Erythrocyte–neutrophil interactions: Formation of leukotriene B₄ by transcellular biosynthesis

(cell–cell communication/leukotriene A₄/epoxide hydrolase “suicide inactivation”/inflammation)

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ABSTRACT

Studies on the mechanism of leukotriene B₄ biosynthesis in suspensions composed of neutrophils plus erythrocytes indicate that human erythrocytes convert neutrophil-derived leukotriene A₄ into leukotriene B₄. Leukotriene B₄ formation by neutrophils in the presence of erythrocytes exceeded that from corresponding suspensions of neutrophils alone. The increase was proportional to the erythrocyte content of the suspension. The erythocyte-dependent increase in leukotriene B₄ biosynthesis did not equal the arithmetic sum of calcium ionophore-dependent biosynthesis by neutrophils plus calcium ionophore-dependent biosynthesis by erythrocytes, since erythrocytes produced no leukotriene B₄ upon incubation with ionophore A23187. Erythrocytes did not stimulate 5-lipoxygenase activity within neutrophils, since the erythrocyte effect was confined to enzymatic hydration: leukotriene B₄ increased coincident with decreased formation of 5,12-dihydroxyicosatetraenoic acids derived from nonenzymatic hydration. Biosynthesis of leukotriene B₄ within the erythrocyte, from neutrophil-derived leukotriene A₄, was established by comparing the effect of normal erythrocytes with erythrocytes containing a leukotriene A₄ hydrodil that was inactivated by the substrate. In the latter case, leukotriene B₄ formation increased by only 30–40%; in the former case, it increased by 100–200%. Transcellular biosynthesis of leukotriene B₄ from erythrocyte–neutrophil interactions (i) explains the paradoxical presence of leukotriene A₄ hydrolase within erythrocytes, a cell incapable of synthesizing leukotriene A₄; (ii) affords a mechanism to overcome rate limitations or “suicide inactivation” of leukotriene A₄ hydrolase in neutrophils; (iii) exploits a cryptic capacity within erythrocytes, provisionally dormant cells in terms oficosanoid biosynthesis; (iv) indicates that the biosynthetic capacity of cell combinations is not necessarily equivalent to the sum of their separate capacities.

Enzymatic cooperation between separate cell types may be a significant, but neglected, mechanism for the biosynthesis of certain prostaglandins or leukotrienes. This concept originated from studies on platelet–endothelial cell interactions, which showed that prostaglandin I₂ (PGI₂), an antithrombotic isocoumarin, was produced by endothelial cells from platelet-derived prostaglandin endoperoxide H₃ (1–5). Analogous transcellular biosynthetic processes are conceivable for other cell combinations and for other arachidonic acid metabolites; however, few biologically relevant examples exist. Requirements for this process include “acceptor” cells with a constitutive enzymatic capacity for the formation of a biologically active isocoumarin product but a deficient, or disabled, capacity for substrate generation, plus auxiliary “donor” cells that can fulfill that corresponding substrate requirement. In this context, neutrophil–erythrocyte interactions attracted our attention as potential sources for transcellular biosynthesis of leukotrienes (6). Ordinarily, biosynthesis of leukotriene B₄ (LTB₄) occurs within an individual cell type via sequential enzymatic reactions including phospholipase-dependent liberation of arachidonic acid, regioselective 5-lipoxygenation, rearrangement into leukotriene A₄ (LTA₄), and enzymatic hydration of LTA₄ by an epoxide hydrolase to yield LTB₄, a biologically active metabolite ([5S,12R]-5,12-dihydroxy-6,14-cis-8,10-trans-icosatetraenoic acid) (7). Spontaneous nonenzymatic hydration yielding two inactive 5,12-dihydroxyicosatetraenoic acid (5,12-diHETE) isomers competes with the enzymatic hydration step. Conceptually, biosynthesis of LTB₄ could occur by a similar enzymatic pathway even if different parts of the enzymatic sequence were segmented within two separate cell types. Neutrophils and erythrocytes in combination possess the enzymatic attributes appropriate for such transcellular biosynthesis of LTB₄. For instance, neutrophils are a prominent source of the substrate, LTA₄, and they secrete it, intact, because of rate limitations in their capacity for LTB₄ formation (8, 9). Erythrocytes, by contrast, lack a demonstrable capacity for biosynthesis of LTA₄, yet they have a paradoxical capacity to convert it, when available, into LTB₄ (6). We aimed to determine whether human erythrocytes could produce the chemotactic myotropic agent LTB₄ from neutrophil-derived LTA₄.

