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# AN IMBALANCE OF ARACHIDONIC ACID METABOLISM IN ASTHMA

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Summary. Metabolism of arachidonic acid via the cyclooxygenase and lipoxygenase pathways was studied in washed platelets from normal and asthmatic subjects. The platelets were incubated with [1-$^{14}$C] arachidonic acid and the metabolites formed were separated by high pressure liquid chromatography (HPLC). The platelets from asthmatic patients had a 40% decrease in cyclooxygenase-derived metabolites and a 70% increase in lipoxygenase-derived product when compared with metabolites generated by platelets from normal subjects. The ratio of cyclooxygenase to lipoxygenase products was 3.24 $\pm$ 0.26 for platelets from normal subjects, and 1.14 $\pm$ 0.15 with platelets from the asthmatic patients. These results indicate an imbalance of arachidonic acid metabolism in platelets from asthmatic patients.

Asthma is a form of reversible airways disease that is characterized by hyperreactive airways and an increase in airway resistance and a decrease in lung compliance (1). While histamine and slow-reacting substance (SRS) have been reported to be two major mediators released during anaphylaxis in human lungs (2), the lack of effect of antihistamines in treatment of asthmatic patients argues against a predominant role of histamine as a mediator in bronchial asthma (3). Conversely, the possible involvement of SRS in the bronchoconstriction of asthma has attracted great attention (4). The contractile effect of SRS on smooth muscle is different from that of histamine. Using a superfusion bioassay system, we observed that the release of histamine from anaphylactic guinea pig lung produced a strong but transient contraction, whereas that of SRS, after a relapse, generated a relatively weak but persistent contraction of guinea pig ileum (5). It is known that SRS is derived from arachidonic acid via the lipoxygenase pathway (6,7).

Overproduction of SRS resulting from excessive lipoxygenase activity could produce the bronchoconstriction in asthma. This excessive activity may occur at the expense of a subnormal activity of cyclooxygenase, exhibiting an imbalance of arachidonic acid

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metabolism via these two pathways in the cells. In order to test this hypothesis, we undertook a series of experiments using a relatively homogenous model of washed platelets from normal subjects and asthmatic patients.

## Materials and Methods

The experiments were designed in a double-blind fashion. Blood samples were collected from normal and asthmatic subjects who had not taken any medications, including non-steroidal anti-inflammatory drugs (NSAIDs), for a minimum of 10 days prior to the experiments. The asthmatic patients involved in this study were of mild to moderate severity, thus allowing them to be off treatment for the required period of time. Ventilatory functions of all subjects were evaluated and recorded in a computerized spirometer (Cyber Diagnostics, Aurora, Colorado). The criterion for asthma was that the value of maximum forced expiratory flow 25-75% (MFEF 25-75%) fell below 80% of the predicted value.

Nineteen normal subjects and 19 asthmatic patients were involved in this study, and an even number of subjects from each of these groups were included in each experiment.

Twenty ml of blood was drawn by puncture of an antecubital vein and mixed immediately with 1/10 volume of 3.8% sodium citrate. The mixture was then centrifuged at 300 x g (1300 rpm, Beckman J-6B centrifuge) for 15 minutes at room temperature. Unless otherwise indicated, only plasticware was used and the temperature was kept at $4^{\circ}C$ throughout the entire experiment. The platelet-rich plasma was made 1 mM with regard to EDTA and divided into fractions which were equivalent to 4 ml of the original blood. The plasma was chilled on ice for 10 minutes and centrifuged at 2,000 x g for 15 minutes. The sedimented platelets were resuspended in an equal volume of Tris-saline EDTA solution containing 134 mM NaCl, 15 mM Tris-HCl buffer (pH 7.4), 5 mM D-glucose and 1 mM EDTA. The platelets were centrifuged at 2,000 x g for 15 minutes and finally suspended in 1 ml of Tris-saline buffer without EDTA. The platelet count was made directly from platelet-rich plasma using a hemocytometer. The final concentration of platelets was 1-3 x $10^8$/ml and the leukocyte contamination was less than 0.1%. The platelet counts in the blood from asthmatic patients were lower, but not significantly so, than those from normal subjects. In three experiments involving six persons, the same number of platelets from normal and asthmatic subjects were used. But all experiments showed that the difference in the ratio of cyclooxygenase to lipoxygenase products between the two groups was not profoundly affected by small differences in the numbers of platelets.

