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12S-HYDROXYEICOSATETRAENOIC ACID PLAYS A CENTRAL ROLE IN THE REGULATION OF PLATELET ACTIVATION

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ABSTRACT. When platelets are activated by the recognition of exposed collagen fibers, they start synthesizing two major arachidonic acid metabolites, i.e. thromboxane A_2 and 12S-hydroxyeicosatetraenoic acid (12-HETE) via cyclooxygenase and 12-lipoxygenase pathways, respectively. Although the physiological role of the former is well established, that of the latter has not been fully elucidated. Recently, we have revealed that 12-HETE interferes with collagen-induced platelet aggregation [Sekiya, F. et al. (1990) Biochim.Biophys.Acta **1044**, 165-168]. In the present paper, we show that this substance enhances thrombin-induced aggregation of bovine platelets, in sharp contrast with the case of collagen. Additionally, 12-HETE is able to prevent the prostaglandin E_1-induced elevation of platelet cAMP level and counteracts its inhibitory effect on platelet aggregations. With these observations, we propose a novel self-regulatory mechanism of platelets where 12-HETE plays a key role; it switches sensitivity of platelets from the primary agonist (collagen) to the secondary one (thrombin), and cancels the inhibitory effect of cAMP elevators.

When vascular wall is damaged, blood platelets are activated by recognizing exposed collagen fibers and undergo dramatic reactions, i.e. adhesion, shape change, aggregation and secretion of the granule contents. These activated platelets contribute to further activation of circulating platelets and enlargement of hemostatic plug either by releasing other chemical mediators, such as ADP and thromboxane A_2 (TXA_2) , or by participating in the conversion of plasma prothrombin into active thrombin. Thrombin thus produced is very essential not only as the catalyst to form fibrin clot but also as the potent inducer of platelet aggregation. Platelets activated by collagen liberate arachidonic acid from membrane phospholipid stores, and liberated arachidonic acid gives rise to two major metabolites, namely TXA_2

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Abbreviations used are: TXA_2, thromboxane A_2; HETE, hydroxyeicosatetraenoic acid; 12-HETE, 12S-hydroxyeicosa-5,8,10,14-tetraenoic acid; PGE_1, prostaglandin E_1; ACD, acid-citrate dextrose; Tris-ACD, 20 mM Tris, 96 mM NaCl, 12 mM KCl, 23 mM glucose, 10 mM citric acid, 14 mM trisodium citrate, pH 7.35.

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via cyclooxygenase pathway and 12-HETE via 12-lipoxygenase pathway (1-3). Potent activity of TXA_2 as an agonist for platelet activation and vasocontraction well explains the physiological significance of platelet cyclooxygenase pathway (1-3). In addition, cyclooxygenase products derived from other tissues are also very important, for instance prostacyclin derived from vascular wall plays a crucial role in suppression of platelet activation (2,4). On the other hand, 12-lipoxygenase products, i.e. 12-HETE and its hydroperoxyl precursor, are presently considered to act negatively on platelet activation (5-7), but some conflicting investigations have been also presented (8). Thus, there is no consensus concerning the physiological role of 12-lipoxygenase products at this time (reviewed in ref.9). We have recently revealed that 12-HETE interferes with arachidonic acid liberation via phospholipase A_2 in bovine platelets which results in reduced production of TXA_2 and inhibition of collagen-induced aggregation, while ADP- or PAF-acether-induced aggregation is little affected, since these agonists do not elicit TXA_2 formation in bovine platelets (10,11). The present study was conducted to clarify further biological action of 12-HETE, and we found that thrombin-induced aggregation was amplified by this substance, in sharp contrast to the case with collagen. We also revealed that 12-HETE could counteract the inhibitory effect of a cAMP elevator, PGE_1.

MATERIALS AND METHODS

Materials Bovine α-thrombin (>2,000 NIH units mg^{-1}, >80% was α-species with small amount of autolysis products, as electrophoretically determined) was obtained from Mochida Pharmaceutical Co. (Tokyo, Japan). 12-Hydroxystearic acid (racemic), linolenic acid and bovine serum albumin (crystalline, fatty acid free) were from Sigma. 12R-HETE, 5-HETE, leukotriene B_4 as well as 12-HETE were chemically synthesized (12). Human fibrinogen was a gift from Green Cross (Osaka, Japan). PGE_1 and 15-HETE were from Cayman Chemical (Ann Arbor, MI).

