A PARTICULATE ARACHIDONATE LIPOXYGENASE IN HUMAN BLOOD PLATELETS

Peter P. K. Ho, C. Patricia Walters, and Hugh R. Sullivan

Lilly Research Laboratories, Eli Lilly and Company, P.O. Box 618, Indianapolis, Indiana 46206

Received April 4,1977

SUMMARY

The microsomal fraction of human platelets contains a lipoxygenase activity in addition to the thromboxane-synthesizing activity. The enzymatic activity was stimulated by tryptophan, but inhibited by catecholamine, methemoglobin, and hydroquinones.

Hamberg and Samuelsson (1) found that human platelets converted added arachidonic acid into three metabolites, i.e., 12L-hydroxy-5,8,10,14-eicosatetraenoic acid (HETE), 12L-hydroxy-5,8,10-heptadecatrienoic acid (HHT), and the hemiacetal derivative of 8-(1-hydroxy-3-oxopropyl)-9,12L-dihydroxy-5,10-heptadecadienoic acid (thromboxane B_2 or TXB_2). HETE was formed by the action of a novel lipoxygenase unrelated to the enzymes involved in prostaglandin biosynthesis, whereas, HHT and TXB_2 were formed by pathways involving the fatty acid cyclo-oxygenase in the initial step with prostaglandin G_2 as the intermediate. This conclusion was supported by the observation that preincubation of platelet suspensions with aspirin or indomethacin resulted in a 3-fold stimulation of the release of HETE during thrombin or arachidonic acid-induced aggregation (2). A soluble arachidonate lipoxygenase was subsequently isolated from bovine platelets and was shown to convert eicosapolyenoic acids with at least two cis double bonds at the 8- and 11-positions into L-12-hydroperoxy acids (3).

Recent studies have also shown that HETE was chemotactic in vitro for human polymorphonuclear leukocytes and eosinophils

ISSN 0006-291X

(4,5). These findings have prompted an attempt to isolate a lipoxygenase from human platelets. In this paper we report that this enzyme activity is found in the particulate fraction. The characterization of this particulate enzyme and the identification of the reaction products are described.

EXPERIMENTAL PROCEDURES

Materials

[1-^{14}C]-Arachidonic acid (sp. act. = 55 mCi/mmole) and [1-14C]-8,11,14-eicosatrienoic acid (sp. act. = 57 mCi/mmole) were obtained from New England Nuclear. The out-dated platelet concentrates were provided by the Community Blood Bank of Marion County (IN). The lyophilized platelet microsomal powder was prepared by the previously described procedure (6). Analytical precoated layers of silica gel G glass plates (LQ6D) for thin-layer chromatography (TLC) were products of Quantum Industries, Fairfield, New Jersey. Whatman Extraction Thimbles (10 x 50 mm) were purchased from Sargent-Welch Scientific, Cincinnati, Ohio.

Enzyme Assay

The reaction mixture contained 50 mM Tris-HCl buffer, pH 8.0, 5 mM L-tryptophan, 0.3 mM indomethacin, and 0.1 mg of lyophilized microsomal powder in a total volume of 0.2 ml. The reaction mixture was initiated by the addition of 10 µl of 100 µM ^{14}C-arachidonic acid (sp. act. = 55 mCi/mmole). After 15 min incubation at 37°C, the reaction was terminated by the addition of 10 µl of 1 M citric acid. The products were separated by TLC with the solvent system of ethyl acetate-2, 2,4-trimethylpentane-acetic acid (100:100:1).

Preparation of the Product of Lipoxygenase

A large-scale reaction mixture (200 ml) was prepared with 50 mM Tris-HCl buffer, pH 8.0, 5 mM L-tryptophan, 0.3 mM indomethacin, 1 mg of arachidonic acid (1.5 µC of ^{14}C-arachidonic acid was added as a tracer), 500 mg of lyophilized microsomal powder and the incubation was performed at 37°C for 60 min. The mixture was extracted twice with 1 liter of ethyl acetate. The residue obtained after the evaporation of the ethyl acetate was dissolved in a small amount of methanol (2 ml). The solution was then cooled to -20°C and the precipitate was removed by centrifugation. This procedure was repeated until the solution was clear at -20°C. It was then applied onto two silica gel plates and subjected to TLC.

