Potentiating Effect of 5,8,11-Eicosatrienoic Acid on Human Platelet Aggregation

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ABSTRACT

5,8,11-Eicosatrienoic acid (20:3ω9), a fatty acid increased in the platelet phospholipids of man and animals fed saturated fats, was either added to human platelets simultaneously with the aggregating agents, or incorporated into the platelet phospholipids by preincubation. 20:3ω9 markedly increased the response of platelets to all aggregating agents tested when added simultaneously with the agent, but solely to thrombin and ionophore, after incorporation into the platelet phospholipids. The potentiating effects of 20:3ω9 on thrombin aggregation do not appear to be related to prostaglandin formation, but rather to the production of a monohydroxy derivative through the lipoxygenase pathway.
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INTRODUCTION

Several polyunsaturated fatty acids have been shown markedly to influence platelet functions. Arachidonic acid (20:4ω6) is a well known (1,2) inducer of platelet aggregation as a precursor of endoperoxides and prostaglandins. By contrast, dihomogammalinolenic acid (20:3ω6) and 5,8,11, 14,17-eicosapentaenoic acid (20:5ω3), also prostaglandin precursors, are inhibitors of platelet aggregation (3,4).

An increase in 5,8,11-eicosatrienoic acid (20:3ω9) in certain blood lipids has been reported in atherosclerotic patients (5), as well as in animals deficient in essential fatty acids (6,7) or fed saturated fats (8). In animals, the higher level of 20:3ω9 in the platelet phospholipids was associated with a higher susceptibility of platelets to thrombin-induced aggregation (7,8). A similar result was also observed recently in farmers from Great Britain and France (9).

The present study determined whether 20:3ω9 in platelets might be responsible for the platelet hyperactivity observed in men and animals fed a saturated fat diet, and, if so, through which mechanism.

MATERIALS AND METHODS

The human serum albumin (essentially fatty acid free), human thrombin and arachidonic acid utilized in these studies were obtained from Sigma Chemical Co. (St. Louis, MO). Collagen was purchased from Horm (Munich) and cation-ionophore A 23187 was given by Lilly Laboratories (Indianapolis). Unlabeled and [^{14}C]20:3ω9 were chemically synthesized according to the technique previously reported for 20:4ω6 (10). Silica Gel G plates for thin layer chromatography (TLC) were purchased from Merck (Darmstadt), and the various solvents and reagents from Prolabo (Paris).

Blood from human volunteers was collected with ACD (citric acid 0.8%, sodium citrate 2.2%, dextrose 2.45%) as the anticoagulant (1/9 in volume). Platelet-rich plasma was obtained by centrifugation (100 G × 15 min); platelets were isolated from their plasma also by centrifugation (900 G × 10 min) and, for studies reported in Figures 1 and 4, resuspended (300,000/μl) in a Tyrode without calcium, containing Hepes buffer (pH = 7.4) as previously described (11).

For incorporation of 20:3ω9 in the platelet phos-

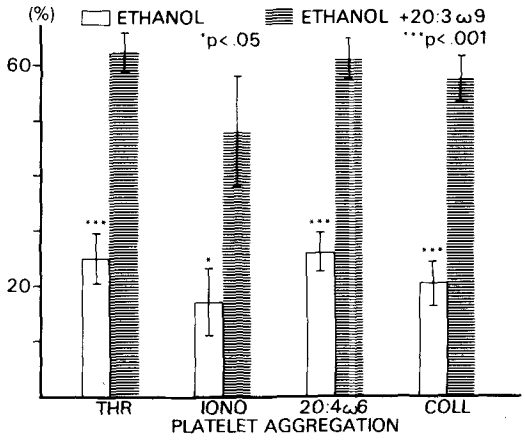

FIG. 1. Influence, on human platelets (resuspended in a Tyrode buffer) of 20:3ω9 (5×10^{-6} M), diluted in ethanol and added simultaneously with the following aggregating agents: thrombin (THR), 0.015 U/ml; ionophore (IONO) A 23187, 0.25×10^{-6} M; arachidonic acid (20:4ω6), 2×10^{-6} M; collagen (COLL), 0.5 μg/ml. Results expressed as percentage of platelet aggregation (mean ± SE of 5 determinations).

