Vol. 107, No. 2, 1982 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
July 30, 1982 Pages 752-759

ARACHIDONIC ACID METABOLISM IN GUINEA PIG MEGAKARYOCYTES

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Received June 21, 1982

SUMMARY Guinea pig megakaryocytes were isolated to 75-90% purity, incubated with 1-^{14}C-arachidonic acid and radioactive metabolites analyzed by thin layer chromatography. Thromboxane B_2, 12-hydroxyheptadecatrienoic acid (HHT), and 12-hydroxyeicosatetraenoic acid (12-HETE) were the major products identified. Pre-incubation with indomethacin inhibited production of thromboxane B_2 and HHT. Comparison of metabolites produced by megakaryocytes and platelets from the same animals indicated that one megakaryocyte produced 2500-4000 times as much thromboxane B_2, HHT, or 12-HETE as did a single platelet. These results suggest that isolated megakaryocytes possess the full complement of cyclooxygenase and lipoxygenase activities found in circulating platelets.

Arachidonic acid metabolism plays an important role in the aggregation and secretory responses of circulating blood platelets (1). Indeed, the use of inhibitors of platelet cyclooxygenase and the beginning work on inhibitors of thromboxane synthetase have direct and significant implications for the treatment of thromboembolic processes in clinical medicine (2). In both designing drug regimens and in assessing response to *in vivo* administration of such agents, it becomes critical to have information as to the operative pathways of arachidonate metabolism in the precursor cells of blood platelets, the megakaryocytes. This work utilizes highly purified suspensions of megakaryocytes from guinea pig bone marrow to characterize both the cyclooxygenase and lipoxygenase activities of bone marrow megakaryocytes.

MATERIALS AND METHODS *Preparation of Megakaryocytes and Platelets.* Albino guinea pigs (Charles River) of either sex (300-450 g) were anesthetized with nembutal (50 mg/kg). Blood taken by cardiac puncture was anticoagulated with 0.38% sodium citrate and washed as described below. The animals were then sacrificed and the humeri, femurs, and tibia removed. Marrow was removed from these bones and processed by minor modifications of the method of Levine and Fedorko (3). In brief, marrow was taken into calcium- and magnesium-free Hanks' balanced salt colution (CMFH) containing 0.38% citrate, 1 mM adenosine, and 2 mM theophylline, washed, and layered onto a discontinuous gradient of bovine serum albumin (Fraction V BSA, Armour) for density gradient centrifugation (adjusted by refractive index, with ascending indices (at 20°C) of 1.3694, 1.3662, 1.3630, and 1.3598). After 30 min. at 10,000 x g, 4°C, all cells remaining above the pellet were pooled, washed in CMFH, and layered on a BSA velocity sedimentation gradient of 1.3430 bottom layer, 2:1 (v/v) of 1.3430: CMFH middle layer, and 1:2 (v/v) of 1.3430: CMFH top layer.

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After 30 minutes at 20°C and unit gravity, cells above the second highest interface were discarded, and the remaining cells washed in CMFH and applied to a second velocity sedimentation gradient. After discarding the top layers once again, the remaining cells were washed in CMFH, counted, and studied.

Isolated marrow cells from each animal were counted in six hemocytometer chambers by phase contrast microscopy. Differential counts of megakaryocytes versus non-megakaryocytes were performed upon Wrights-Giemsa stained cytocentrifuge (Shandon Corp.) preparations, in order to maximize discrimination between the smaller megakaryocytes and cells of other lines. Total megakaryocytes as well as megakaryocyte purity were calculated by multiplying the differential counts by the total cell counts obtained by phase contrast.

Citrated guinea pig blood and human blood (drawn by venipuncture from volunteers giving informed consent) was centrifuged for 20 min. at 100 x g to produce platelet-rich plasma (PRP). The cells were pelleted at 4°C in the presence of 1% EDTA and resuspended in CMFH. The procedure was repeated with successively decreasing concentrations of EDTA, until the platelets were firally resuspended in CMFH alone. The final platelet suspension was counted either by phase contrast or electronically (Model S Plus, Coulter).

