TRANSFER OF ARACHIDONIC ACID TO HUMAN PLATELET PLASMALOGEN IN RESPONSE TO THROMBIN

Susan Rittenhouse-Simmons, Francis A. Russell, and Daniel Deykin*

Boston VA Hospital and Departments of Medicine and Biochemistry, Boston University School of Medicine, Boston, Mass. 02130

Received March 16,1976

SUMMARY. We have examined the turnover of phosphatide [^{3}H]-arachidonic acid in human platelets exposed to thrombin and have found: a) a decrease in the radioactivity of phosphatidyl choline and phosphatidyl inositol b) no alteration in the radioactivity of phosphatidyl serine or diacyl phosphatidyl ethanolamine and c) a marked increase in the specific activity of plasmalogen phosphatidyl ethanolamine, attributable to labelled fatty acid in ester linkage. The increase was not depressed by the presence of unlabelled arachidonic acid in the medium when the platelets were exposed to thrombin. Experiments with the non-penetrating reagent trinitrobenzene sulfonic acid revealed a) no preferential exposure of plasmalogen phosphatidyl ethanolamine to the medium during incubation with thrombin, and b) an inhibitory effect of the reagent on the release of arachidonic acid and transfer to plasmalogen. We suggest that plasmalogen phosphatidyl ethanolamine is a receptor for arachidonic acid transferred from phosphatidyl choline and/or phosphatidyl inositol within the membrane and that the enzyme(s) involved in this transfer is (are) accessible at the surface of the platelet.

Recently, both the thrombin-induced release of arachidonic acid from human platelet phospholipids (1,2,3) and the subsequent production of labile arachidonic acid metabolites (4,5) have been of particular interest to those concerned with platelet aggregation and hemostasis. We report some observations resulting from experiments with gel-filtered human platelets which have been preincubated with [3H]-arachidonic acid in order to label the phosphatides, and subsequently exposed to thrombin. In confirmation of the findings of investigators working with washed human platelets (1,2,3), we note most of the release of labelled material to be from PC and PI. However, we have also found a phospholipid component which undergoes a dramatic increase in specific activity in response to thrombin: plasmalogen phosphatidyl ethanolamine. Previous failure to detect

* Supported by an award from the Veterans Administration [MRIS #5231822]
Abbreviations: DPE=diacyl phosphatidyl ethanolamine; PPE=plasmalogen phosphatidyl ethanolamine; TNBS=2, 4, 6-trinitrobenzene sulfonic acid; [3H]-GFP=gel filtered platelets loaded with [^{3}H]-arachidonic acid; TNB-PPE, TNB-DPE=TNB derivatives of PPE and DPE; PC=phosphatidyl choline; PI=phosphatidyl inositol; PS=phosphatidyl serine; SM=sphingomyelin; PE=phosphatidyl ethanolamine.

such an increase may be attributable to the known lability of plasmalogen in the usual silica gel systems (6) and subsequent co-chromatography of the lysophosphatide product with other highly labelled phospholipids.

We have also made use of the non-penetrating reagent TNBS (7,8) to label plasmalogen and diacyl phosphatidyl ethanolamine at the surface of our platelets. We observe that neither the diacyl nor the plasmalogen PE reacts preferentially with TNBS after exposure to thrombin. There is thus no disproportionate exposure of either of these phospholipids upon thrombin treatment. However, TNBS, applied after thrombin, does inhibit the release of radio-label from PC and PI, and, more conspicuously, the transfer of arachidonic acid to PPE, without preventing aggregation.

METHODS. Blood was obtained from normal human donors and processed as described elsewhere (9). Bovine serum albumin was delipidated according to the procedure of Chen (10). [5,6,8,9,11,12,14,15-^{3}H]-arachidonic acid in hexane was obtained from New England Nuclear (Boston) and its purity (> 98%) checked by adsorption thin layer chromatography. The hexane solution, containing 0.25 mCi, 72Ci/mmole [^{3}H]-arachidonic acid was evaporated under N_2 flow, after which 40 ml of 10% (delipidated) bovine serum albumin, pH 6.5, were added. Aliquots of this solution were stored frozen at -70°C.

Portions of platelet rich plasma (5.5 ml) were incubated with 1.0 ml [^{3}H]-arachidonic acid-albumin at 37°C for 15 min., after which time maximum uptake had occurred (~50%). The incubated platelet rich plasma was then passed through a 0.5 ml top layer of 2mM EDTA on a Sepharose 2B column (9) at pH 6.8, which removed external [^{3}H]-arachidonic acid and plasma proteins. Incorporation of [^{3}H]-arachidonic acid was measured per 10^9 cells.