Platelet preparation and aggregation assay Platelets were isolated from bovine blood anticoagulated with ACD that was obtained from a local slaughterhouse, washed twice with Tris-ACD buffer (20 mM Tris, 96 mM NaCl, 12 mM KCl, 23 mM glucose, 10 mM citric acid, 14 mM trisodium citrate, pH 7.35) and finally suspended in the same buffer (13). The concentration of platelet suspension was adjusted to 1 x 10^9 ml^{-1} in a total volume of 250 µl, and $CaCl_2$ was added to give 8 mM. After a few minutes-preincubation at 37°C, agonists were added and platelet aggregation was monitored turbidimetrically in an aggregometer (Hematracer 601, Niko Bioscience, Tokyo, Japan) with stirring at 1,000 rpm. As platelet response differs depending on the preparations, assays were repeated at least three times using independent preparations, and representative results are shown.

Quantification of cAMP Two hundred microliters of washed platelet suspension in Ca^{2+}-free Tris-ACD buffer (2 x 10^9 ml^{-1}) were exposed to PGE_1 and 12-HETE (added in 1 µl of ethanol) and incubated at 37°C. The reaction was terminated by the addition of equal volume of ice cold 0.2 N HCl, and samples were stored at -80°C until the assay. cAMP in the samples was quantified with a specific radioimmunoassay using a commercial kit (Yamasa Shoyu, Choshi, Japan), after removal of insoluble materials by centrifugation, appropriate dilution and neutralization.

RESULTS AND DISCUSSION

When added with thrombin, 12-HETE showed marked potentiating effect on aggregation of bovine washed platelets, although it alone did not cause aggregation (10). Figure one illustrates representative aggregation traces in the presence of different concentrations of 12-HETE. Response of platelets toward a threshold concentration of thrombin, which induced only weak aggregation, was amplified depending upon 12-HETE concentration. Similar potentiating effect on thrombin-induced aggregation was also observed in human washed platelets. 12-HETE did not affect the proteolytic activity of thrombin, which is indispensable to induce platelet activation (14), at least when fibrinogen was used as the substrate (not shown). Therefore, the potentiating effect of 12-HETE on thrombin-induced platelet aggregation did not seem to be on thrombin itself, but presumably on signal transduction pathways in thrombin-stimulated platelets. Among many intraplatelet biochemical reactions elicited by thrombin, activation of inhibitory G-protein for adenylate cyclase, denoted as G_i, is reported to be one of the most early steps for the signaling by thrombin (reviewed in refs.15,16) and it is also known that thrombin among many physiological stimuli specifically provokes adenylate cyclase inhibition (17,18). We speculated that this is the step probably stimulated by 12-HETE.

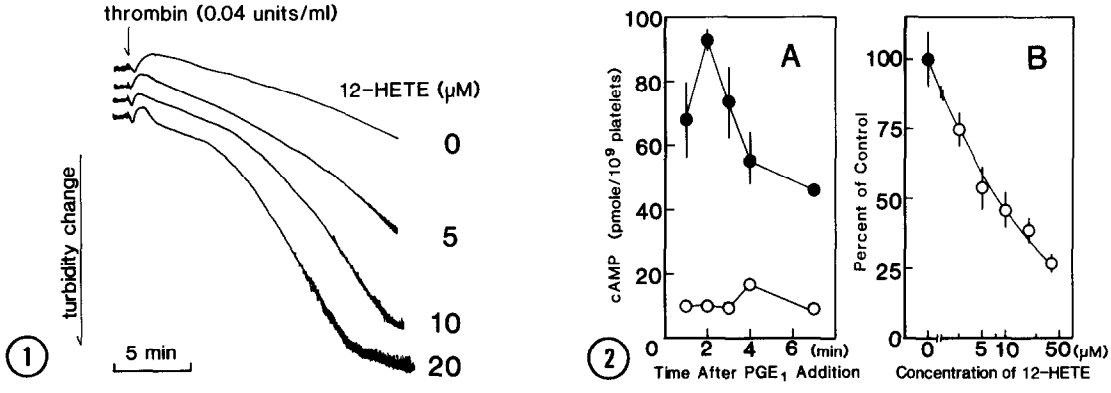

Figure 1. 12-HETE potentiates thrombin-induced platelet aggregation. Washed bovine platelet suspension was exposed to respective concentration of 12-HETE added in ethanol (final concentration 0.4%) for 1 min. The aggregation was initiated by the addition of 0.04 units ml^{-1} thrombin. Decrease in turbidity was not due to platelet lysis, since no significant leakage of lactate dehydrogenase, a cytosolic enzyme marker, was observed.

