic leukotriene A₂-derived metabolites were used as a quantitative index of the amount of leukotriene A₂ released into the extracellular milieu. The results obtained demonstrated that in human PMNL, the relative amounts of nonenzymatic versus enzymatic leukotriene A₂-derived metabolites increased with decreasing cell concentrations. After a 20-fold dilution of PMNL in cell preparations, a doubling in the amount of nonenzymatic leukotriene A₂-derived metabolites was observed following challenge (from 53.9 ± 1.3 to 110.4 ± 8.9 pmol/10⁶ PMNL, p < 0.01). Reduction of possible cell-cell interactions by dilution suggested that over 50% of leukotriene A₂ synthesized is released from the PMNL. These data provide evidence that, in human PMNL preparations, transfer of leukotriene A₂ to neighboring PMNL is taking place, resulting in additional formation of leukotriene B₄ and its ω-oxidized metabolites 20-hydroxy- and 20-carboxy-leukotriene B₄. Neutrophil reuptake of extracellular leukotriene A₂ leads to an underestimation of the fraction of leukotriene A₂ that is in fact available for transcellular metabolism when tight cell-cell interactions occur, such as during PMNL adhesion to the microvascular endothelium and diapedesis.

Arachidonic acid oxidation, catalyzed by cyclooxygenase or 5-lipoxygenase, leads to potent biologically active molecules such as thromboxane, prostacyclin, and leukotrienes (1, 2). While most biochemical studies have focused on cells that possess cyclooxygenase or 5-lipoxygenase (5-LO),¹ it is now clear that the formation of eicosanoids is not strictly limited to those cells which have these primary oxidative enzymes. The discovery of reactive intermediate transfer in eicosanoid biosynthesis was made by Marcus (3) who showed that platelet-derived endoperoxides could be transformed into prostacyclin by adjacent endothelial cells. More recently, conversion of LTA₄ to LTD₄ and cysteinyl leukotrienes LTC₄, LTD₄, and LTE₄ 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).

Polymorphonuclear leukocytes (PMNL) possess relatively large amounts of 5-lipoxygenase, the enzyme catalyzing the sequential conversion of arachidonic acid to 5-hydroperoxyeicosatetraenoic acid (5-HPETE) and LTA₄ (9). Upon cell activation, significant amounts of LTD₄ and its ω-oxidized metabolites 20-hydroxy- and 20-carboxy-LTB₄ are released into the extracellular milieu together with nonenzymatic breakdown products of LTA₄, namely Δ⁶-trans-LTB₄ isomers and 5,6-dihydroxyeicosatetraenoic acids (5,6-diHETEs) (10–12). Recent studies in complex organ systems (13–18) showed that perfusion of PMNL in the isolated lung or heart of the rabbit only caused a significant increase in the production of cysteinyl leukotrienes when PMNL were activated during the perfusion process. These data suggest that transcellular biosynthesis of cysteinyl leukotrienes might indeed be of physiopathological relevance when tight cell-cell interactions occur, such as during adhesion and diapedesis of PMNL through the microvascular endothelium of a functioning organ system.

In light of these observations it was of interest to assess the relative amount of LTA₄ released from PMNL and therefore available for transcellular biosynthesis of cysteinyl leukotrienes, with respect to total LTA₄ synthesized. The release of LTA₄ into the extracellular milieu would remove this intermediate from intracellular LTA₄ hydrolase that catalyzes conversion of LTA₄ into LTD₄. Intracellular LTD₄ can be further metabolized by a specific cytochrome P-450 to 20-hydroxy-LTB₄ and 20-carboxy-LTB₄ (19). The extracellular (released) LTD₄ will react with water with a half-life lower than 30 s (20) to yield the nonenzymatic products, Δ⁶-trans-LTB₄, Δ⁴-trans-12-epi-LTB₄, and 5,6-dihydroxyeicosatetraenoic acid isomers. But PMNL are able to take up exogenously added LTA₄ (21) and metabolize it into LTD₄, thus reducing the fraction of released LTA₄ that is actually available for transcellular metabolism (or nonenzymatic hydrolysis).

In the present study experiments were designed to test the effect of dilution on the quantitative profile of LTA₄ metabolites produced after challenge with the Ca²⁺ ionophore A23187. The hypothesis that in diluted cell preparations LTA₄ would have less chance of being reabsorbed and metabolized by vicinal PMNL has been tested. In a previous study, Cluzeau et al. (22) showed that the use of diluted cell suspensions provided important information concerning the amount of platelet activating factor and LTD₄ released by PMNL. Using a similar approach, we provide evidence that significant transcellular...