In Experiment I, [^{3}H]-GFP were incubated with 5mM$CaCl_2$ and buffer or highly purified bovine thrombin (IU.ml^{-1}, kindly provided by Dr. D. Waugh of MIT) for 15 minutes at 37°C. A solution of EDTA, pH 6.5, was added after this at a final concentration of 5mM, and the cells were sedimented by centrifugation. Release of radio-label to the supernatant was determined and pelleted cells were drained.

In Experiment II [^{3}H]-GFP were incubated as above with 0.93% delipidated bovine serum albumin preincubated with unlabelled arachidonic acid (Sigma) to give a final concentration of 10^{-6} or 10^{-7} M arachidonic acid, and treated as above.

In Experiment III, a solution of TNBS (pH 6.8) was made up just before use and added 1 min or 3 min after the addition of buffer or thrombin as described in Experiment I. TNBS, final concentration 3mM, was incubated with the cells for 15 min. The remaining procedure was as described above.

The lipids of pelleted cells were extracted into chloroform/methanol (2:1) for three hours and the upper aqueous layer, formed after shaking the extract with 2/10 volume of 0.73% NaCl, was removed. Lipids were chromatographed in two directions on Whatman SG81 paper as described by Wuthier (11), using solvent systems II and V (11) at 19°C. After chromatography had been completed in direction 1, the lipid track was sprayed with 5mM $HgCl_2$, as described by Cohen and Derksen (12). Papers were dried and developed in the second direction. Lipids were stained with Rhodamine 6G (11). The radioactivity of the spots was counted in Aquasol/H_2O in a Packard Scintillation Counter and the phosphorus of duplicates was quantitated (11). Using this system it was possible to resolve not only SM, PC, PS, PI and PE, but also lysoPPE breakdown product and the TNB derivatives of DPE and lysoPPE.

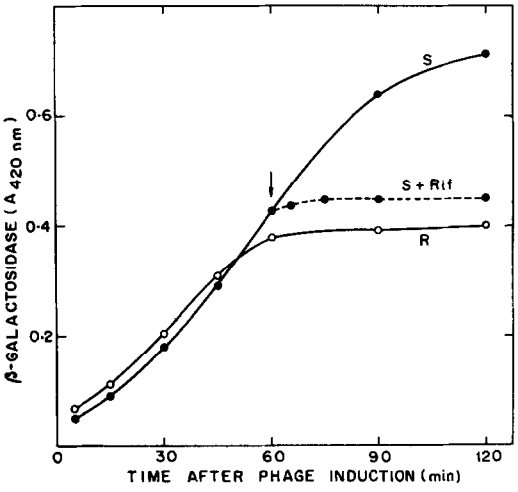

Fig. 1.

Synthesis of β-galactosidase in induced R and S cells. Cultures containing 1 x 10^9 cells/ml growing in M9-glycerol medium were heat induced at 42° and shifted to 37° shaker bath. Thiomethyl-β-D-galactoside was added to a final concentration of 3 x 10^{-3}M. At different periods, 1 ml aliquots were withdrawn, shaken with 0.02 ml of toluene for 20 min and then incubated with 0.2 ml of 0.4% ONPG for 30 min more. The reaction was stopped by addition of sodium carbonate (0.25 M final) and the color was read in Beckman DU spectrophotometer at 420 nm. Rifampicin was added (final conc. 50 μg/ml) to an aliquot of S cells at the time indicated by the arrow. -O-, R cells; -●-, S cells; --●--, S cells plus rifampicin.

cells required continued transcription of host DNA was shown by rifampicin sensitivity of the process. The addition of rifampicin to the S cells at any time after induction stopped further increase of β-galactosidase activity within minutes (Fig. 1).

Phage induced S cells have been found to be more fragile than R cells with respect to treatment with detergents and freezing-thawing (12). On the other hand, release of 260 nm UV-absorbing material was more in R cells than S cells. This indicated a change in the permeability barrier in R cells

TABLE I

CHANGE IN THE CONTENT OF [^{3}H]-LABELLED ARACHIDONIC ACID IN VARIOUS PHOSPHOLIPID FRACTIONS OF PLATELETS INCUBATED WITH THROMBIN*

