Preferential utilization of endogenous arachidonate by cyclo-oxygenase in incubations of human platelets

L. Sautebin, D. Caruso, G. Galli* and R. Paoletti

Institute of Pharmacology and Pharmacognosy, University of Milan, Via A. del Sarto 21, Milan, Italy

Received 18 April 1983

Thromboxane B_2 (TXB_2) and 12-hydroxy-5,8,10,14-eicosatetraenoic acid (12-HETE) formed from the endogenous and exogenous arachidonate during human platelet incubation, was evaluated by selected ion monitoring (SIM). TXB_2 formed from endogenous substrate accounted for about one third of the total, whereas the great part of 12-HETE derived from exogenous arachidonate. These data indicate that under the tested conditions the pool of arachidonate that acts as substrate for cyclo-oxygenase is different from the pool that acts as substrate for lipoxygenase and that the arachidonate released from phospholipids is preferentially utilized by cyclo-oxygenase.

TXB_2 *12-HETE* *Arachidonic acid* *Cyclo-oxygenase* *Lipoxygenase* *Human platelets*

1. INTRODUCTION

The metabolic pathway of arachidonic acid in human platelets involves the oxygenation of the acid released from phospholipids, catalyzed by two enzymes, fatty acid cyclo-oxygenase and lipoxygenase. The cyclo-oxygenase pathway generates thromboxane A_2 (TXA_2) and 12-hydroxy-5,8,10-heptadecatrienoic acid (HHT). The lipoxygenase pathway generates 12-hydroperoxy-5,8,10,14-eicosatetraenoic acid (12-HPETE) which is reduced to the corresponding 12-hydroxy acid (12-HETE) by a peroxidase [1].

While TXA_2 has been shown to induce aggregation, the biological functions of the lipoxygenase metabolites in platelets are not fully understood.

12-HPETE and 12-HETE appear to be chemotactic agents for human polymorphonuclear leukocytes [2,3]. It has also been suggested that the lipoxygenase products in human platelets may act as regulators of the arachidonate metabolism because 12-HPETE was shown to stimulate in vitro its own production by increasing lipoxygenase activity [4]. In addition, it has been reported [5] that the hydroperoxy acid inhibits the TXA_2 synthesis.

In view of the possible interference of arachidonate metabolites produced via the lipoxygenase pathway on the cyclo-oxygenase pathway, we investigated the competition of cyclo-oxygenase and lipoxygenase for the common substrate, the arachidonate released from phospholipids.

2. MATERIALS AND METHODS

2.1. *Materials*

[1-^{14}C]Arachidonic acid (54 mCi/mmol) was purchased from Amersham (International plc). *N,O*-bis-(trimethylsilyl)-trifluoroacetamide (BSTFA) and pyridine were from Carlo Erba

* To whom correspondence should be addressed

Abbreviations: TXA_2, thromboxane A_2; TXB_2, thromboxane B_2; 12-HETE, 12-hydroxy-5,8,10,14-eicosatetraenoic acid; 12-HPETE, 12-hydroperoxy-5,8,10,14-eicosatetraenoic acid; HHT, 12-hydroxy-5,8,10-heptadecatrienoic; PG_s, prostaglandins; PRP, platelet-rich plasma; SIM, selected ion monitoring; RIA, radioimmunoassay; TLC, thin-layer chromatography; RGLC, radiogaschromatography; BSTFA, *N,O*-bis-(trimethylsilyl)-trifluoroacetamide

Published by Elsevier Science Publishers B.V.
00145793/83/$3.00

(Milan); Biosil-A 100–200 mesh from BioRad Laboratories (Richmond CA); thrombin from Topostasin, Roche. Other reagents and solvents were all of analytical grade.

2.2. *Platelet preparation*

Blood was collected in 0.15 volumes of an anticoagulant dextrose citrate from healthy donors who had not taken aspirin or any other antiinflammatory drug during the week before collection. After centrifugation at 200 × *g* for 15 min platelet-rich plasma (PRP) was obtained and aliquots were subjected to platelet counting by contrast phase microscopy. Platelets recovered by centrifugation of the PRP at 800 × *g* for 15 min were suspended in 1 vol. Tris–saline buffer [6] in order to obtain a final concentraiton of 10^9 platelets/ml.

