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FORMATION OF LEUKOTRIENES AND OTHER HYDROXY ACIDS DURING PLATELET-NEUTROPHIL INTERACTIONS IN VITRO

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SUMMARY: Interactions of human platelets with neutrophils were studied in suspensions of [^{3}H]arachidonate-labeled platelets and unlabeled neutrophils stimulated with ionophore A23187. Several radioactive arachidonate metabolites, not produced by platelets alone, were detected, including [^{3}H]-labeled leukotriene B_4 (LTB_4), dihydroxyeicosatetraenoic acid (DHETE) and 5-hydroxy-eicosatetraenoic acid (5-HETE). When [^{3}H]12-HETE, a platelet product, was added to stimulated neutrophils, DHETE was formed. Similarly, when [^{3}H]5-HETE, a neutrophil product, was added to stimulated platelets, DHETE was the major product. These results suggest that upon stimulation: 1) platelet-derived arachidonate may serve as precursor for the neutrophil-derived eicosanoids LTB_4 and 5-HETE, and 2) that platelet-derived 12-HETE can be converted to DHETE by human neutrophils. The present investigation documents cell-cell interactions via the lipoxygenase pathway, which may be important in hemostasis, thrombosis and inflammation.

Neutrophils may participate along with platelets in hemostasis and thrombosis (1). In platelets the cyclooxygenase pathway of arachidonic acid metabolism has been implicated in these processes. Platelets also have a pathway for lipoxygenation of arachidonate, which forms 12-hydroxy acids. The main pathway of arachidonic acid metabolism in neutrophils involves 5-lipoxygenation, leading to formation of eicosanoids such as leukotrienes and other hydroxy acids (2, 3). Studies on the origin and control of these arachidonate metabolites are of interest, since these compounds initiate significant

ABBREVIATIONS: DHETE (5S,12S-DHETE): 5S,12S-dihydroxy-6-trans,8-cis,10-trans,14-cis-eicosatetraenoic acid; 5-HETE: 5S-hydroxy-6-trans,8,11,14-cis-eicosatetraenoic acid; 12-HETE: 12S-hydroxy-5,8-cis,10-trans,14-cis-eicosatetraenoic acid; LTB_4 (leukotriene B_4): 5S,12R-dihydroxy-6-cis,8,10-trans,14-cis-eicosa-tetraenoic acid; THETE: trihydroxyeicosatetraenoic acids.

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biological activities including chemotaxis, neutrophil activation, and smooth muscle contractility (3-5).

It has been suggested that two cell types may be involved in the formation of leukotrienes and other dihydroxy acids (5-7). Since we previously demonstrated that platelet-derived precursors (endoperoxide and arachidonate) are utilized by endothelial cells in the formation of prostacyclin (8), we examined the interactions of platelets and neutrophils. In the present study we document that stimulated platelets interact with neutrophils through release of [^{3}H]arachidonate and [^{3}H]12-HETE which serve as substrates for eicosanoid formation by neutrophils.

METHODS AND MATERIALS

Preparation of platelet suspensions. Blood was collected and processed (8, 9) from donors who had ingested 650 mg aspirin 12 and 2 hr prior to venipuncture. Platelets were labeled (8) with 50 μCi [^{3}H]arachidonate added during the first wash (9).

Preparation of neutrophil suspensions. Suspensions containing 98+1% neutrophils were prepared (10). Cells were suspended in buffer (Hepes, 5 mM; NaCl, 140 mM; KCl, 5 mM; $CaCl_2$, 1.29 mM; $MgCl_2$, 1.20 mM; pH 7.45). Lactic acid dehydrogenase release, a measure of cell viability, was less than 3.5%.