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Experimental Procedures

Chemicals and Reagents—All chemicals used were reagent-grade and obtained from commercial sources. Eicosanoids were purchased from Cayman Chemical Co. (Ann Arbor, MI). 12-O-Methyl-all-trans-LTB₄ derivatives were prepared by reacting LTA₄ (20 nmol) with methanol (1 ml) acidified with HCl and purified by RP-HPLC. HPLC-grade solvents were obtained from Merck (Darmstadt). Type I “plus” water was obtained using a MILLIPORE Plus water purifier (Millipore, Molsheim), fed with double distilled water.

Preparation of Human Polymorphonuclear Leukocytes—Human polymorphonuclear leukocytes (PMNL) were obtained from blood withdrawn from healthy donors that had not taken medications for at least 1 week; blood was collected into a 50-ml polypropylene centrifuge tube containing 5.7 ml of ACD (41 mM citric acid/36 mM sodium citrate × 2H₂O, 136 mM glucose) and carefully mixed. After centrifugation for 15 min at room temperature (RT) and 280 × g, platelet-rich plasma was removed, and residual blood was combined with an equal volume of saline and 0.5 volume of dextran T-500 (6%, w/v, in saline), followed by thorough mixing, and allowed to stand at RT for 30 min. The leukocyte-enriched upper phase was centrifuged for 15 min at RT and 280 × g. The pellets cells were then subjected to erythrocyte lysis by gentle resuspension in 1 volume of a 0.2%, w/v, NaCl solution and further dilution with 1 volume of a solution of the following composition, 3.98 g of NaCl + 0.5 g of sucrose in 250 ml of distilled water at +4°C. Mononuclear cells were separated by centrifugation on Ficoll cushions (density 1.077 g/ml) for 30 min at RT and 400 × g. The pellet was then resuspended and obtained PMNL resuspended twice with 10–15 ml of phosphate-buffered saline without Ca²⁺ and Mg²⁺ (PBS-). Cells were finally resuspended at a final concentration of approximately 20–30 × 10⁶ cells ml⁻¹ in PBS⁻ and kept on ice until used. This preparation contained more than 95% PMNL as assessed by differential count on May-Grunwald/Giemsa-stained cytocentrifugates.

Cell Incubations—Challenge of PMNL samples at different concentrations with the calcium ionophore A23187 (Calbiochem, 5 µM) for 2 or 10 min at 37°C, after addition of Ca²⁺ (2 mM) and Mg²⁺ (0.5 mM) and 5 min of thermal equilibration. In selected experiments, human serum albumin (Sigma) was added to a final concentration of 10 mg ml⁻¹ in order to achieve 5-LO activation, concentration of A23187 was raised to 50 µM. LTA₄ free acid was obtained through base hydrolysis of LTA₄ methyl ester using ice-cold acetone/NaOH 0.25 M (4:1, v/v) at 0°C, and kept on ice until used. This preparation contained more than 95% PMNL as assessed by differential count on May-Grunwald/Giemsa-stained cytocentrifugates.

Results

Human Polymorphonuclear Leukocyte Cell Incubations—Administration of synthetic LTA₄ free acid to human PMNL resulted in a cell number-dependent formation of enzymatic-LTA₄ metabolites (data not shown).

Purified human PMNL preparations (20 × 10⁶ cells ml⁻¹) produced the expected profile of LTA₄-derived metabolites (19, 23) after challenge with the calcium ionophore A23187 (5 µM, 10 min, 37°C), identified by HPLC-UV spectral analysis. The major LTA₄-derived metabolite detected was 20-hydroxy-LTB₄ (20-OH-LTB₄), accounting for more than 60% of LTA₄-derived products. The remaining 40% was composed of 20-COOH-LTB₄ (20-COOH-LTB₄), and nonenzymatic-LTA₄ metabolites. LTC₄ was present in minor and variable amounts (<1–20 pmol/10⁶ PMNL), possibly arising from eosinophils or platelet contamination (5, 24) (Fig. 1, panel A).

Decreasing PMNL concentration from 20 to 1 × 10⁶ cells ml⁻¹ resulted in a significant increase in nonenzymatic-LTA₄ metabolites (from 53.9 ± 1.9 to 110.4 ± 8.3 pmol/10⁶ PMNL, p < 0.01) (Fig. 1, panel B), whereas the total LTA₄-derived metabolites, on a per cell basis, did not change (252.4 ± 11.3 and 272.6 ± 23.8 pmol/10⁶ PMNL, at 20 × 10⁶ and 10 × 10⁶ PMNL ml⁻¹, respectively). The amount of nonenzymatic-LTA₄ metabolites expressed as a percent of the total LTA₄-derived metabolites showed a progressive increase from 21.5% at 20 × 10⁶ PMNL ml⁻¹ to 41.1% at 1 × 10⁶ cells ml⁻¹.