2.3. *Platelet incubation*

Aliquots of the platelet suspension were preincubated for 2 min at 37°C and then incubated for 5 min at the same temperature with various concentrations of [1-^{14}C]arachidonic acid (1,5,10,100 nmol/10^9 cells = 1–100 μM) dissolved in ethanol. In some experiments thrombin (5 units/ml) and [1-^{14}C]arachidonic acid (5 μM) were added simultaneously to aliquots of the preincubated platelet suspension and the mixture was incubated for 5 min at 37°C.

Incubations were stopped by the addition of 20 vol. of $CHCl_3$:CH_3OH (2:1; v/v). 12-[$^2H_8$]HETE (156 pmol/ml) and [$^2H_8$]TXB_2 (280 pmol/ml) were also added to the samples before any other manipulation. Blank samples incubated in the absence of any added substrate were also analyzed.

2.4. *Analysis of the distribution of radioactivity in lipid extracts*

Total lipid extracts were counted for their radioactivity and then fractionated by column chromatography (0.5 g Biosil-A). Fraction A (hexane:diethyl ether; 85:15; v/v; 15 ml) contained arachidonate; 12-HETE and HHT were recovered in fraction B ($CHCl_3$; 15 ml); TXB_2 and PG_s in fraction C ($CHCl_3$:CH_3OH; 95:5; v/v; 15 ml) and phospholipids in fraction D (CH_3OH; 9 ml, CH_3OH:H_2O; 99:1; v/v; 6 ml). Fractions were counted for radioactivity and aliquots were subjected to thin-layer chromatography (TLC) ($CHCl_3$:CH_3OH:CH_3COOH:H_2O; 90:8:1:0.8; by vol.). The distribution of radioactivity on the plates was analyzed using a radiochromatoscanner (Packard model 7201). In order to check the identity of the radioactive compounds aliquots of the fraction were also analyzed by radiogaschromatography (RGLC) using a Carlo Erba gaschromatograph model GV connected with a Nuclear Chicago radioactivity counter model 4998 after esterification with an ethereal solution of diazomethane and silylation with a mixture of BSTFA and pyridine (4:1; v/v). Analytical conditions were as described below.

2.5. *Evaluation of TXB_2 and 12-HETE formed from endogenous and exogenous arachidonate*

Aliquots of the esterified residue of fractions B and C were dissolved in BSTFA:pyridine (4:1, v/v) and injected into a Varian Mat 112 S gaschromatograph-mass spectrometer. An 1 m × 3 mm glass silanized column packed with 1% SE-30 on Gaschrom Q was used; oven, molecular separator and ion source were at 260, 300 and 290°C, respectively; electron energy 70 eV; electron multiplier 3.5 kV; helium flow rate 15 ml/min; resolution 300. For the analysis of TXB_2 the spectrometer was focused on ions at m/e 256 and 258 for the detection of unlabelled and ^{14}C-labelled species and at m/e 260 for the deuterated internal standard [7]. For quantitation of TXB_2 a linearity curve obtained from the analysis of standard samples containing deuterated and unlabelled TXB_2 was used.

The curve showed a linear increase ($y = 0.83x \pm 0.16$; $r^2 = 0.999$) of the ratio of the intensities of the peaks in the traces of the ions at m/e 256 and 260 (y) with the increase of the ratio between the amount of unlabelled TXB_2 and the deuterated compound (x). A constant value (0.15) was instead observed for the ratio of the intensities of ions 258/256 which derives from the natural isotopic abundance. Total amount of TXB_2 (nmol/10^9 cells) in the samples was calculated from the sum of the intensities of the peaks at the retention time of the TXB_2 derivative (5 min 20 s) in the trace of the ions at m/e 256, originating from endogenous substrate and in the trace of the ion at m/e 258 originating from TXB_2 containing one atom of ^{14}C per molecule:

TXB_2 (nmol/10^9 cells) =

$$1/a \times [^2H_8]TXB_2 \times I_{256} + I_{258}/I_{256}$$

where:

I = intensity of the peak at the retention time of TXB_2;

a = slope of the standard curve;

$[^2H_8]TXB_2$ = nmol/10^9 cells of deuterated standard added after incubation.