Zones with an R_f value of 0.41 were collected into Whatman Extraction Thimbles and extracted with methanol (50 ml). After evaporation to dryness, the product was dissolved in 5 ml of chloroform and subjected to silica gel column chromatography (2 x 45 cm) as described previously (6). HETE was eluted with 500 ml of chloroform:methanol (95.5, v/v) and showed a single spot with an R_f = 0.41 in TLC. The methyl ester of this product was then prepared by treatment with diazomethane. The

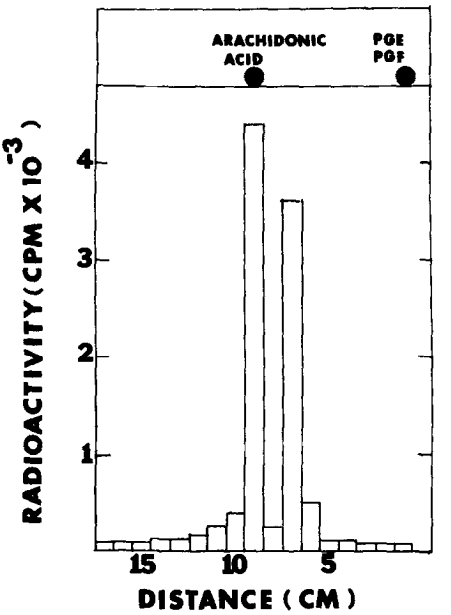


Fig. 1. Thin-layer chromatography of labeled products in reaction mixture. Human platelet microsomes (0.1 mg) were incubated with ^{14}C-arachidonic acid in the presence of L-tryptophan and indomethacin and the reaction was performed as described in METHODS. Solvent system = ethyl acetate-2,2,4-trimethylpentane-acetic acid (100:100:1 v/v/v).

use of column chromatography was necessary in order to remove the colloidal gel particles which filtered through the thimbles during extraction.

Combined Gas Chromatography-Mass Spectrometry

The separation and identification of the trimethylsilyl (Me_3SiO) derivative of HETE methyl ester were accomplished utilizing an LKB-900 combined gas chromatograph-mass spectrometer. Separations were accomplished using a 1.2 M siliconized glass column (1.2 mm i.d.) packed with 1% UV-W98 methylvinyl silicone gum rubber on 800-100 mesh Gas Chrom Q (Applied Science Lab., State Collete, PA). The column temperature was maintained isothermally at 210°, while the flash heater and separator were held at 250°. The carrier gas (helium) flow was 30 ml/min. The ionization potential and trap current were 70 eV and 60 uA, respectively. The entrance and collector slits of the MS were adjusted to 0.1 and 0.3 mm.

The preparation of the trimethylsilyl derivative of the HETE methyl ester was accomplished by reaction of the product with bis(trimethysilyl)trifluroacetamine (Regisil-Regis Chemical Co.) in the presence of triethylamine (1:1).

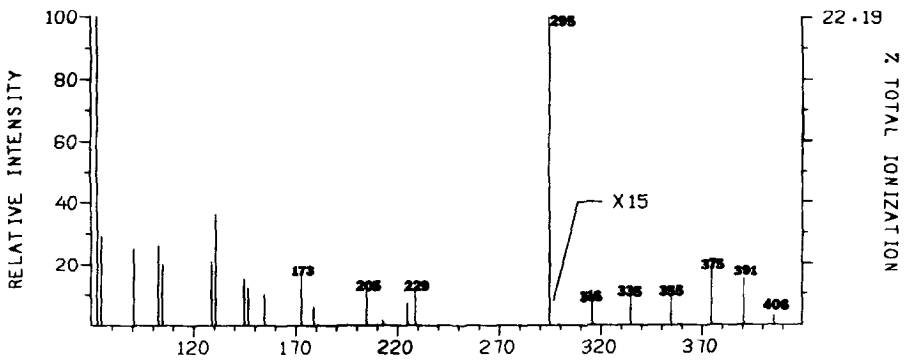


Fig. 2. Mass spectrum recorded on the Me_3SiO derivative of the methyl ester of the product isolated from the reaction mixture by TLC.