The correlation between the concentration of PMNL ml⁻¹ and the ratio of (enzymatic-LTA₄ metabolites)/(nonenzymatic-LTA₄ metabolites) best fitted a square polynomial correlation (r² = 0.72, p < 0.0001) (Fig. 2), indicating a possible saturation of enzymatic metabolism of LTA₄ in concentrated PMNL preparations. To test this hypothesis, increasing concentrations of synthetic LTA₄were added to PMNL (20 × 10⁶ cells ml⁻¹), resulting in preferential nonenzymatic metabolism at concentrations of LTA₄ higher than 1 µM (Fig. 3). In fact, nonenzymatic-LTA₄ 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 (25), identified by RP-HPLC retention time and on-line UV spectral analysis, were observed when synthetic LTA₄ was used at concentrations higher than 1 µM.

Decreasing the concentration of PMNL caused a marked increase of LTB₄ with respect to 20-OH- and 20-COOH-LTB₄ (Figs. 1 and 4), in agreement with previous data (22, 26) and suggesting that ω-oxidation of LTB₄ is mainly carried out after reuptake of released LTB₄.

Reducing the incubation time for A23187 challenge from 10 to 2 min resulted in the appearance of methyl trapping metab-
The relative amounts of nonenzymatic-LTA₄ metabolites represented 41.1 ± 1.6 and 50.3 ± 1.5% at 10 and 2 min, respectively, in diluted PMNL incubations (p < 0.01). A similar shift toward nonenzymatic-LTA₄ metabolites was observed in concentrated PMNL incubations (Table I), where nonenzymatic metabolites represented 23.1 ± 1.3% at 10 min and 37.7 ± 1.4% at 2 min after challenge (p < 0.001). Total amounts of LTA₄ metabolites per million cells were also significantly lower at the shorter incubation time studied (Table I).

Human serum albumin, at a final concentration of 10 mg ml⁻¹, totally inhibited the production of 5-LO metabolites after challenge with A23187 (5 mM for 10 min at 37°C), possibly due to binding to albumin itself (28). Increasing the concentration of A23187 to 50 mM restored a well-detectable production of LTA₄-derived metabolites (Table I). Interestingly, the quota of nonenzymatic metabolites was significantly decreased in diluted cell incubations (43.4 ± 1.8 versus 50.2 ± 1.5%, p < 0.05 versus without albumin) but increased in concentrated PMNL preparations (43.5 ± 2 versus 37.7 ± 1.4%, p < 0.05 versus without albumin) if compared with samples without albumin. In agreement with the stabilizing effect of albumin, intact LTA₄, represented by 12-O-methyl-all-trans-LTB₄-derivatives, was 18.4 ± 0.8 and 26.5 ± 1.8% of total LTA₄-derived metabolites, in diluted and concentrated PMNL preparation, respectively.

**DISCUSSION**

Over the last 5 years an increasing body of evidence has indicated the importance of transcellular metabolism of leukotriene A₄ in complex organ systems. Grimminger and co-workers (13–15) showed that perfusion and activation of PMNL in the isolated lung of the rabbit resulted in the production of significantly increased amounts of cysteinyl leukotrienes, with...
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The data presented indicate that the amount of nonenzymatic-LTA₄ metabolites observed in PMNL challenged by calcium ionophore A23187 in vitro, at the commonly used concentrations of 10–20 × 10⁶ cell/ml, results in a substantial underestimation of the fraction of LTA₄ that is indeed available for transcellular metabolism. In the past, the fact that PMNL themselves act as acceptor cells for released LTA₄, as well as cells that convert released LTB₄ to ɷ-oxidized metabolites, was evidently overlooked (Fig. 5). The use of diluted human PMNL preparations permitted the estimate that over 50% of the LTA₄ synthesized through activation of 5-lipoxygenase by the use of the calcium ionophore A23187 is actually released into the extracellular milieu. Preliminary data, using a different approach to obtain a PMNL-perfused isolated rabbit heart (16, 18). These reports have raised the issue of determining how much of the LTA₄ synthesized by PMNL can be made available to adhering cells (namely endothelial or smooth muscle cells). The overall potential for LTA₄ transfer from PMNL (donor cell) to acceptor cells (29) has usually been quantitated by evaluating the production of nonenzymatic-LTA₄ metabolites in purified PMNL incubations (13, 14, 16). Nevertheless, given the capacity of PMNL to actively take up LTA₄ from the extracellular milieu, and convert it to LTB₄ (21), such a calculation would still underestimate the amount of LTA₄ provided by PMNL to neighboring cells. The evidence presented in this report clearly demonstrates that LTA₄ represents the major metabolite released from PMNL to the extracellular milieu. Diluted cell suspensions have been used as an approach to limit the ratio of LTA₄ that, once released into the extracellular milieu, is available for further enzymatic metabolism by neighboring PMNL. Challenge of sequentially diluted PMNL preparations with the calcium ionophore A23187 resulted in increased amounts of nonenzymatic-LTA₄ metabolites (namely ω-oxidized metabolites 20-hydroxy-LTB₄ and 20-carboxy-LTB₄) with respect to enzymatic-LTA₄ metabolites (LTB₄ and its ω-oxidized metabolites). 5-Keto-(7E,9E,11Z,14Z-)eicosatetraenoic acid (25), a reported nonenzymatic-LTA₄ metabolite, was not observed in A23187-challenged PMNL preparations, whereas it was present in detectable amounts when synthetic LTA₄ was used at concentrations higher than 1 μM. The decrease in the ratio of (enzymatic metabolites)/(nonenzymatic metabolites) was well correlated with the decreased concentration of LTA₄ observed at higher PMNL concentrations used. Administration of increasing concentrations of synthetic LTA₄ to PMNL at a concentration of 20 × 10⁶ ml⁻¹ supported this hypothesis, in agreement with previous data indicating that the LTA₄ hydrolase, and not 5-lipoxygenase, is the limiting factor in the synthesis of LTB₄ in human leukocytes (30). Concentrations of LTA₄ upon challenge with A23187, as estimated from the total amount of LTA₄-derived metabolites observed, resulted in approximately 1.13, 2.3, and 5 μM in PMNL preparations at 5, 10, and 20 × 10⁶ PMNL ml⁻¹, respectively. These LTA₄ concentrations are very compatible with saturation of the enzymatic metabolite formation observed with exogenous LTA₄.