Experimental design: Labeled platelets and unlabeled neutrophils. For TLC studies $3x10^8$ labeled platelets were preincubated at 37°C for 5 min. $3x10^7$ unlabeled neutrophils were then added (total volume, 1 ml). One min later 1 or 2 μM ionophore (in 1 μl ethanol) was added. After 5 min, reactions were stopped and lipids extracted (11). For TLC plus HPLC studies, incubations contained $1.8x10^9$ platelets/ml and $1.8x10^8$ neutrophils/ml in 3-5 ml, stimulated with 2-5 μM ionophore. Experiments with neutrophils or unlabeled platelets alone contained $3x10^8$ platelets/ml or $3x10^7$ neutrophils/ml. [^{14}C]arachidonic acid (0.32 μCi, 5.7 nmoles), [^{3}H]12-HETE (0.45 μCi, 9.4 pmoles), or [^{3}H]5-HETE (0.40 μCi, 6.6 pmoles) were added as sodium salt 15 sec after cell stimulation. This concentration of [^{14}C]arachidonic acid was non-lytic and non-stimulatory for neutrophils (12).

Thin-layer radiochromatography. Lipid extracts were chromatographed on activated (1 hr, 110°C) silica gel G plates (Analtech) along with standards using chloroform/ methanol/acetic acid/water (90:8:1:0.8) (13) as solvent. Other TLC procedures have been described (14). Where specified, LTB_4 and DHETE were separately scraped from unstained plates (maintained under N_2), using external standards stained with a fine stream of I_2 vapor as guides, prior to elution with ethyl acetate.

HPLC analysis of eicosanoids. Lipids from combined platelet and neutrophil suspensions were fractionated on silicic acid columns and purified by reverse-phase HPLC (2). Products were identified by UV absorption spectra (2), comparison of elution times with those of authentic standards, and their ability to provoke neutrophil aggregation (4,15).

Materials. [1-^{14}C]Arachidonic acid, [^{3}H]arachidonic acid, [^{3}H]12-HETE and [^{3}H]5-HETE: New England Nuclear. Eicosanoid standards were gifts: 5S,12S-DHETE; 5-HETE; LTB_4; Dr Pierre Borgeat. 5S,12R-all trans LTB_4 and 5S,12S-all trans LTB_4; Dr Edward J. Goetzl. 5S,12S-DHETE; Dr Robert R. Gorman. 5-HETE and synthetic LTB_4; Dr J. Rokach. Ionophore A23187; Dr Robert L. Hamill. Additional standards; Dr John E. Pike.

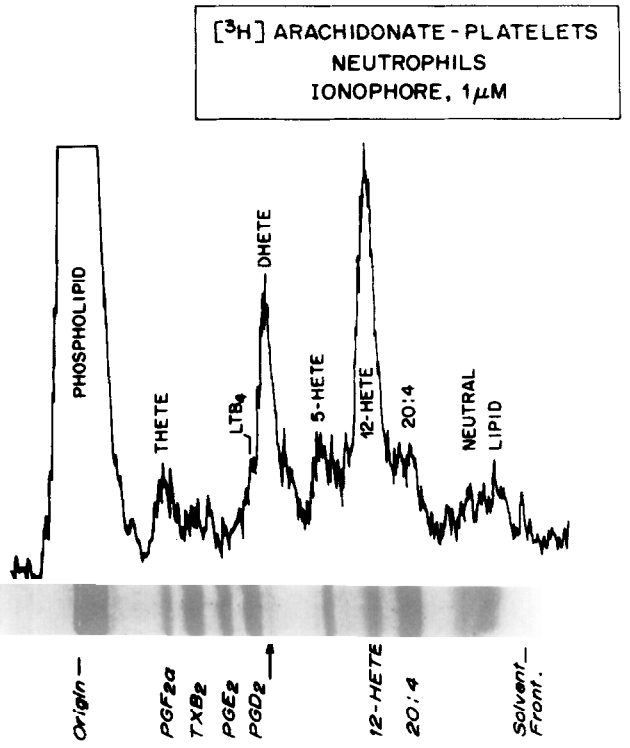

Fig.1 TLC radiochromatogram of lipids extracted from combined suspensions of stimulated [^{3}H]arachidonate-labeled platelets and unlabeled neutrophils. The tracing shows distribution of radioactivity derived from [^{3}H]arachidonate-prelabeled platelets. Shown below the radioactivity tracing is a photograph of the iodine-stained TLC plate. Prior to chromatography unlabeled standards were added to the lipid extract. In this system synthetic LTB_4 standards consistently co-migrated with PGD_2. The arrow points to iodine-stainable endogenous lipid which co-migrated with authentic DHETE, and was always above LTB_4. The data are representative of 3 separate experiments.