MATERIALS AND METHODS

Materials. Synthetic LTA₄ methyl ester (Upjohn) was purified prior to use (10). LTA₄ lithium salt was prepared as described (11). Synthetic leukotrienes and prostaglandin B₂ (PGB₂; Upjohn) and microcrystalline cellulose, α-cellulose, and calcium ionophore A23187 (Sigma) were used as received. Water, ethyl acetate, and methanol, all distilled in glass (Burck and Jackson, Muskegon, MI), and C₁₈ reversed-phase HPLC (RP-HPLC) columns (250 × 6 × 4 mm) (Rainer Instruments, Woburn, MA) were used for metabolite isolation and quantification.

Preparation of Purified Human Erythrocyte Suspensions. Human venous blood (50 ml) collected in sterile 3.8% (wt/vol) sodium citrate (5.5 ml) was centrifuged at 200 × g for 30 min, and the platelet-rich plasma was removed. Sedimented erythrocytes, leukocytes, and platelets were resuspended in sterile 0.9% (wt/vol) NaCl (50 ml) and centrifuged at 200 × g for 20 min. The supernatant was discarded. After two washes the cell suspension was depleted of residual leukocytes and platelets by filtration through columns containing microcrystalline cellulose and α-cellulose (1:1, wt/wt) (12). Erythrocytes were resuspended at 2 × 10⁶ cells per ml. The purified erythrocyte suspension contained ≤5 × 10⁴ leuko-

Abbreviations: LTB₄ and LTA₄, leukotriene B₄ and A₄; diHETE, dihydroxyicosatetraenoic acid; PGI₂ and PGB₂, prostaglandin I₂ and B₂; RP-HPLC, reversed-phase HPLC.

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cytes per ml and \(5 \times 10^6\) platelets per ml. Differential cell counts were determined with a Coulter Diffz system.

**Isolation of Human Polymorphonuclear Neutrophils.** Polymorphonuclear neutrophils were isolated from human whole blood by dextran sedimentation, centrifugation over Ficoll/Hypaque (Sigma) gradients, and hypotonic lysis (13). Neutrophils were resuspended (2 x 10^9 cells per ml) in Hanks’ balanced salt solution (HBSS), 0.02 M Hepes buffer, pH 7.2, with 1.4 mM CaCl_2 and 0.8 mM MgCl_2.

**Transcellular Metabolism of Neutrophil-Derived LTA_4.** Neutrophils alone (1.5 ml, 2 x 10^6 cells per ml), erythrocytes alone (1.5 ml, 2 x 10^6 cells per ml), or neutrophils plus erythrocytes (1.5 ml, 2 x 10^6 neutrophils per ml, 1 x 10^6 erythrocytes per ml) were incubated at 37°C for 5 min, then calcium ionophore A23187 (5 \(\mu\)M) was added to stimulate leukotriene biosynthesis. After 2.5 min at 37°C biosynthesis was quenched by adding ethyl acetate (5.0 \(\mu\)l) containing PGB, (0.5 \(\mu\)g) as a quantitative internal standard for RP-HPLC. LTB_4 and related diols were extracted three times with 5 ml of ethyl acetate each time. Solvent was evaporated and the residue was reconstituted in methanol/water (70:30, vol/vol) (1.0 ml). Leukotrienes were determined by RP-HPLC (7). To measure erythrocyte concentration dependence, the neutrophil concentration was fixed at 16 x 10^6 cells per ml while the erythrocyte concentration was varied between 0 and 0.8 x 10^6 cells per ml. To establish neutrophil concentration dependence, the neutrophil content was varied from 0 to 20 x 10^6 cells per ml in the presence or absence of a fixed erythrocyte content (0.8 x 10^6 cells per ml). For kinetic studies samples were quenched at intervals between 0 and 30 min.