Platelets obtained from the same volumes of blood were incubated at $37^{\circ}C$ with [1-$^{14}C$] arachidonate (0.1 μCi/10 μM) for 30 minutes, so that a steady state of metabolite formation was reached. The labeled arachidonate (52.7 mCi/mmol) was obtained from New England Nuclear, Boston, MA, and unlabeled arachidonate (>99%) was purchased from NuChek, Elysian, Michigan. Both arachidonate preparations were purified by silica column chromatography prior to use and were dissolved in a small amount of dimethylsulfoxide (DMSO). The final concentration of DMSO in each sample was less than 0.2%. The metabolites, as well as non-metabolized arachidonate, were extracted by the method of Sun (8). The reactions were terminated by the addition of two volumes of acetone. The acetone in the supernatant was removed by flash evaporation under $N_2$. The aqueous solution was acidified to pH 3.0 and the metabolites were extracted 3 times with 3 volumes of diethyl ether. The ether extracts were pooled, dehydrated and evaporated to dryness under $N_2$. The residue was taken up immediately in 100 μl of acetonitrile and kept frozen at $-70^{\circ}C$ until analyzed.

HPLC separation of arachidonate metabolites was performed by a method previously described (9). Our Waters HPLC system (Milford, MA) consisted of two 6000 A solvent delivery systems with a 660 solvent programmer, a U6K universal sample injector, a 450

variable wavelength U.V. detector and a Model 730 data module. An Ultrasphere-ODS (4.6 x 250 mm) (Beckman, Palo Alto, California) reverse phase column was used to separate the metabolites produced by human platelets. The solvent, delivered at 1 ml/min, spanned a linear gradient of 35% to 95% acetonitrile in $H_2O$ in 25 min, and then maintained an isocratic elution of 95% for 5 min. The solvent contained 0.01% acetic acid to maintain the pH at 4.8. The pump back-pressures of 2,000 lb/in$^2$ (psi) for 35% acetonitrile and 1,000 psi for 95% acetonitrile were noted carefully, as was the complete return to baseline pressure between samples.

The U.V. absorption of the effluent was monitored at 192 nm for the fatty acid and its derivatives and data were recorded on a data module. The monitoring of U.V. absorption also enabled us to maintain consistent column elutions. Fractions were collected at 0.5 min intervals. Each fraction was mixed with 4 ml Aquasol and counted for 2 min in a liquid scintillation counter (Beckman Instruments). The counting efficiencies were 90% for $^{14}C$ and 29% for $^{3}H$, and these counting efficiencies did not appear to vary significantly with different concentrations of acetonitrile. The radioactivity was plotted as a function of retention time. Each radioactive peak was integrated and corrected for recovery. Recovery was calculated as the ratio of the total radioactivity recovered from HPLC to the total radioactivity added to the incubation medium. The recovery rates of $TXB_2$, HETE and arachidonate during the ether extraction were 68, 65, and 70%, respectively; the recovery rate of the HPLC step was 99.5%. The overall recovery rate of all the fractions was 59 $\pm$ 0.8% (n=60). Thus the final corrected radioactivity of each peak formed the quantitative basis of this method.

Each metabolite had its own retention time on the column, which reflected its polarity and lipid solubility. The metabolites were identified by the injection of the authentic standards alone, and by co-chromatography of duplicate samples with the standards. To confirm the identity of the peaks, thin layer chromatography (TLC) was also used for identification of these metabolites. The retention times of $TXB_2$, HHT, HETE, and arachidonate were 8.5, 19.5, 24.0, and 32.0 min respectively. These metabolites accounted for 80-95% of the substrate conversion. During incubation of arachidonate at 37°C without platelets, less than 0.5% of added substrate was spontaneously degraded, indicating the requirement of the cells for the arachidonate to be metabolized. Furthermore, the results obtained from the assay of triplicate samples were within 5% error, suggesting that this radiometric-HPLC method was highly reproducible.