Additions to Medium	PC	PI	PS	lysoPPE**	DPE
None					
(average of 6 experiments)	0.89±0.05 (65.2)	0.64±0.13 (7.9)	1.16±0.15(11.5)	3.9±0.77(1.4)	1.2±0.24 (11.4)
Cold Arachidonic acid, 10^{-7}M	0.84 (65.9)	0.51 (9.3)	1.29 (10.9)	4.8 (2.0)	1.22 (10.2)
Cold Arachidonic acid, 10^{-6}M	0.81 (66.2)	0.56 (8.2)	1.41 (11.4)	6.0 (1.0)	1.49 (11.9)
TNBS, 3 x 10^{-3}M, 1 min.	0.96 (65.1)	1.0 (7.3)	1.04 (11.1)	1.2 (0.91)	1.0 (10.6)
TNBS 3 x 10^{-3}M, 3 min.	0.96 (66.5)	0.97 (6.2)	1.02 (10.8)	2.5 (0.68)	0.89 (12.3)

*Values are expressed as % of ^{3}H activity in the phospholipid after thrombin treatment÷ % of ^{3}H activity in the phospholipid for controls (-thrombin). Values in parentheses represent the % of ^{3}H activity in control (-thrombin) phospholipids.

**PPE was detected on chromatography paper as lysoPPE, following hydrolysis with $HgCl_2$ as described in text.

TABLE II

CHANGES IN SPECIFIC ACTIVITY OF PLASMALOGEN AND DIACYL PHOSPHATIDYL ETHANOLAMINE*

	lyso PPE			DPE		
Additions	Control	+Thrombin	$\frac{+\text{Thrombin}}{\text{Control}}$	Control	+Thrombin	$\frac{+\text{Thrombin}}{\text{Control}}$
None						
(average of 4 experiments)	4.10 ± 0.70	16.2 ± 2.7	4.17 ± 0.80	34.3 ± 3.8	35.4 ± 3.9	1.04 ± 0.11
TNBS, 1 min.	4.69	5.84	1.25	39.5	40.8	1.03
TNBS, 3 min.	4.36	10.5	2.4	38.3	32.3	0.84

*Specific activities are expressed as dpm $^{3}H/\mu g$ P x 10^{-3}. The plasmalogen activity was detected in the lysoPPE derivative (see text). The extent of reaction of PE with TNBS was 8% for controls and 15% for platelets exposed to thrombin.

in control platelets. No reaction with PS occurred. The proportion of lyso-PPE/DPE phosphorus remained constant (0.46 $\pm$ 0.02) for control platelets, thrombin-treated platelets, and control and thrombin-treated platelets exposed to TNBS. In addition, the ratios of radioactivity of the TNB derivatives (lyso-PPE/DPE) were found to be identical to those of residual (unreacted with TNBS) lysoPPE/DPE. When TNBS was added three minutes after thrombin and a significant increase in radioactive labelling of PPE was observed, a similar increase was also observed in the TNB derivative of PPE. The specific activities of PPE and TNB-PPE were the same. This result shows that TNBS produced a uniform effect on the transfer of radio-label, rather than a specific effect on the plasmalogen fraction which had reacted with TNBS. It seems likely that TNBS is exerting its inhibitory action by reacting with an exposed protein (TNBS has a general reactivity with amines).

To our knowledge, this is the first observation of a transfer of arachidonic acid to a phospholipid species following the exposure of platelets to thrombin. It is not yet known which phospholipids serve as a direct source for arachidonic acid which is converted by fatty acid and cyclo-oxygenase and/or lipoxygenase (4,5) to biologically active metabolites. Conceivably, PPE could be such a source.

References

1. Russell, F.A., and Deykin, D. (1976). Amer. J. Hematol., in press.

2. Bills, T.K., Smith, J.B., and Silver, M.J. (1976) Biochem. Biophys. Acta. 424, 303-314.

3. Schoene, N.W., and Iacono, J.M. (1975) Fed. Proc. 34, 257.

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

5. Hamberg, M., Svensson, J., and Samuelsson, B. (1975) Proc. Nat. Acad. Sci. US 72, 2994-2998.

6. Owens, K. (1966) Biochem. J. 100, 354-361.

7. Giordesky, S.E. and Marinetti, G.V. (1973) Biochem. Biophys. Res. Commun. 50, 1027-1031.

8. Schick, P.K., Kurica, K., and Chacko, G.K. (1975) Fed. Proc. 34, 241.

9. Simmons, S. Rittenhouse-, and Deykin, D. (1976) Biochem. Biophys. Acta, in press.

10. Chen, R.F. (1967) J. Biol. Chem. 242, 173-181.

11. Wuthier, R.E. (1966) J. Lip. Res. 7, 544-550.

12. Cohen, P. and Derksen, A. (1969) Brit. J. Haemat. 17, 359-371.