^{14}C-Enrichment was determined as the ratio of the intensity of ion at m/e 258 to the sum of intensities of ions at m/e 258 and 256. From this fraction the molar radioactivity (m.r.) of TXB_2 isolated from the lipid extract after incubation was also calculated as follows:

m.r. (dpm/nmol) = 64 nCi/nmol ×

$$2200 \text{ dpm/nCi} \times I_{258}/I_{258} + I_{256}$$

The intensity of the ion at 258 (I_{258}) was corrected for the contribution to the signal of the natural isotope determined in the analysis of the derivative of standard TXB_2. In some experiments the addition of deuterated TXB_2 was avoided and molar radioactivity was evaluated from the measurement of the radioactivity associated with the compound after separation by TLC, as previously described, and the mass of the compound by radioimmunoassay (RIA). Levels and isotopic enrichment of 12-HETE formed during platelet incubation were determined in fraction B as described for TXB_2 from the intensities of the peaks in the traces of the ions at 295, 297 and 301 [7] corresponding to unlabelled, labelled and deuterated species, at the retention time of the 12-HETE derivative (3 min 40 s).

3. RESULTS AND DISCUSSION

Most of the arachidonate is present in tissues as an acyl-group in phospholipids and the compound is released in the free form and acts as the substrate for cyclo-oxygenase [8] when synthesis of prostanoids is required. In view of the finding that exogenous arachidonate added to tissue homogenate or cultured cells [9,10] is converted to prostanoids less efficiently than the endogenous substrate, a direct transfer of the acyl-group of phospholipids to the enzyme catalyzing its transformation without previous mixing with the free form present in the tissue could be hypothesized.

Moreover lipoxygenase, catalyzing the formation of 12-HETE is thought to be a cytoplasmic enzyme [11] and could therefore be more accessible to the pool of free arachidonate than the membrane-bound cyclo-oxygenase. In order to test this hypothesis we have studied the catabolism of exogenous and endogenous arachidonate in human platelets both to TXB_2 and to 12-HETE.

For this purpose using [1-^{14}C]arachidonate with high molar radioactivity as the exogenous substrate the ^{14}C-enrichment in TXB_2 and 12-HETE formed during platelet incubation was evaluated by the SIM. This approach allows to differentiate the contribution of the endogenous and exogenous substrates in the formation of the metabolites. Because 64 mCi/mmol corresponds to the molar radioactivity of a compound containing one atom of ^{14}C per molecule with 100% isotopic abundance, commercial [1-^{14}C]arachidonic acid with 50–55 mCi/mmol molar radioactivity has an isotopic enrichment of 78–85%.

The isotopic abundance of [1-^{14}C]arachidonic acid used in the described experiments was determined by SIM analysis of its methyl ester from the ratio of the intensities of the peaks on the traces of ions at m/e 322 and 320, corresponding to the M^+ of 1-^{14}C-labelled and unlabelled species, respectively. Ratio was found to be 5.4, in good agreement with the theoretical value of 5.2 calculated on the basis of the 54 mCi/mmol molar radioactivity of the compound. In unlabelled arachidonic methyl ester the ratio was found to be 0.07. The latter value should be found for TXB_2 and 12-HETE originating from the endogenous precursor whereas an increase of the isotopic enrichment should be observed if exogenous arachidonate participates to the formation of these metabolites too.

The amount and the degree of isotopic enrichment of TXB_2 formed in incubations of human platelets with various concentrations of [1-^{14}C]-arachidonate is reported in table 1. Cyclo-oxygenase activity measured as TXB_2 formation increased with the concentration of the labelled substrate and seemed to reach the saturation gradually [12].