RESULTS

Products of the Incubated Reaction Mixture

Since the platelet microsomes also contain the enzyme complex, fatty acid cyclo-oxygenase and thromboxane synthetase (6), indomethacin was included in the reaction mixture to completely inactivate the cyclo-oxygenase. As shown in Fig. 1, the thin-layer radiochromatogram of the incubated reaction mixture contains two mjaor peaks which correspond to arachidonic acid and HETE. The identity of HETE was based on its R_f value, gas-liquid chromatography, and mass spectrometry.

Two peaks were observed with the Me_3SiO derivative of the methyl ester of HETE and the mass spectrum (Fig. 2) of the major peak (90%, retention time = 2.5 min) is identical to that reported by Hamberg and Samuelsson (1). A molecular ion at m/e 406 was obtained and ions of high intensity were seen at m/e 391, 375, 335, 316, 295, 229, 225, 205, and 173.

Cofactor Requirements

Table 1 shows the effect of various components on enzymatic formation of HETE. L-tryptophan stimulated the enzymatic

Table 1. Cofactors Requirements for HETE Formation

Cofactors	HETE (pmoles)
None	134
2 μM Methemoglobin	57
5 mM L-tryptophan	200
1 mM Epinephrine	61
5 mM L-tryptophan + 1 mM Epinephrine	94
1 mM L-tryptophan + 2 μM Methemoglobin	52
1 mM Epinephrine + 2 μM Methemoglobin	16
5 mM L-tryptophan + 2 μM Methemoglobin + 1 mM GSH	26

The reaction mixture contained 5 μM ^{14}C-arachidonic acid, 0.3 mM indomethacin, and 0.1 mg of lyophilized microsomes in 0.2 ml of 50 mM Tris buffer, pH 8.0. After incubation for 15 min at 37°C, the HETE was assayed as described in METHODS.

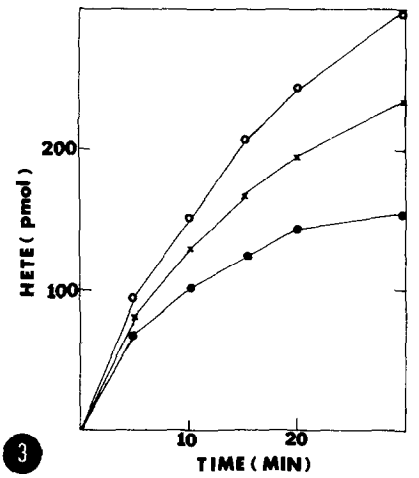
reaction, while epinephrine and methemoglobin inhibited the reaction. Reduced glutathione had no effect on HETE synthesis either alone or in combination with methemoglobin, L-tryptophan, or epinephrine. The rates of HETE formation were maximal at pH = 6.0 - 9.0.

Time Course of HETE Formation

At 10 μM arachidonic acid, the formation of HETE was only linear for the first 15 min using L-tryptophan as a cofactor. The degree of HETE synthesis was also dependent upon the amount of microsomal protein added (Fig. 3).

Substrate Specificity

^{14}C-8,11,14-Eicosatrienoic acid at 5 μM (sp. act. = 57 μCi/mole) was substituted for arachidonic acid in the reaction mixture and product formation was examined by TLC. A product



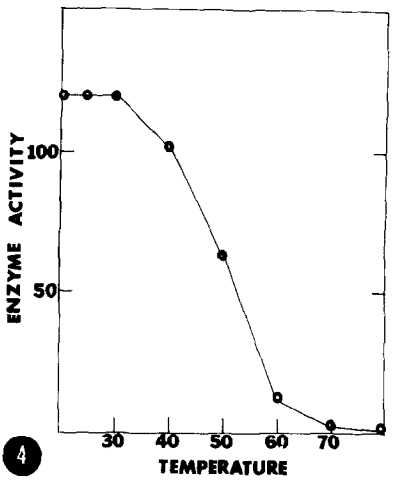


Fig. 3. Time course of HETE formation. The assays were performed under standard assay conditions with 10 μM arachidonic acid and various amounts of microsomal powder per 0.2 ml of reaction mixture.