The significance of difference was evaluated by the Student's t-test.

## Results

Fig. 1 shows that the platelets from normal and asthmatic subjects had different capacities and patterns in the metabolism of exogenously added arachidonate. The platelets from asthmatic patients converted 68%, whereas those from normal subjects degraded 81% of added substrate into metabolites ($p < 0.025$). The production of metabolites via the two pathways by platelets from normal and asthmatic subjects was also quite different. Based on the radioactivity obtained, the platelets from asthmatic patients produced 40% less $TXB_2$ and HHT, and 70% more HETE than the cells from normal subjects ($P < 0.0005$). These data were expressed in an additional way by calculating the ratios of cyclooxygenase metabolites, including $TXB_2$ and HHT, and the major lipoxygenase product, HETE. It is apparent that the ratio of cyclooxygenase and lipoxygenase metabolites of the platelets from asthmatic

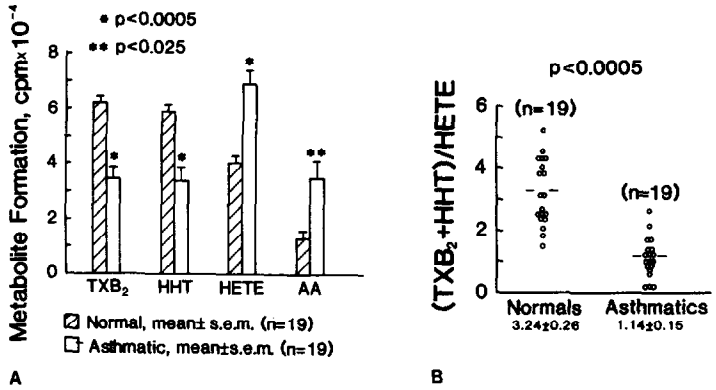


Figure 1: Metabolism of arachidonic acid via cylooxygenase and lipoxygenase pathways in platelets from normal and asthmatic subjects.

Washed platelets were isolated and incubated with [1-$^{14}$C] arachidonate (0.1 μCi/10μM). The metabolites formed and non-metabolized arachidonate were extracted and separated by high pressure liquid chromatography. The major metabolites of cyclooxygenase include thromboxane $B_2$($TXB_2$) and 12-hydroxy-5,8,10-hepatadecatrienoic acid (HHT), whereas the major lipoxygenase product consists mainly of 12-hydroxy-5,8,10,14-eicosatetraenoic acid (12-HETE). (A) The production of cyclooxygenase and lipoxygenase metabolites by the platelets from normal and asthmatic subjects. (B). The ratio of cyclooxygenase and lipoxygenase products in the platelets from these two different groups.

patients was 1.14 ± 0.15 (mean ± S.E.M, n=19) compared with 3.24 ± 0.26 (n=19) for the cells from normal subjects ($p < 0.005$). These results suggest an imbalance of arachidonate metabolism via cyclooxygenase and lipoxygenase pathways in platelets from asthmatic patients.

## Discussion

Arachidonic acid (5,8,11,14-eicosatetraenoic acid) is released from the phospholipids of plasma membranes upon activation of phospholipases (10,11). The arachidonate released in platelets, neutrophils, and lung is metabolized via two enzymatic pathways (12-18). In one, arachidonate is converted by a fatty acid enzyme, cyclooxygenase, into endoperoxides and subsequently into thromboxanes (TXs) and prostaglandins (PGs), retaining three oxygen atoms. In the other pathway, arachidonate is metabolized via a series of lipoxygenases that peroxidize arachidonate at different carbon atoms, e.g. 5,11, and 12, retaining one or two oxygen atoms. Platelets have a C-12 lipoxygenase, neutrophils have C-5, and the lung has C-11 and C-12 lipoxygenases (19). These enzymes catalyze the formation of unstable hydroperoxides (HPETEs) which then degrade to the stable hydroxy acids (HETEs) or are further transformed into leukotrienes (12-18).