The ratio between labelled and unlabelled species of TXB_2 decreased from 5 μM to 10 μM arachidonate and then remained almost constant. Molar radioactivity determined by TLC and RIA

Table 1

TXB_2 formed from [^{12}C] and [^{14}C]arachidonate in incubations of human platelets

Substrate [1-^{14}C]AA (54 nCi/nmol) μM	TXB_2[a] nmol/10^9 cells by RIA	Ratio of intensities[b] m/e 258 (from [^{14}C]AA) m/e 256 (from [^{12}C]AA)	Molar radioactivity dpm/nmol 10^{-3} by RIA	by SIM
1	0.05	–	–	–
5	0.43	4.6	115	120
10	1.30	1.3	80	80
50	3.53	1.7	77	89
100	4.60	1.8	81	90
5 + Thrombin (5 units/ml)	8.50	–	5	–

[a] Values are subtracted from the mean TXB_2 concentration (0.01) nmol/10^9 cells obtained in blank samples in which platelets were incubated in the absence of added arachidonate

[b] Mean ratios in blank samples (0.15) were subtracted

Values are means of 3 experiments with different platelet preparations. Each assay was made in triplicate

showed the same trend. These data indicate an increasing grade of dilution of the added substrate with endogenous non-labelled arachidonate released from phospholipids. It appears, therefore, that exogenous arachidonate induces the release of the acid esterified in phospholipids in a concentration-dependent manner.

When 5 μM labelled arachidonate was incubated in the presence of thrombin, the obtained TXB_2 did not contain detectable amounts of labelled species as expected from the massive release of arachidonate induced by thrombin. Nevertheless, the amount of TXB_2 was only twice that obtained in incubations carried out with 100 μM exogenous arachidonate, which is in agreement with previous data showing the saturation of cyclo-oxygenase at 100 μM substrate concentration [12].

A different picture was obtained for 12-HETE (table 2). Its formation increased linearly (r^2 = 0.998) with the labelled substrate concentration as expected from results indicating that saturation of lipoxygenase occurs at a higher concentration of arachidonate [12]. The isotopic enrichment of 12-HETE also increased with the substrate concentration being almost 10-times that of TXB_2 at arachidonate concentrations higher than 10 μM. This suggests that arachidonate released from phospholipids is poorly utilizied for the production of 12-HETE by lipoxygenase. The experiments in the presence of thrombin confirm this conclusion: the amount of 12-HETE formed was only 30% higher than that observed at 10 μM exogenous arachidonate despite the release of large amounts of substrate from phospholipids described above.

An unexpected result in the analysis of the isotopic enrichment of 12-HETE was the absolute

Table 2

12-HETE formed from [^{12}C] and [^{14}C]arachidonate in incubations of human platelets

Substrate [1-^{14}C]AA (54 nCi/nmol) (μM)	12-HETE nmol/10^9 cells by SIM	Ratio of intensities[a] m/e 297 (from [^{14}C]AA) / m/e 295 (from [^{12}C]AA)
1	0.09	1.7
5	0.74	4.9
10	1.90	10.8
50	7.10	12.0
100	13.40	15.0
5 + thrombin (5 units/ml)	2.90	0.2

[a] Mean ratios (0.1) in blank samples obtained in incubations of human platelets without added arachidonate were subtracted

Values are means of 3 experiments with different platelet preparations. Each assay was made in triplicate

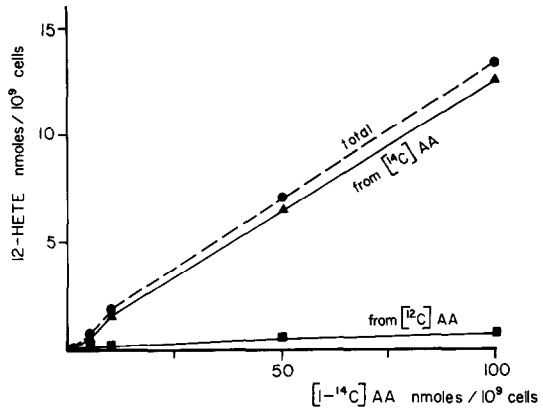

Fig.1. 12-HETE formed from [^{12}C]- and [^{14}C]arachidonate in incubations of human platelets.

value of the ratio of intensities of ions at m/e 297 and 295, which exceeded that of the added substrate. Since arachidonate is likely to be involved in metabolic pathways other than those catalyzed by cyclo-oxygenase and lipoxygenase, the isotopic enrichment of the substrate giving rise to 12-HETE may depend upon an isotopic effect in any unidentified fast reaction utilizing free arachidonate.