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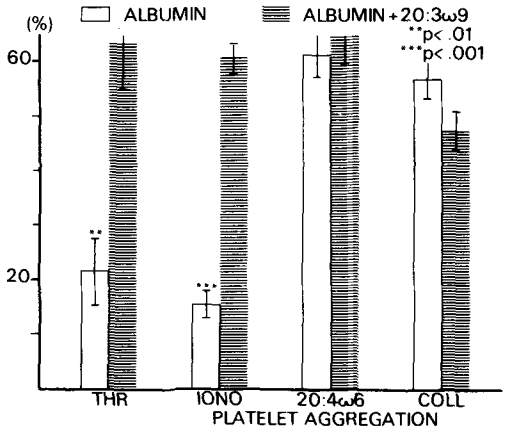

FIG. 2. Influence of 20:3ω9 incorporated into the platelet phospholipids (by a 2-hr incubation period of platelets with 20:3ω9 bound to albumin and resuspension in Tyrode/Hepes) on aggregation induced by the agents as in Figure 1.

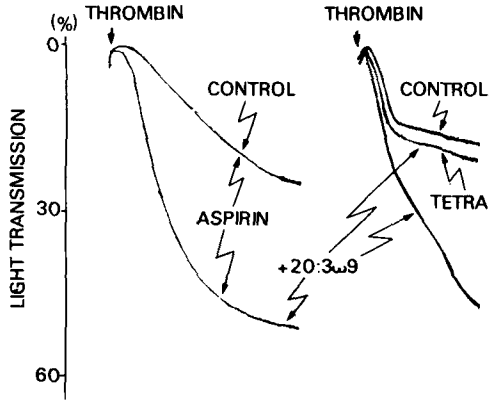

FIG. 3. Influence of aspirin (left tracings) at 2×10^{-4} M and of 5,8,11,14-heneicosatetraynoic acid (TETRA) (an inhibitor of platelet lipoxygenase) (right tracings) at 5×10^{-6} M, on the aggregation induced by thrombin (0.015 U/ml). Before testing the effect of aspirin and TETRA on thrombin aggregation, platelets were incubated for 2 hr in tyrode albumin not containing (controls) or containing 20:3ω9 to enrich platelets with this fatty acid.

pholipids (studies of Figures 2, 3 and Table 1), platelets were incubated for 2 hr in a shaker bath at 37C in a Tyrode/Hepes buffer containing albumin (3.5 g/l) and 20:3ω9 bound to albumin (12). The binding of 20:3ω9 to albumin in a molar ratio of 1 was done by overnight incubation at 37C of the fatty acid in a Tyrode/Hepes buffer/albumin (pH: 7.4) solution. Then, the platelet suspension was acidified (pH = 6.4) with citric acid and centrifuged at 700 G for 10 min. Finally, the platelets were resuspended in the Tyrode/Hepes buffer without albumin, to perform platelet aggregation or to analyze by gas liquid chromatography (GLC), fatty

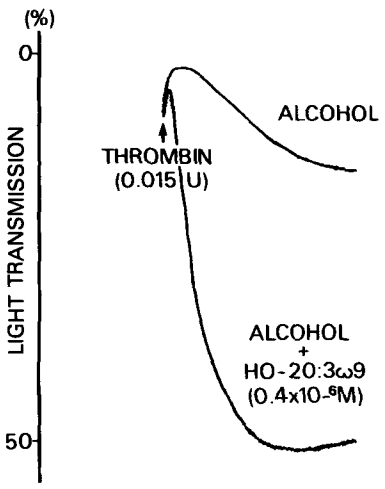

FIG. 4. One typical recording observed in 3 different series of the potentiating effect of monohydroxy-20:3ω9 (0.4×10^{-6} M, final concentration) diluted in ethanol, added simultaneously with thrombin. This response of human platelets to thrombin (0.015 U/ml) was performed under similar conditions to those reported in Figure 1.

acid composition of the platelet phospholipids after TLC separation (13).