Metabolism of 1-^{14}C-Arachidonic Acid and Analyses of Metabolites. 1-^{14}C-arachidonic acid (50-58 Ci/mol) was purchased from New England Nuclear or Amersham and stored as an ethanol solution at -5 mM. Cells were suspended at 2-5 x 10^8 platelets/ml or 0.5-2.5 x 10^5 megakaryocytes/ml in CMFH containing 25 mM Tris-HCl, pH 7.4. Suspensions were incubated at 37°C for 5 minutes (in the presence or absence of 30 μM indomethacin), made 1.0 mM in $CaCl_2$, and arachidonic acid was then added to a final concentration of 1 to 30 μM. Cells were incubated at 37°C with gentle shaking for the indicated times. Metabolism was terminated by the addition of three volumes of a 1:2 (v/v) mixture of chloroform:methanol.

Metabolites were extracted essentially as described previously (4). Thromboxane B_2 (TXB_2), prostaglandin E_2, prostaglandin D_2, and prostaglandin $F_{1\alpha}$ (PL Biochemical), 12-HETE (New England Nuclear), 5-HETE (a gift from Dr. P. Borgeat) and 15-HETE (prepared with soybean lipoxygenase) were used as internal standards. Three volumes of chloroform were added, and the extract was acidified with 0.2 volumes of 0.5 N HCl. Phases were thoroughly mixed, then separated by centrifugation. The lower phase was removed, dried over anhydrous magnesium sulfate and evaporated in conical vials under a stream of nitrogen.

The extracts were analyzed by thin layer chromatography on Silica Gel G in chloroform:methanol:glacial acetic acid:water (90:8:1:0.8 v/v/v/v) (System A) which resolves all metabolites of human platelets (5); or ether:petroleum ether:glacial acetic acid (100:50:1 v/v/v) (6) (System B) which resolves positional isomers of hydroxy fatty acids (7). Extracts were also analyzed by conversion of free fatty acids to methyl esters with diazomethane (8) and separation in System C (upper phase of water:ethyl acetate:iso-octane (100:50:100 v/v/v)) (9). Chromotograms were analyzed by radioautography on Kodak XAR film developed in an X-Omat processor. Incorporation of radioactivity into metabolites was measured by scraping regions of the chromatogram indicated by radioautography and liquid scintillation counting of the scraped adsorbent in 83% Aquasol (New England Nuclear), 12% water, 5% methanol.

RESULTS Megakaryocyte Yields and Purity. By utilizing two velocity sedimentation steps, we routinely achieved yields of 1.4-5.0 x 10^5 megakaryocytes per animal, at megakaryocyte purities ranging from 75 to 90% of nucleated cells by cell number. Due to the greater average size of megakaryocytes as compared with the contaminating marrow cells, megakaryocyte purity in terms of cell volume would be higher still. Utilizing the relative volume estimates (10,11) of 15,000 μm^3 for megakaryocytes and 420 μm^3 for non-megakaryocytes,