Figure 2. 12-HETE abolishes PGE_1-induced cAMP elevation in platelets. PGE_1-induced cAMP elevation in platelets was investigated as described under METHODS. Presented results are the mean (± SE) of duplicate determinations which are representative of four independent experiments. Open circles; in the presence of 12-HETE, closed circles; in the absence of 12-HETE. (A) Platelets were incubated with 20 μM 12-HETE or ethanol alone for 1 min, the reaction was initiated by the addition of 2 μM PGE_1, and terminated at indicated times. (B) Platelets were incubated with respective concentrations of 12-HETE for 1 min, then exposed to 2 μM PGE_1 for a further 2 min.

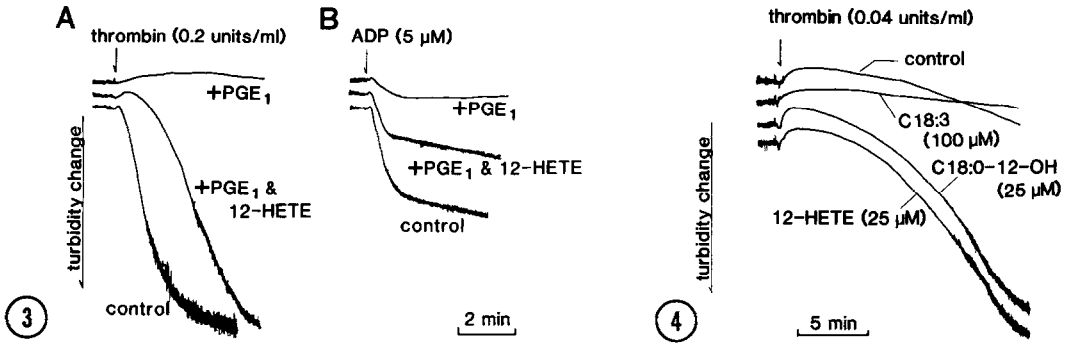

Figure 3. 12-HETE counteracts the inhibitory effect of PGE_1 in platelet aggregations. Washed platelets were exposed to 12-HETE (20 μM) or ethanol alone for 1 min, and to PGE_1 for a further 1 min, then stimulated by thrombin (A) or ADP (B). The concentrations of thrombin and PGE_1 were 0.2 units ml^{-1} and 2 μM, respectively (A), and those of ADP and PGE_1 were 5 μM and 40 nM, respectively (B). In the case of ADP-induced aggregation, 1 mg ml^{-1} fibrinogen was included in the incubation mixture. Other conditions were same as described in the legend to Fig.1. Since ADP-induced aggregation was more susceptible to PGE_1 than that by thrombin, a lower concentration of PGE_1 was used.

Figure 4. Hydroxyl group in 12-HETE molecule is responsible for the potentiating effect on thrombin-induced platelet aggregation. Platelets were exposed to either 12-HETE (25 μM), 12-hydroxystearic acid (C18:0-12-OH, 25 μM), linolenic acid (C18:3, 100 μM) or ethanol alone for 1 min, and stimulated by 0.04 units ml^{-1} thrombin. In this experiment, 1 mg ml^{-1} serum albumin was included in the medium, since high concentration of linolenic acid sometimes caused platelet lysis in the absence. At a lower concentration (25 μM), linolenic acid also showed no potentiating effect even in the absence of albumin.

We decided to look into the effect of 12-HETE on cAMP elevation in platelets induced by PGE_1, which is known to stimulate adenylate cyclase via stimulative G-protein, G_s (19), and shares a common receptor with prostacyclin (20). As is shown in Fig.2A, PGE_1 promoted rapid and marked accumulation of cAMP that peaked at 2 min and then gradually declined presumably by the action of cAMP-phosphodiesterase, but in the presence of 12-HETE almost no accumulation was seen. This inhibition of PGE_1-induced accumulation of cAMP was dependent on 12-HETE concentration (Fig.2B), and its effectiveness to reduce cAMP level was paralleled with its potentiating effect on thrombin-induced aggregation (compare Fig.2B to Fig.1). Furthermore, 12-HETE was effective even in lowering the already elevated cAMP level by preexposure to PGE_1 (not shown). The effect of 12-HETE toward the basal cAMP level was unable to be demonstrated, because it is already too low to be quantified unequivocally.

The effect of 12-HETE on cAMP suggested an interesting possibility that it might counteract the inhibitory effect of PGE_1 (or prostacyclin in vivo) on platelet activation, and we tested this idea. Upon the exposure to PGE_1, platelet response toward thrombin was decreased, but when 12-HETE was included, the inhibition was partly canceled and strong aggregation was observed (Fig.3A). Furthermore, although 12-HETE alone poorly affected aggregations induced by weak agonists, such

as ADP (10), it could overcome the inhibitory effect of PGE_1 on ADP-induced aggregation, partly if not all (Fig.3B). This phenomenon was again observed in human platelets.