Platelet aggregation was studied with a turbidimetric method (14). In the first series of studies (Fig. 1), 20:3ω9 was added simultaneously with the aggregating agent into the cuvette of the aggregometer. For these experiments, 20:3ω9 was dissolved in ethanol and 2 μl of this solution (0.5%) were added to 0.4 ml of the platelet suspension (5×10^{-6} M). In studies reported in Figure 3, aspirin at 4×10^{-3} M (20 μl) diluted in Tyrode/ethanol (9:1 in volume) was added to the platelet suspension (0.4 ml) 3 min before the aggregating agent. 5,8,11,14-Heneicosatetraynoic acid, a specific inhibitor of human platelet lipoxygenase (15), was added in ethanol and used at 5×10^{-6} M.

The metabolism of [^{14}C]20:3ω9 was studied after double extraction of lipids as reported recently (13). The lipid extract was analyzed by TLC. Monohydroxy-eicosanoic acids were separated from fatty acids by elution with hexane/diethyl ether/acetic acid (60:4:1, v/v). Prostaglandins and thromboxane B were separated by a second elution with diethyl ether/methanol/acetic acid (90:1:2, v/v), phospholipids staying at the origin (16). Finally, the different phospholipids were separated by a third elution with chloroform/methanol/acetic acid/water (85:15:14:4, v/v). After each elution, a quantitative radiochromatogram was performed. Thus, the amount of each compound and the incorporation into phospholipids could be calculated.

Finally, the monohydroxy-20:3ω9 was prepared

TABLE 1

Amounts of 20:3ω9 in Different Platelet Fractions Before and After Stimulation by Thrombin and Ionophore

Inductor	Free	Monohydroxy	Cyclooxygenase Products	PC	PE	PI + PS	Total phospholipids
None (Tyrode)	3.2 ± 0.90	0.17 ± 0.08	ND	11.53 ± 1.26	1.63 ± 0.31	3.51 ± 0.68	16.6 ± 1.8
Thrombin (0.1 U/ml)	2.99 ± 0.98	2.27[a] ± 0.45	ND	10.95 ± 0.98	1.46 ± 0.66	2.91 ± 0.23	15.3 ± 1.2
Ionophore A 23187 (10^{-6}M)	1.49 ± 0.42	5.78[b] ± 0.64	ND	9.56 ± 0.99	1.17 ± 0.16	2.16 ± 0.29	12.9 ± 1.2

[a] $p<0.005$.
[b] $p<0.001$.
Results expressed in nmol/10^9 platelets are mean ± SE of 5 experiments. ND = not detectable; PC = phosphatidylcholine; PE = phosphatidylethanolamine; PI + PS = phosphatidylinositol + phosphatidylserine.

from exogenous [^{14}C]20:3ω9 as follows: 20:3ω9 (final concentration 10^{-5} M) dissolved in ethanol was incubated for 10 min at 37C with a suspension of washed platelets as a source of enzymes. Lipids were extracted at pH = 3 by chloroform/ethanol (2:1). The monohydroxy derivative was separated and purified by two successive runs with hexane/diethyl ether/ acetic acid (80:20:1, v/v) as eluent.

RESULTS

As shown in Figure 1, 20:3ω9, added simultaneously with the aggregating agents, markedly potentiated platelet aggregation induced by all agents. In contrast to this, when platelets were preenriched with 20:3ω9 (Fig. 2), solely the aggregation to thrombin and ionophore was increased. The aggregations to arachidonate and collagen were not modified (Fig. 2).