RESULTS and DISCUSSION

Stimulation of [^{3}H]arachidonate-labeled platelets and unlabeled neutrophils with ionophore A23187. Cell suspensions containing radiolabeled platelets and unlabeled neutrophils were stimulated in order to determine whether precursors originating from platelets contribute to production of neutrophil-derived eicosanoids. Several radioactive metabolites, not produced by stimulated platelets alone, were detected in the combined system. These included LTB_4 (5.5% of non-phospholipid radioactivity recovered from the TLC plate), DHETE (19.7%), 5-HETE (7.7%), and, possibly, THETE (6.9%) (Fig. 1). If ionophore was omitted, 98.4% of radioactivity remained associated with phospholipids. When combined platelet-neutrophil suspensions were stimulated with ionophore, LTB_4 and DHETE were produced in quantities which could actually be visualized

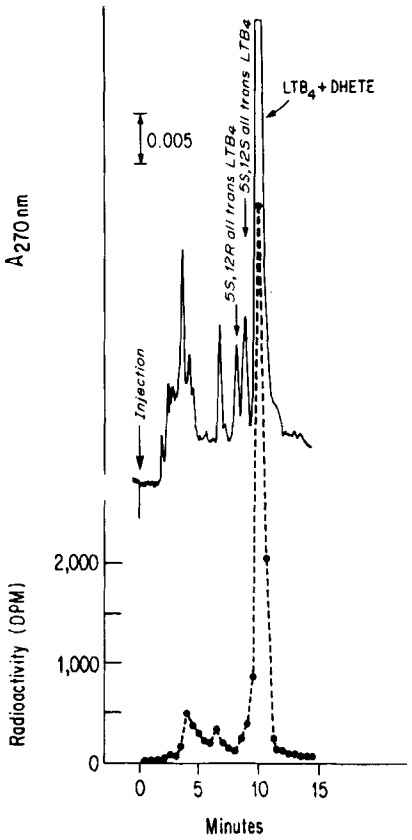

Fig.2 HPLC separation of lipoxygenase products obtained from a combined suspension of stimulated [^{3}H]arachidonate-labeled platelets with unlabeled neutrophils. UV absorbance (270 nm) is shown in the top trace. The corresponding radioactivity elution profile is shown below. Cells were incubated and lipids were extracted and separated by HPLC as described under "Materials and Methods". Authentic standards were run prior to injection of sample. The data are representative of 3 separate experiments in which samples were examined in duplicate or triplicate. DHETE co-chromatographed with synthetic LTB_4 (5,16). The peak shown here was further resolved into LTB_4 and DHETE by radio-TLC (see text and Fig. 3).

on stained TLC plates (Fig. 1). In the absence of platelets these metabolites were not visualized.

Lipid extracts were also analyzed by HPLC. As shown in Fig. 2, radioactivity was associated with the HPLC peak co-migrating with synthetic LTB_4. The material in this peak also demonstrated a UV spectrum similar to that of synthetic LTB_4 and provoked aggregation when added to neutrophils. Upon TLC, however, this material was resolved into two peaks which migrated as LTB_4 (R_f = 0.38) and DHETE (R_f = 0.42). The same proportion of these 2 compounds was obtained when the extract was chromatographed directly on TLC plates.