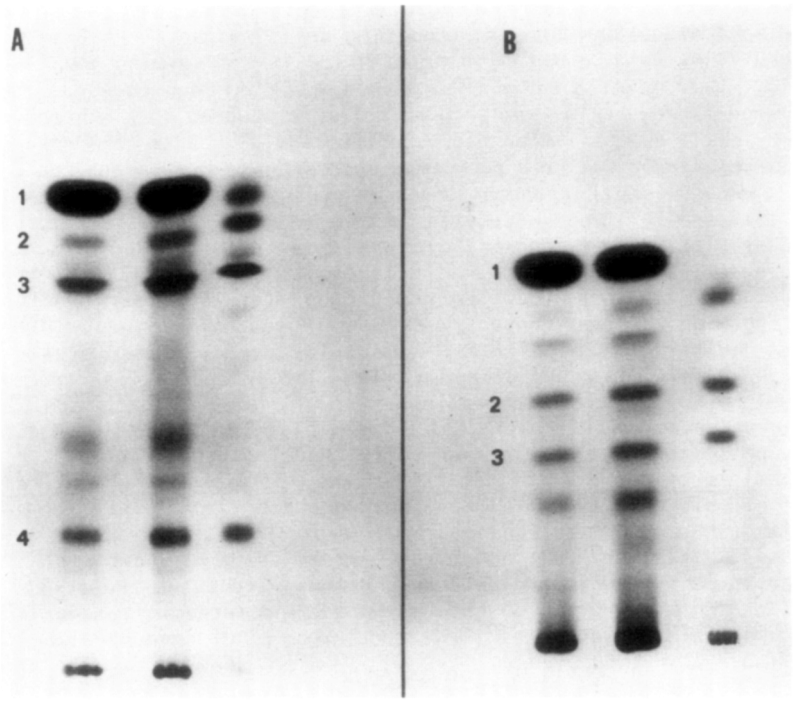

Figure 1 - Products of arachidonic acid metabolism by guinea pig platelets and megakaryocytes. Autoradiographs of thin layer chromatograms run in system A (plate A) or system B (plate B). On each plate the samples from left to right were guinea pig megakaryocyte products, guinea pig platelet products, and human platelet products. Presumptive identity of bands: (1) arachidonic acid; (2) 12-HETE; (3) HHT; (4) TXB_2.

megakaryocyte cell mass in our studies would comprise over 99% of total cell mass.

Identification of Arachidonic Acid Metabolites. Guinea pig megakaryocytes and platelets produced a similar spectrum of products when incubated with 1-^{14}C-arachidonic acid (Fig. 1). The major radioactive products included a band which co-migrated with the TXB_2 internal standard and two bands which migrated in the hydroxyacid region of the chromatogram in system A (Fig. 1A). The slower of these two moved with an R_f of 0.71, identical to that of 12-hydroxyheptadecatrienoic acid (HHT) produced by human platelets. The faster of the two migrated with an R_f of 0.78, identical to that of 12-hydroxyeicosatetraenoic (12-HETE) from human platelets. In system B, which resolves even positional isomers of HETES, these two major products migrated with R_f's (0.37 and 0.49) identical to HHT and 12-HETE from human platelets (Fig. 1B) and distinct from 15-HETE (0.52) or 5-HETE (0.34). Additional, as yet unidentified, products with mobilities differing slightly from 12-HETE and HHT were also routinely observed in both guinea pig megakaryocytes and platelets. These metabolites did not correspond to major products of human platelets. Minor components with mobilities similar to prostaglandins D_2 and

Table 1. Effect of Indomethacin On the Production of Arachidonic Acid Metabolites by Guinea Pig Megakaryocytes and Platelets*

	TXB_2	HHT	12-HETE
Megakaryocytes	34 ± 9.8	42 ± 4.6	98 ± 17.3
Platelets	8.8 ± 2.6	18.2 ± 4.4	110 ± 36.3

*Suspensions of guinea pig megakaryocytes or platelets were incubated at 37°C for 5 minutes in the presence or absence of 30 μM indomethacin prior to the addition of 1-^{14}C-arachidonic acid (5 μM final concentration). After an additional 5 minutes incubation with the radioactive precursor, products were extracted and analyzed as described in the text, using solvent system A. Results are expressed as $(CPM_{Indo}/CPM_{Control}) \times 100$. (Mean ± S.E. for three experiments.)

E_2 in system A (R_f's between 0.4 and 0.5) did not co-migrate with these compounds when they were included as internal standards. The mobilities of methyl esters of these major products from guinea pig platelets and megakaryocytes were also identical to those of human platelet products in system C.

The identification of TXB_2 and HHT was supported by experiments in which guinea pig platelets or megakaryocytes were preincubated for 5 minutes with 30 μM indomethacin. This compound inhibits the production of both TXB_2 and HHT in human platelets, but does not inhibit production of the lipoxygenase product 12-HETE (12,13). Qualitatively similar results were seen for the guinea pig platelets and megakaryocytes (Table 1). In these experiments indomethacin inhibited the production of both TXB_2 and HHT in platelets (over 80% inhibition of both) and in megakaryocytes (66% and 58% inhibition respectively), without inhibition of 12-HETE.