**“Suicide Inactivation” of Erythrocyte LTA_4 Epoxide Hydrolase.** Erythrocytes (1.0 ml, 2 x 10^6 cells per ml) were incubated with 30 \(\mu\)M LTA_4 for 2.0 min at 37°C. Under these conditions the epoxide hydrolase inactivates itself. Erythrocytes were then washed twice with 10 ml of 0.9% (wt/vol) NaCl containing human serum albumin at 25 mg/ml to sequester metabolites of synthetic LTA_4. The content of LTB_4 in the supernatant was determined as described (6), and the erythrocytes were resuspended in 0.9% (wt/vol) NaCl (2 x 10^6 cells per ml). The extent of suicide inactivation of LTA_4 epoxide hydrolase in erythrocytes was determined by comparing LTB_4 biosynthesis from control erythrocytes incubated with 10 \(\mu\)M LTA_4 to LTB_4 biosynthesis from suicide-inactivated erythrocytes incubated with 10 \(\mu\)M LTA_4. Transcellular metabolism of LTA_4 from neutrophils incubated with 5 \(\mu\)M A23187 was characterized as described above. Control erythrocytes were compared to erythrocytes with suicide-inactivated LTA_4 epoxide hydrolase to establish the involvement of erythrocytes as acceptor cells for LTA_4 donated by neutrophils.

**RESULTS**

Human neutrophils stimulated with calcium ionophore A23187 produced three 5,12-diol metabolites of LTA_4, including the enzymatic hydration product LTB_4 and the two nonenzymatic products (5S,12R)-5,12-dihydroxy-6,8,10-trans-14-cis-icosatetraenoic acid (peak I) and (5S,12S)-5,12-dihydroxy-6,8,10-trans-14-cis-icosatetraenoic acid (peak II) (Fig. 1A), consistent with the established characteristics of these cells (7). Human erythrocytes incubated with A23187 did not produce detectable amounts of these or other leukotrienes, confirming that erythrocytes alone did not synthesize LTA_4 from endogenous arachidonic acid (Fig. 1C). When erythrocytes and neutrophils together were incubated with A23187, LTB_4 biosynthesis increased with a corresponding decrease in the formation of nonenzymatic isomers I and II, relative to suspensions of neutrophils alone (Fig. 1B). Ten separate experiments gave equivalent chromatographic profiles. Quantitative and qualitative results were confirmed by UV spectrophotometry, radioimmunoassay, and bioassay. Results were descriptively similar for stimuli other than calcium ionophore A23187. For instance,
human neutrophils (2 × 10^7 cells per ml) incubated for 5 min at 37°C with 0.2 μM formylmethionylleucylphenylalanine (fMet-Leu-Phe) produced 4 pmol of LTB4 in the absence of erythrocytes and 54 pmol of LTB4 in the presence of 0.8 × 10^9 erythrocytes per ml. Erythrocytes alone, incubated with 2 μM fMet-Leu-Phe, produced no detectable hydration products of LTA4.

LTB4 biosynthesis was directly proportional to the neutrophil content of cell suspensions in the presence or absence of erythrocytes (Fig. 2). However, LTB4 biosynthesis in suspensions containing neutrophils and erythrocytes together consistently exceeded that from a corresponding suspension with neutrophils alone. For 2, 5, 10, 15, and 20 × 10^6 neutrophils per ml, LTB4 biosynthesis increased 76 ± 10%, 87 ± 10%, 109 ± 15%, 156 ± 17%, and 202 ± 30% (mean ± SEM, n = 7), respectively, in the presence of 0.8 × 10^9 erythrocytes per ml.