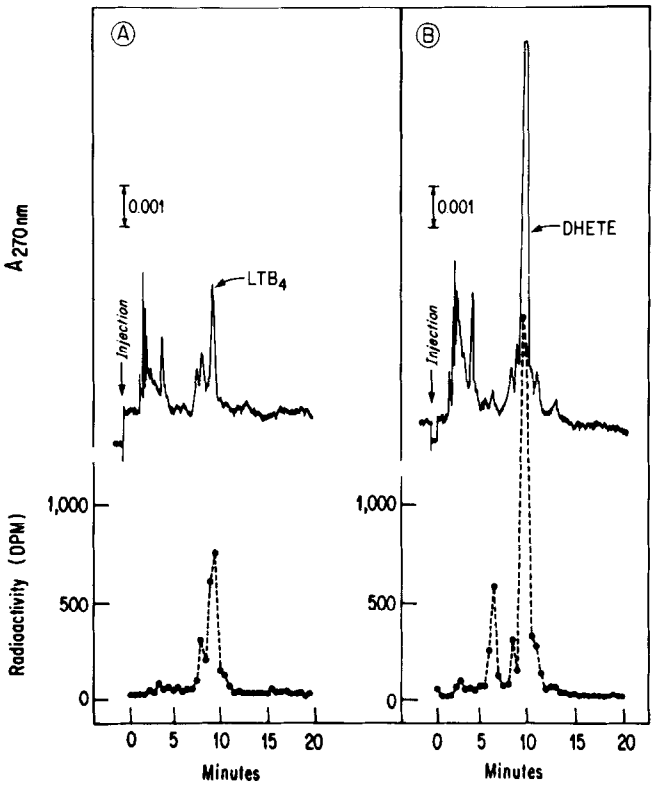

Fig.3 HPLC analysis of LTB_4 (A) and DHETE (B) following their separation by TLC. Panel A shows both UV absorbance at 270 nm (top) and radioactivity elution profile of LTB_4 (bottom). Prior to injection onto the HPLC column, lipids were eluted from the LTB_4 region on the TLC plate. Panel B represents the corresponding profiles for lipids eluted from the DHETE region of the TLC plate. Data shown are representative of 2 individual experiments.

In a separate experimental approach LTB_4 and DHETE were separated from an aliquot of the total lipid extract by TLC, then separately eluted and subsequently analyzed by reverse-phase HPLC (Fig. 3). Platelet-derived radioactivity was associated with the HPLC peaks obtained from each of the two TLC areas. The peaks had the same elution time on HPLC as that of synthetic LTB_4.

Since platelets were the sole source of radioactivity in these studies, LTB_4, DHETE, and 5-HETE were produced from precursor(s) originating from platelets upon ionophore stimulation. This is the first direct demonstration of the platelet contribution to formation of neutrophil-derived leukotrienes and hydroxy acids.

Platelets utilized in the above experiments were obtained from donors who had ingested aspirin, therefore only 3 possible platelet-derived precursors

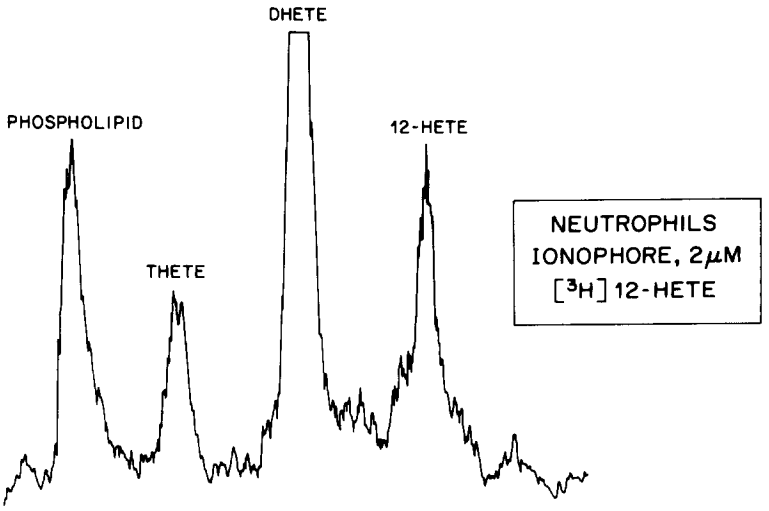

Fig.4 TLC radiochromatogram of lipids extracted from neutrophils incubated with ionophore and [^{3}H]12-HETE. DHETE was the major metabolite when ionophore and [^{3}H]12-HETE were added to neutrophils. Data are representative of 2 experiments. Analogous results were obtained when the lipid extract was chromatographed on HPLC (4 separate experiments). DHETE was also the major metabolite when [^{3}H]5-HETE and ionophore were added to platelets (not shown).

were available to the neutrophils -- arachidonic acid, 12-hydroperoxyeicosatetraenoic acid (12-HPETE) and 12-HETE. The effects of adding 2 of these compounds to stimulated neutrophil suspensions were studied.