Kinetic Analyses of Metabolite Production. Incubation of megakaryocytes with 5 μM 1-^{14}C-arachidonic acid led to a linear accumulation of TXB_2 and HHT for up to five minutes (Fig. 2). The production of 12-HETE continued at a high rate for fifteen minutes. Guinea pig platelets were more variable in their metabolism. Platelets from some animals terminated production of all products within one minute of addition of arachidonate even though significant quantities of non-metabolized arachidonic acid remained, while others were similar to the megakaryocytes in their time course of metabolism.

Five minute incubations were selected to approximate initial rate of production of all metabolites. The production of thromboxane by megakaryocytes from exogenously provided arachidonic acid appeared saturable, with half-maximal production occuring at approximately 5 μM (Fig. 3). By

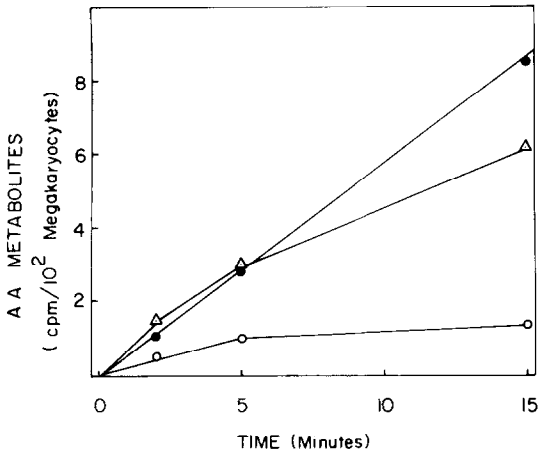

Figure 2 - Time course of arachidonic acid metabolism. Guinea pig megakaryocytes ($0.5 - 1.0 \times 10^5$) were incubated with 5 μM 1-^{14}C-arachidonic acid for the indicated times. Radioactivity incorporated into TXB_2 (o), HHT (Δ) and 12-HETE (•) was determined. (Data represent means of two experiments.)

contrast, the production of 12-HETE did not appear to saturate at concentrations of up to 30 μM. Similar saturation kinetics were observed for guinea pig platelets. While considerable animal to animal variation existed in the amount of products produced, data obtained with platelets from individual guinea pigs suggested that thromboxane production was saturable between 3 and 10 μM arachidonate, while 12-HETE production was not saturated by 30 μM arachidonate.

In an effort to compare the relative amount of metabolism of arachidonic acid by guinea pig platelets and their progenitor cells, metabolism of 5 μM

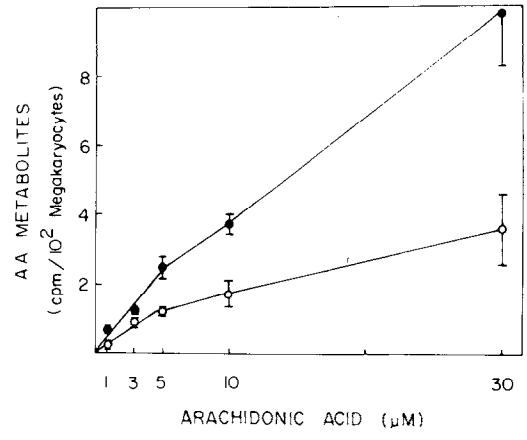

Figure 3 - Concentration dependence of metabolite production. Megakaryocytes were incubated with the indicated concentrations of 1-^{14}C-arachidonic acid for five minutes and the incorporation of radioactivity into TXB_2 (o) and 12-HETE (•) determined. (Mean ± S.E. for four experiments.)

Table 2. Relative Production of Cyclooxygenase and Lipoxygenase Metabolites by Guinea Pig Megakaryocytes and Platelets.*

	TXB_2	HHT	12-HETE	
Megakaryocytes	3.2 ± 0.7	10.0 ± 2.6	15.8 ± 2.0	(pmoles/10^5 cells)
Platelets	8.3 ± 2.0	39 ± 7.4	48 ± 9.2	(pmoles/10^8 cells)

*Megakaryocytes and platelets from the same guinea pigs were incubated with 5 μM 1-^{14}C-arachidonic acid for 5 minutes, products analyzed, and pmoles calculated from the specific activity of the arachidonic acid provided, any contribution of endogenous arachidonic acid being ignored. HHT values may include unidentified hydroxy acids. (Mean ± S.E. for eight experiments.)