The O-methyl trapping products of LTA₄ were observed in incubations terminated 2 min after A23187 challenge, indicating the presence of intact LTA₄ even after this time period. The total amounts of LTA₄-derived metabolites was significantly lower if compared with amounts observed after 10 min, both in diluted and in concentrated PMNL preparations. However, shortening the incubation time after challenge led to a significant shift toward nonenzymatic-LTA₄ metabolites at both concentrations studied. This would be consistent with a time-dependent reuptake of LTA₄ into the PMNL and subsequent conversion into LTB₄.

It is known that albumin is able to stabilize LTA₄, increasing its half-life at physiological pH from a few seconds to over 20 min (20). The effect of human serum albumin, at a concentration of 10 mg ml⁻¹, was studied in diluted and concentrated PMNL preparations, after challenge with A23187 for 2 min. The results obtained showed that in diluted cell preparations, stabilization of LTA₄ by albumin was able to partially revert the effect of dilution, allowing intact LTA₄ to travel to distant PMNL and be enzymatically transformed into LTB₄. On the other hand, in the presence of higher concentrations of LTA₄ such as in concentrated cell preparations, a favorable competition by the bound LTA₄ versus that LTA₄ which can be taken up from the albumin complex by the human PMNL exists, resulting in the trapping of intact LTA₄ in the extracellular milieu. In addition to affecting LTA₄ metabolism, dilution of PMNL preparations influenced the amounts of LTB₄ relative to the ω-oxidized metabolites 20-hydroxy-LTB₄ and 20-carboxy-LTB₄ observed upon A23187 challenge. Decreasing the PMNL concentration, linked with the increase in nonenzymatic-LTA₄ metabolites, also resulted in a 4-fold increase in LTB₄ with a complementary decrease in 20-COOH- and 20-OH-LTB₄. These results, in agreement with previous studies (22, 26), indicate that LTB₄ synthesized in purified PMNL preparations is first released and then ω-oxidative metabolism occurs after reuptake by the cells.

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approach, suggested that the fraction of LTA₄ secreted by PMNL could represent up to 80% of the total (31).

At variance with what is generally accepted, the data reported here indicate that the majority of LTA₄ is released before being metabolized to LTB₄ and is therefore available for transcellular biosynthesis to cysteinyl leukotrienes by proximal cells. Inhibition of LTC₄ formation arising from the interaction of human PMNL and glomerular endothelial cells, by antibodies against CD18 and L-selectin, has recently been reported (32). Adhesion of PMNL to potential LTA₄ acceptor cells would therefore appear to be a key step toward an efficient transfer of LTA₄ resulting in the formation of cysteinyl leukotrienes. Close interaction may cause direct transfer of LTA₄ from the PMNL to the LTC₄ synthase-carrying endothelial cells, resulting in substantial changes in the metabolic profile of 5-lipoxygenase-derived products. It has been shown that LTB₄ may enhance its own biosynthesis in an autocrine fashion (33); similarly, cysteinyl leukotrienes may amplify their own biosynthetic mechanisms, inducing endothelial cell-dependent transcellular metabolism to cysteinyl leukotrienes (34). Metabolism of LTA₄ to cysteinyl leukotrienes within the microvasculature may have considerable pathophysiological consequences, in light of the ability of LTC₄ and LTD₄ to induce profound modification of vascular permeability leading to edema formation (35).

The data presented in this work indicate that LTA₄, the main 5-LO-derived metabolite released by PMNL, can therefore 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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