Stimulation of neutrophils with ionophore A23187 in the presence of either [^{14}C]arachidonate or [^{3}H]12-HETE. To examine possible pathways involved in formation of labeled neutrophil eicosanoids, [^{3}H]12-HETE or [^{14}C]arachidonate were incubated with neutrophils. Addition of 1 μM ionophore and 5.7 μM [^{14}C]arachidonate to neutrophils resulted in synthesis of LTB_4 (15.3% of recovered non-phospholipid counts), DHETE (6.0%) and 5-HETE (23.3%). The possible THETE (R_f = 0.17-0.21) were also observed (4.0%). The DHETE formed in this experiment is attributable to the presence of platelets in this particular neutrophil preparation, as borne out by subsequent studies, reported below. Neutrophils exposed to 5.7 μM [^{14}C]arachidonate but not stimulated with ionophore did not produce these metabolites.

Addition of 2 μM ionophore and 9.4 nM [^{3}H]12-HETE to neutrophils resulted in production of DHETE (46.4% of recovered non-phospholipid counts) which was synthesized from this platelet lipoxygenase product (Fig. 4). Essentially no

LTB_4 was formed from [^{3}H]12-HETE since, in contrast to the DHETE area, the LTB_4 area on the plate contained only 2.3% of recovered counts. Once again, 7.2% of the radioactivity was recovered in the THETE zone. HPLC analyses similarly indicated that radioactive metabolites, obtained from incubation of neutrophils with ionophore and [^{3}H]12-HETE, eluted in the LTB_4-DHETE region.

The above results suggest that radiolabeled platelet-derived 12-HETE serves as precursor of labeled DHETE synthesized by neutrophils when [^{3}H]-arachidonate-labeled platelets and unlabeled neutrophils are stimulated by ionophore. Moreover, these studies indicate that labeled platelet-derived arachidonate is the precursor of labeled LTB_4 and 5-HETE synthesized by unlabeled neutrophils (Fig. 1).

Stimulation of unlabeled platelets with ionophore A23187 in the presence of [^{3}H]5-HETE. Since DHETE is formed via double oxygenation (5, 16) of arachidonate, two pathways of its formation are possible: a) neutrophil lipoxygenation of platelet-derived 12-HETE, and b) platelet lipoxygenation of neutrophil-derived 5-HETE (5, 16). The first of these pathways was demonstrated by the experiments reported in the previous section. The second pathway was investigated by addition of 2 μM ionophore to platelets in the presence of 6.6 nM [^{3}H]5-HETE. A TLC peak containing 28.0% of recovered non-phospholipid radioactivity co-migrated in two solvent systems (second system: organic phase of ethyl acetate/acetic acid/ isooctane/water 110:20:50:100, v/v) with a DHETE standard, as well as with DHETE derived from incubation of neutrophils with [^{3}H]12-HETE and ionophore. These studies further confirmed the identity of DHETE and the dual pathways by which it is formed in a combined cell system. Interestingly, despite formation of DHETE by platelets from 5-HETE, no detectable THETE were produced in the absence of neutrophils.

CONCLUSIONS

Functional aspects of DHETE reported to date include chemotactic activity comparable to that of 5-HETE (17) and possible regulation of leukotriene formation (18). The present study indicates a potential for significant production of DHETE during platelet-neutrophil interactions, since it can be formed

by each of these cells from precursors supplied by the other (Figs. 1-4).

Since LTB_4 is a powerful chemoattractant (3) and a complete secretagogue for neutrophils (4), the platelet contribution to its formation documented here for the first time (Fig. 1), is of considerable interest. Thus platelet-neutrophil interactions generate products capable of playing a role in normal or abnormal homeostasis. Moreover, production of these metabolites continues unabated in subjects ingesting aspirin.

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