The biochemical and pharmacological properties of cyclooxygenase and lipoxygenase metabolites vary greatly. Because of the oxygen constituents, products of cyclooxygenase are polar and are likely to be released immediately after synthesis, whereas those of lipoxygenase are less polar and may accumulate in the cells after synthesis. The two enzymes also differ in substrate requirement for activation. When exogenously added, substrate concentration was in low μM range, cyclooxygenase is a major metabolic pathway. Increasing the substrate concentration to high μM range, lipoxygenase became the predominate pathway (9). Also, the rate of metabolite formation via cyclooxygenase was hyperbolic with rapid formation of products (20), whereas soybean lipoxygenase metabolites exhibited a lag followed by a slow development to maximal concentration (21). Previously, we reported that inhibition of cyclooxygenase enhanced, whereas inhibition of lipoxygenase reduced, the amplitude and the duration of anaphylactic contraction of guinea pig lung strips (22). Furthermore, we observed that products of cyclooxygenase were involved in the early phase, whereas those of lipoxygenase were related to the late phase of arachidonate-induced contraction of guinea pig lung strips (23). Because of the differences in the properties of cyclooxygenase to lipoxygenase products, we postulated that the ratio of cyclooxygenase and lipoxygenase products was important in the maintenance of homeostasis.

The abnormally low ratio of cyclooxygenase to lipoxygenase products in platelets from asthmatic patients, such as we observed in this report, may be important in the pathogenesis of various diseases. The exact causes of the apparent imbalance in arachidonic acid metabolism remain unknown. We postulate that inhibition of lipoxygenase may be capable of restoring the balanced metabolism of arachidonate via cyclooxygenase and lipoxygenase. Thus, a selective inhibitor of lipoxygenase may be efficacious in the future treatment of asthma and other diseases.

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References

1. McFadden, E.R., (1975) J. Allergy Clin. Immunol. 56, 18-26.

2. Brocklehurst, W.E. (1960) J. Physiol. (London) 151, 416-435.

3. Altounyan, R.E. (1964) Thorax 19, 406-415.

4. Parker, C.W. (1979) J. Allergy Clin. Immunol. 63, 1-145.

5. Yen, S.S. and Kreutner, W. (1979) The Pharmacologist 21, 154.

6. Bach, M.K. and Brashler, J.R. (1974) J. Immunol. 113, 2040-2047.

7. Jakschik, B.A., Falkenhein, S. and Parker, C.W. (1977) Proc. Natl. Acad. Sci. U.S.A. 74, 4577-4581.

8. Sun, F.F. (in press) Methods of Enzymology, Academic Press, New York.

9. Yen, S.S. (submitted) Effect of substrate concentration on arachidonic acid metabolism in washed human platelets.

10. Samuelsson, B. (1969) Progr. Biochem. Pharmacol. 5, 109-128.

11. Kunze, H. and Vogt, W. (1971) Ann. N.Y. Acad. Sci. 180, 123-125.

12. Hamberg, M. and Samuelsson, B. (1974) Proc. Natl. Acad. Sci. U.S.A. 71, 3400-3404.

13. Nugteren, D.H. (1975) Biochim. Biophys. Acta 380, 299-307.

14. Borgeat, P., Hamberg, M. and Samuelsson, B. (1976) J. Biol. Chem. 251, 7816-7820 and correction (1977) 252, 8772.

15. Borgeat, P. and Samuelsson, B. 1979) Proc. Natl. Acad. Sci. U.S.A. 76, 3213-3217.

16. Borgeat, P. and Samuelsson, B. (1979) J. Biol. Chem. 254, 2643-2646.

17. Borgeat, P. and Samuelsson, B. (1979) J. Biol. Chem. 254, 7865-7869.

18. Murphy, R.C., Hammarstrom, S. and Samuelsson, B. (1979) Proc. Natl. Acad. Sci. U.S.A. 76, 4275-4279.

19. Moncada, S. and Vane, J.R. (1979) Pharmacol. Rev. 30, 293-331.

20. Smith, W.L. and Lands, W.E.M. (1971) J. Biol. Chem. 246, 6700-6702.

21. Smith, W.L. and Lands, W.E.M. (1972) J. Biol. Chem. 247, 1038-1047.

22. Yen, S.S. and Kreutner, W. (1980) Agents and Actions 10, 274-278.

23. Yen, S.S. (1981) Prostaglandins 22, 183-194.