O——O 0.2 mg
x——x 0.1 mg
●——● 0.05 mg

Fig. 4. Effect of temperature on the stability of the lipoxygenase. The assays were performed under standard assay conditions, and the lipoxygenase activity was expressed as pmoles of HETE formed per 15 min.

similar in R_f value to hydroxy fatty acids was found. The rate of reaction using ^{14}C-8,11,14-eicosatrienoic acid as the substrate, however, was approximately 40% of that for arachidonic acid.

Enzyme Stability and Inhibitors

The lipoxygenase could not be released from the membrane component by the technique of freezing and thawing. Although the enzyme activity was stable to the treatment of freezing and thawing, it lost most of its activity in the presence of 4 M KCl. The heat stability of lipoxygenase is shown in Fig. 4. The lipoxygenase was incubated at various temperatures

for 5 min and then assayed at 37°C. The enzyme is very unstable at 60° and lost 50% activity at 50°C.

Certain transition metal ions (e.g., Cu^{2+}) and hydrogen donors (e.g., hydroquinone) were inhibitors of lipoxygenase with a I_{50} similar to those found in the thromboxane-synthesizing systems (6,7).

DISCUSSION

The present paper demonstrates the presence of a lipo-oxygenase in the membrane fraction of human platelets. This finding was based on an earlier observation in which compounds with R_f values between the methyl esters of arachidonic acid and thromboxane B_2 were obtained in the reaction mixture incubated with human platelet microsomes (8). We, therefore, added an excess amount of indomethacin, a potent inhibitor of fatty acid cyclo-oxygenase, to the reaction mixture, and HETE was isolated as the only product synthesized by the microsomal preparation.

In separate experiments with freshly prepared human platelets we could not detect any enzymatic activity in the supernatant fraction. It is not clear whether the discrepancy between our results and that of Nugteren (3) is due to species difference or methods in breaking up the platelets. Nevertheless, the enzyme cannot be released from the membrane component by freezing and thawing, a method employed by Nugteren to obtain the soluble lipoxygenase from bovine platelets.

The effects of tryptophan as well as the rapid inactivation and inhibition by hydroquinones are similar to those observed with the cyclo-oxygenase (6). However, the fact that epinephrine is an inhibitor rather than an activator suggests that mechanisms of lipoxygenation and cyclo-oxygenation are different.

More recently, Borgeat et al. (9) reported that addition of arachidonic acid and homo-γ-linolenic acid to a suspension of rabbit peritoneal neutrophils led to the synthesis of 5-L-hydroxy-6,8,11,14-eicosatetraenoic acid and 8-L-hydroxy-9,11,14-eicosatrienoic acid, respectively. We have also detected a lipoxygenase activity as well as the thromboxane-synthesizing activity in the microsomal preparation of human polymorphonuclear leukocytes (P. K. Ho and C. P. Walters, unpublished results). The characterization of this lipoxygenase is now in progress.

REFERENCES

1. Hamberg, M. and Samuelsson, B. (1974) Proc. Nat. Acad. Sci. (USA) 71, 3400-3404.

2. Hamberg, M., Svensson, J., and Samuelsson, B. (1974) Proc. Nat. Acad. Sci. (USA) 71, 3824-3828.

3. Nugteren, D. H. (1975) Biochim. Biophs. Acta 380, 299-307.

4. Turner, S. R., Tainer, J. A., and Lynn, W. S. (1975) Nature, 257, 680-681.

5. Goetzl, E. J., Woods, J. M., and Gorman, R. R. (1977) J. Clin. Invest. 59, 179-183.

6. Ho, P. P. K., Walters, C. P., and Sullivan, H. R. (1976) Prostaglandins 12, 951-970.

7. Ho, P. P. K., and Walters, C. P. (1976) Res. Comm. in Chem. Pharmacol. and Pathol. 15, 673-687.

8. Ho, P. P. K., Walters, C. P., and Herrmann, R. G. (1976) Biochem. Biophys. Res. Comm. 69, 218-224.

9. Borgeat, P., Hamberg, M., and Samuelsson, B. (1976) J. Biol. Chem. 251, 7816-7820.