Fig. 3 depicts the relative hydration of neutrophil-derived LTA4 as a function of erythrocyte concentration. For a constant neutrophil concentration, enzymatic conversion of LTA4 into LTB4 increased in proportion to the erythrocyte concentration in the suspension. Neutrophils alone produced 0.49 ± 0.044 nmol of LTB4 (mean ± SEM, n = 15) compared to 0.67 ± 0.05, 0.77 ± 0.06, 0.94 ± 0.09, 1.03 ± 0.10, and 1.10 ± 0.10 nmol of LTB4 in the presence of 16, 32, 48, 64, and 80 × 10^6 erythrocytes per ml, respectively. The erythrocyte-dependent increase in LTB4 biosynthesis was statistically significant (P < 0.01). Erythrocytes alone produced no detectable LTB4, or related metabolites, confirming, again, that the increases were not attributable to ionophore-dependent production by separate cell types. Nonenzymatic hydration of LTA4, reflected by the formation of 5,12-diHETE isomers I and II, was inversely proportional to the erythrocyte concentration (Fig. 3).

Results in Figs. 1–3 suggested that the erythrocyte-dependent increase in LTB4 biosynthesis occurred by transcellular metabolism of LTA4 derived from neutrophils. A distinctive feature of the erythrocyte epoxide hydrolase that catalyzes LTB4 formation allowed a rigorous test of this proposal. Specifically, LTA4 epoxide hydrolase in erythrocytes is susceptible to suicide inactivation (14). Our model for transcellular metabolism predicts that suicide inactivation of epoxide hydrolase by exposure of erythrocytes to synthetic LTA4 would diminish their capacity to produce LTB4 upon subsequent exposure to neutrophil-derived LTA4. Our results confirm this prediction. Neutrophils alone incubated with 5 μM A23187 produced 0.606 ± 0.040 nmol of LTB4 (mean ± SEM, n = 8); neutrophils plus autologous control erythrocytes produced 1.29 ± 0.060 nmol of LTB4; neutrophils plus autologous erythrocytes with an epoxide hydrolase that had been inactivated by prior exposure to 10 μM LTA4 produced 0.83 ± 0.050 nmol of LTB4. Thus, the relative increase in LTB4 biosynthesis was 0.68 nmol of LTB4 (Fig. 4), or 112%, with normal erythrocytes, which may be compared to 0.22 nmol of LTB4, or 37%, with erythrocytes containing suicide-inactivated LTA4 hydrolase. Control experiments with two successive additions of synthetic LTA4 confirmed that erythrocytes were suicide inactivated by the first addition of synthetic LTA4 (Fig. 4). It was noteworthy that nonenzymatic hydration products were relatively constant, or slightly increased, in suspensions of suicide-inactivated erythrocytes, indicating that the 5-lipoxygenase enzyme in neutrophils was not inactivated.

LTB4 levels in suspensions containing erythrocytes remained high for at least 20 min. For example, at 5, 10, and 20 min, suspensions of neutrophils alone (1.5 × 10^7 cells per ml) contained 0.56, 0.46, and 0.21 nmol of LTB4, which may be compared to 1.25, 1.25, and 0.87 nmol of LTB4 in corresponding suspensions containing 6.4 × 10^9 erythrocytes per ml.
**DISCUSSION**