[^{14}C]20:3ω9, when incubated with platelets, was predominantly incorporated into the glycerophospholipids (Table 1) since 83.4 ± 9.0% (mean ± SE of 5 experiments) of the 20:3ω9 was found in the glycerophospholipids, representing 16.6 nmol/10^9 platelets. Among the various fractions, it was in the phosphatidylcholine that the bulk (69.5%) of the radioactive 20:3ω9 was incorporated. GLC analysis of total phospholipid fatty acids of platelets incubated with or without 20:3ω9 confirmed the incorporation into phospholipids of this fatty acid. In this fraction, the level of 20:3ω9 passed from 0.2 to 1.9% (mean of 5 experiments). However, the increase found by GLC analysis was slightly lower than this observed by radioactivity determination. Incubation of 20:3ω9-rich platelets in the presence of thrombin or cation ionophore A 23187 induced the formation of a monohydroxy-20:3ω9. The formation of this substance was accompanied by a decrease of 20:3ω9, both free and bound to phospholipids.

As shown in Figure 3, aspirin added to the platelet suspension at a concentration (2×10^{-4} M) which completely inhibits prostaglandin formation from arachidonic acid did not modify the potentiating effect of 20:3ω9 on thrombin-induced aggregation. By contrast, this potentiating effect was completely suppressed by 5,8,11,14-heneicosatetraynoic acid (Fig. 3).

Finally, the monohydroxy-20:3ω9, added to a human platelet suspension simultaneously with thrombin as done for 20:3ω9 in the study reported in Figure 1, markedly increased the response of platelets to thrombin (Fig. 4), as observed with 20:3ω9. However, to achieve the same effect, the concentration of the hydroxy derivative was 0.4×10^{-6} M, whereas it was 5×10^{-6} M for the fatty acid. Besides, 5,8,11,14-heneicosatetraynoic acid was added at 5×10^{-6} M (as in the study of Figure 3) to human platelet suspensions at the same time that the monohydroxy-20:3ω9 and aggregation induced by thrombin as above. In 6 experiments, the increased response (mean ± SE) of platelets with the monohydroxy alone was 59.9 ± 9.4% whereas it was 61.2 ± 6.4% in the presence of heneicosatetraynoic acid.

DISCUSSION

The present experiments appear to confirm previous results suggesting that the fatty acid 20:3ω9 in the platelet phospholipids might be one of the factors responsible for the increased response of platelets to thrombin aggregation in animals and men on a saturated fat diet (8,9).

The marked potentiating effect of 20:3ω9 on the response to all agents, when added simultaneously with this agent, suggests that 20:3ω9 has to be present under its free form to increase platelet aggregation. Concordant with this hypothesis are the results obtained after incorporation of 20:3ω9 into the platelet phospholipids which have shown

that it was solely the aggregation to thrombin and ionophore which was increased. Thrombin and ionophore (17,18) are known to be able to release consistent amounts of polyunsaturated fatty acids from the platelet phospholipids, while collagen appears to be much less efficient in that respect (1). Arachidonic acid does not release the esterified fatty acids at all.

It seems that the increased response to aggregation of 20:3ω9-rich platelets may be explained by the monohydroxy derivative produced in substantial amounts (ca. 1 nmol/ml of platelet suspension) after stimulation by thrombin or ionophore. This monohydroxy derivative appears to be formed from 20:3ω9 both free and bound to phospholipids. By contrast, no cyclooxygenase products derived from 20:3ω9 could be detected. This was expected since cyclooxygenase needs a substrate with at least 3 double bonds at 8,11,14 positions on C_{20} fatty acids (19).

The observation that the potentiating effect of 20:3ω9 on platelet aggregation was not inhibited by aspirin at 2×10^{-4} M suggests that this effect was not due to an increase in prostaglandin formation. By contrast, 5,8,11,14-heneicosatetraynoic acid at a concentration (5×10^{-6} M), known to inhibit specifically the lipoxygenase pathway in human platelets (15), inhibited the potentiating effect of 20:3ω9 but not that of the monohydroxy-20:3ω9. Thus, it seems that lipoxygenase is involved in the potentiating effect of 20:3ω9. In addition, the monohydroxy derivative of 20:3ω9 appears to be at least 10 times as potent as the acid itself to increase in a similar way the response of platelets to thrombin. Consequently, the amount of the monohydroxy-20:3ω9 needed to induce the potentiation observed is consistent with the amount synthesized (1 nmol/ml of platelet suspension) in the presence of thrombin.