1-^{14}C-arachidonic acid for 5 minutes was studied in platelets and megakaryocytes isolated from the same animals. The data presented in Table 2 suggest that each megakaryocyte produced approximately 2500 times as much TXB_2, 3900 times as much HHT, and 3000 times as much 12-HETE as each platelet.

DISCUSSION We have demonstrated that a highly purified preparation of guinea pig megakaryocytes metabolizes arachidonic acid via the major pathways seen in guinea pig and human platelets -- cyclooxygenase to yield thromboxanes and HHT, and lipoxygenase to yield 12-HETE. The high purity of the megakaryocyte preparation allows the unequivocal attribution of these synthetic pathways to the megakaryocytes rather than to contaminating cell types.

In this study the identity of the products was established on the basis of chromatographic mobility in various solvent systems, co-chromatography with authentic purified standards, and identity with the well-defined products of human platelets. The effects of indomethacin (a selective inhibitor of cyclooxygenase) (13) on the synthesis of the various arachidonic acid metabolites was an additional criterion used for identification.

Utilizing centrifugal elutriation to purify megakaryocytes from the femoral marrow of rats (5-10% final megakaryocyte purity), Worthington and Nakeff have recently described the production of TXB_2 by these cells (14). The present studies, employing quite different isolation techniques, as well as a different animal species, provide confirmation that megakaryocytes possess the capacity for thromboxane production. Through the use of the chromatographic techniques employed in the present work, moreover, it is now clear that TXB_2 is only one of several identifiable products formed when radiolabeled arachidonic acid is incubated with isolated megakaryocytes. In fact, as shown in Table 2, the relative production of TXB_2 appears to be less than that of both 12-HETE and HHT.

The ability to study the effects of metabolic inhibitors of these pathways in megakaryocytes should prove useful. The use of cyclooxygenase inhibitors both therapeutically and as a diagnostic aid for the estimation of platelet survival (15) and production (16) has generated considerable speculation as to the effect such agents may have on the progenitor cell of the platelet, the megakaryocyte (17,18). We have found an immediate inhibition of TXB_2 and HHT production when indomethacin was administered *in vitro* to highly purified suspensions of guinea pig megakaryocytes. This suggests that the megakaryocytes from these animals share with platelets, as well as with most other cells studied, a common susceptibility of their cyclooxygenase to inhibitors of this type. Of further interest, and a subject of continuing study, is the determination of the ability of megakaryocytes cultured *in vitro* to recover from inhibition by cyclooxygenase inhibitors such as indomethacin or aspirin. The establishment of baseline values of arachidonic acid metabolism in the present paper should prove useful for such studies as well as for studies involving the effects upon megakaryocytes of cyclooxygenase inhibitors administered *in vivo* (19,20).

The relative proportions of cyclooxygenase and lipoxygenase metabolites appear similar in megakaryocytes and in their progeny, as the ratio of TXB_2:HHT:12-HETE for platelets (1:4.7:5.7) was similar to that for megakaryocytes (1:3.1:4.9) (Table 2). Additionally, the production of metabolites was 2500-4000 times as great per megakaryocyte as per platelet. Since megakaryocytes are estimated to produce approximately 1000-5000 platelets (21), these results suggest that the megakaryocytes we have studied already contain the full complement of cyclooxygenase and lipoxygenase activities necessary for their eventual platelet progeny.

ACKNOWLEDGMENTS

The authors wish to thank John Dusse, Carolyn Ganley, and Maria Suchomski, for their excellent technical assistance. This research was supported by N.Y.S. Health Research Council Grant 11-037 and a grant from the Pharmaceutical Manufacturers Association Foundation to JLM, and USPHS Grant HD-14405 to MJS.

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