Fig.1 and 2 show the contribution of labelled and unlabelled substrate in the formation of the total amount of TXB_2 and 12-HETE after incubation of human platelets, with the result that the TXB_2 formed from the endogenous substrate accounts for about 1/3 of the total, whereas

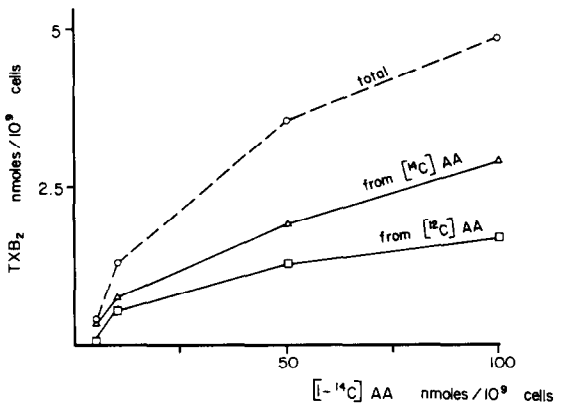

Fig.2. TXB_2 formed from [^{12}C]- and [^{14}C]arachidonate in incubations of human platelets.

12-HETE derives almost quantitatively from exogenous arachidonate.

In conclusion these experiments indicate that under the tested conditions the pool of arachidonate that acts as a substrate for the cyclo-oxygenase differs from that accessible to the lipoxygenase. This corresponds well with data presented in [13] showing that platelet lipoxygenase can utilize arachidonic acid derived from plasma phospholipids whereas platelet cyclo-oxygenase preferentially utilizes that derived from platelet phospholipids.

These findings may suggest that the enzyme and phospholipids from which the substrate is released are confined to the same or to closely related membranes.

ACKNOWLEDGEMENTS

The excellent technical assistance of Mrs Maria Cancellieri and Mr Amelio Toia is appreciated.

REFERENCES

[1] Hamberg, M. and Samuelsson, B. (1974) Proc. Natl. Acad. Sci. USA 71, 3400–3404.
[2] Turner, S.R., Teiner, J.A. and Lynn, W.S. (1976) Nature 257, 680–681.
[3] Goetzl, E.J., Hill, H.R. and Gorman, R.R. (1980) Prostaglandins 19, 71–85.
[4] Siegel, M.I., Mc Connel, R.T., Abrahams, S.L., Porter, N.A. and Cuatrecasas, P. (1979) Biochem. Biophys. Res. Commun. 89, 1273–1280.
[5] Hammarstrom, S. and Falardeau, P. (1977) Proc. Natl. Acad. Sci. USA 74, 3691–3695.
[6] Blackwell, G.J., Duncombe, W.G., Flower, R.J., Parson, M.F. and Vane, J.R. (1977) Br. J. Pharmacol. 59, 353–366.
[7] Hamberg, M., Svensson, J. and Samuelsson, B. (1974) Proc. Natl. Acad. Sci. USA 10, 3824–3828.
[8] Vogt, W. (1978) in: Advances in Prostaglandins and Thromboxane Research (Samuelsson, B. and Paoletti, R. eds) vol.3, pp.89–95, Raven Press, New York.
[9] Wolfe, L.S., Pappius, H.M. and Marion, J. (1976) in: Advances in Prostaglandins and Thromboxane Research (Samuelsson, B. and Paoletti, R. eds) vol.1, pp.345–355, Raven Press, New York.

[10] Coene, M.C., Van Hove, C., Claeys, M. and Herman, G.A. (1981) Biochim. Biophys. Acta 710, 437–445.
[11] Nugteren, D.H. (1975) Biochim. Biophys. Acta 380, 299–307.
[12] Sun, F.F., McGuire, J.C. and Metzler, C.M. (1981) in: Progress in Lipid Research (Holman, R.T. ed) vol.20, pp.275–278, Pergamon Press, New York.
[13] Hwang, D.H. (1982) 5th International Conference on Prostaglandins, abstract book, p.188.