Another monohydroxy fatty acid (12-OH-20:4) has been found by other investigators (20,21) to play an essential role in the irreversible platelet aggregation induced in rat by arachidonic acid. Our present results indicate that a polyunsaturate fatty acid such as 20:3ω9, which apparently is not a prostaglandin precursor, may play an agonist role in platelet aggregation.

Finally, it seems of interest to underline the remarkably different effect on platelet functions of the 20:3 isomers. When tested under the present experimental conditions, instead of the potentiating effect described here for the ω9, 20:3ω3 presents a moderate, and 20:3ω6 a strong, inhibitory effect on platelet functions (12), in confirmation of the work performed by previous investigators (22).

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REFERENCES

1. Samuelsson, B. (1977) in Prostaglandins in Hematology, pp. 1-10, Spectrum Publication, New York.
2. Smith, J.B., Ingerman, C.M., and Silver, M.J. (1977) in Prostaglandins in Hematology, pp. 277-292, Spectrum Publication, New York.
3. Willis, A.L., Comai, K., Kuhn, D.C., and Paulsrud, J. (1974) Prostaglandins 8, 509-519.
4. Needleman, P., Raz, A., Minkes, M.S., Ferrendelli, J.A., and Sprecher, H. (1979) Proc. Natl. Acad. Sci. USA 76, 944-948.
5. Kingsbury, K.J., Brett, C., Stovold, R., Chapman, A., Anderson, J., and Morgan, D.M. (1974) Postgrad. Med. J. 50, 425-440.
6. Holman, R.T. (1960) J. Nutr. 70, 405-410.
7. McGregor, L., and Renaud, S. (1978) Thrombos. Res. 12, 921-927.
8. McGregor, L., Morazain, R., and Renaud, S. (1980) Lab. Invest. 43, 438-442.
9. Renaud, S., Dumont, E., Godsey, F., Morazain, R., and McGregor, L. (1981) in Nutrition in the 1980's, pp. 361-381, Alan R. Liss, New York.
10. Sprecher, H. (1971) Lipids 6, 889-894.
11. Lagarde, M., Bryon, P.A., Guichardant, M., and Dechavanne, M. (1980) Thrombos. Res. 17, 581-588.
12. Lagarde, M., Burtin, M., Dechavanne, M., Sicard, B., and Coiffier, B. (1980) Prostaglandins Med. 4, 177-183.
13. Lagarde, M., Guichardant, M., Menashi, S., and Crawford, N. (1982) J. Biol. Chem. 25, 3100-3104.
14. Mustard, J.F., Hegardt, B., Rowsell, H.C., and McMillan, R.L. (1964) J. Lab. Clin. Med. 64, 548-559.
15. Wilhelm, T.E., Sankarappa, S.K., Van Rollins, M., and Sprecher, H. (1981) Prostaglandins 21, 323-332.
16. Lagarde, M., Gharib, A., and Dechavanne M. (1977) Clin. Chim. Acta 79, 255-259.
17. Bills, T.K., Smith, J.B., and Silver, M.J. (1977) in Prostaglandins in Hematology, pp. 27-55, Spectrum Publication, New York.
18. Knapp, H.R., Oelz, O., Roberts, J.L., Sweetman, B.J., Oates, J.A., and Reed, P.W. (1977) Proc. Natl. Acad. Sci. USA 74, 4251-4255.
19. Raz, A., Minkes, M., and Needleman, P. (1977) Biochim. Biophys. Acta 488, 305-311.
20. Dutilh, C.E., Haddeman, E. Jouvenaz, G.H., TenHoor, F., and Nugteren, D.H. (1979) Lipids 14, 241-246.
21. Dutilh, C.E., Haddeman, E., Don, J.A., and TenHoor, F. (1981) Prostaglandins Med. 6, 111-126.
22. Willis, A.L., Stone, K.J., Hart, M., Gibson, V., Marples, P., Botfield, E., Comai, K., and Kuhn, D.C. (1977) in Prostaglandins in Hematology pp. 371-310, Spectrum Publication, New York.

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