Collectively, these results demonstrate that transcellular biosynthesis of LTB₄ occurs along an erythrocyte-neutrophil axis. Neutrophils can produce and release both LTB₄ and LTA₄; erythrocytes can convert a portion of neutrophil-derived LTA₄ into additional LTB₄. Certain points support this conclusion. First, the erythrocyte-dependent increase in LTB₄ biosynthesis did not originate from the arithmetic sum of ionophore stimulation of neutrophil arachidonic acid metabolism plus ionophore stimulation of erythrocyte arachidonic acid metabolism. Human erythrocytes, lacking detectable phospholipase A2 and 5-lipoxygenase activity, did not produce LTB₄ or related LTA₄ metabolites upon stimulation with ionophore A23187. Second, the increase in LTB₄ formation did not originate from erythrocyte-dependent stimulation of neutrophil metabolism. If erythrocytes had stimulated arachidonic acid release and 5-lipoxygenase metabolism within the neutrophil, increased nonenzymatic formation of 5,12-diHETE isomers would have accompanied the increase in LTB₄ biosynthesis. This was not the case. Instead, the erythrocyte effect was confined to enzymatic hydration of LTA₄; LTB₄ formation increased at the expense of nonenzymatic hydration. Third, a role for biosynthesis of LTB₄ by erythrocytes was established by comparing normal erythrocytes with erythrocytes whose LTA₄ epoxide hydrolase had been inactivated by the substrate. Erythrocytes with a disabled LTA₄ epoxide hydrolase did not augment LTB₄ biosynthesis as effectively as normal erythrocytes did. In view of these points, and the fact that neutrophils are the exclusive source of LTA₄ under our experimental conditions, one may conclude that erythrocytes convert neutrophil-derived LTA₄ into LTB₄.

Our results on erythrocyte-neutrophil interactions and transcellular biosynthesis provide one plausible explanation for the otherwise paradoxical presence of LTA₄ epoxide hydrolase within human erythrocytes. Furthermore, transcellular biosynthesis of LTB₄ affords a mechanism to supersede rate limitations or suicide inactivation within the neutrophil, and to recruit a provisionally inactive cell, the erythrocyte, for active contributions to leukotriene formation. The cellular and enzymatic traits that permit transcellular biosynthesis from erythrocyte-neutrophil interactions also facilitated a rigorous evaluation of the hypothesis. These traits include the compartmentalization of phospholipase and 5-lipoxygenase activities within neutrophils but not erythrocytes, the susceptibility of LTA₄ hydrolase to suicide inactivation, and the hydration of LTA₄ by both enzymatic and nonenzymatic processes. These or comparable traits may be useful for identifying other model systems of cell-cell interactions.

It is interesting to compare erythrocyte-neutrophil interactions with existing examples of transcellular metabolism of eicosanoids. For instance, platelet-neutrophil interactions can also increase LTB₄ biosynthesis and form (5S,12S)-diHETE and 12,20-diHETE (15–18) products of uncertain biological significance. In contrast, neutrophil-erythrocyte interactions increase only LTB₄, a leukotriene with chemotactic and myotropic effects (19). Platelet-endothelial cell interactions yield PGJ₂, an antithrombotic substance, but proofs that PGJ₂ originates from platelet-derived prostaglandin H₂ have depended upon the use of pharmacological agents to modify platelet thromboxane synthetase or endothelial cell cyclooxygenase. In contrast, a transcellular origin for LTB₄ and a need for such an origin can be established exclusively in terms of cellular, enzymatic, and chemical attributes of erythrocyte and neutrophil LTA₄ hydrolase. The biological significance of transcellular metabolism remains to be established, but our results support Marcus’ advocacy for cell–cell interactions as an important but novel form of eicosanoid biosynthesis (3, 16). Since cell combinations encountered in disease may differ from those encountered in normal circumstances the study of transcellular metabolism assumes some significance (20).

An important point from our results is that the biosynthetic capacity achieved by combining separate cell types is not simply the quantitative or qualitative sum of their individual capacities. In the extreme case represented, cells such as erythrocytes with “zero” capacity alone can contribute substantially when their constitutive biosynthetic capacity is allied with that of another cell type. This phenomenon is not necessarily restricted to erythrocytes and neutrophils. Furthermore, this type of cell–cell interaction is not necessarily restricted to eicosanoids. The results for leukotrienes may
have heuristic value for studies of cell-cell interactions in other biosynthetic contexts.