The structure/function relationship of 12-HETE molecule was also investigated. As is presented in Fig.4, the potentiating activity was also found in a saturated hydroxyfatty acid, whereas polyunsaturated fatty acids without a hydroxyl group, such as linolenic acid, was not effective but rather inhibitory at the concentrations up to 100 μM as reported (21). Though not presented here, naturally occurring HETEs, including 12R-HETE (the stereo isomer of 12-HETE), 5-HETE, 15-HETE and leukotriene B_4 (5S,12R-diHETE), are also all effective at the concentrations comparable to that of 12-HETE (around 20 μM). Therefore, the activity of 12-HETE should be attributable to the hydroxyl group within the molecule.

12-Lipoxygenase activity and its product in platelets were first described more than 15 years ago (22), but their roles have been obscure (9) and little attention has been paid compared with the product of the other pathway, TXA_2. Our previous finding (11) and present results provide an evidence that 12-HETE is actively involved in the self-regulatory mechanism of platelet activation.

Although it is not easy to address the precise action of 12-HETE *in vivo*, it might be reasonable to propose the following scheme of biological sequence where 12-HETE participates in. When platelet activation is initiated by recognizing exposed collagen fibers at the site of vascular injury, vigorous arachidonic acid metabolism is undertaken. TXA_2 is immediately formed but readily disappears due to its lability. In addition, production period of TXA_2 is restricted by the suicide self-inactivation of cyclooxygenase (23) and TXA_2 synthase (24). The production of 12-HETE by 12-lipoxygenase, however, proceeds linearly (8), and leads to an accumulation of considerable amount. Accumulated 12-HETE would suppress arachidonic acid liberation and reduce platelet aggregability toward TXA_2-dependent agonists, such as collagen. Meanwhile, thrombin is produced on the surface of activated platelets, and 12-HETE does enhance platelet activation induced by this thrombin. Thus, switching of major responsible agonists, from the primary one (collagen) to the secondary one (thrombin), takes place. Furthermore, 12-HETE counteracts prostacyclin derived from vascular wall, of which action is mediated by cAMP elevation. This effect should promote an increase in aggregability toward weak agonists, such as ADP, and accelerates the thrombus formation.

We have used 12-HETE throughout the present study despite of similar activity of other hydroxyfatty acids including those synthesized *in vivo*. Among HETEs 12-HETE should be present in blood circulation most abundantly considering the population of platelets and their high activity of 12-lipoxygenase. In addition, 12-HETE should act predominantly by its autocrine and paracrine mechanism, for platelets themselves produce it and its local concentration must be high at the site where platelet activation occurs. However, it may be also possible that other

HETEs, such as endothelium-derived 15-HETE (25) and leukocyte-derived 5-HETE and leukotriene B_4 (26), participate in the potentiation of platelet activation. This interesting point is left to be studied.

In conclusion, we propose that 12-HETE plays a central role in the regulation of platelet activation. The precise mechanism how the action of 12-HETE is brought about remains to be further elucidated. Two effects of 12-HETE, i.e. the potentiation of the action of thrombin and the reduction of the cAMP level, are likely to correlate each other, considering the mechanism of thrombin-induced platelet activation in which the putative G_i plays a crucial role(s) (15,16,18). We are currently investigating the detailed biochemical basis of these phenomena in order to clarify this possible linkage.

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REFERENCES

1. Rittenhouse-Simmons, S. & Deykin, D. (1981) in Platelets in biology and pathology 2, (J.L. Gordon, Ed.) pp.349-372. Elsevier/North-Holland, Amsterdam.
2. Needleman, P., Turk, J., Jakschik, B.A., Morrison, A.R. & Lefkowith, J.B. (1986) Annu.Rev.Biochem. **55**, 69-102.
3. Marcus, A.J. (1987) in Hemostasis and thrombosis: Basic principles and clinical practice, 2nd edition, (R.W. Colman, J. Hirsh, V.J. Marder & E.W. Saltzman, Eds.) pp.676-688. Lippincott, Philadelphia.
4. Moncada, S., Gryglewski, R., Bunting, S. & Vane, J.R. (1976) Nature **263**, 663-665.
5. Hammarström, S. & Falardeau, P. (1977) Proc.Natl.Acad.Sci.USA **74**, 3691-3695.
6. Siegel, M.I., McConnell, R.T., Abrahams, S.L., Porter, N.A. & Cuatrecasas, P. (1979) Biochem.Biophys.Res.Commun. **89**, 1273-1280.
7. Aharony, D., Smith, J.B. & Silver, M.J. (1982) Biochim.Biophys.Acta **718**, 193-200.
8. Dutilh, C.E., Haddeman, E., Jouvenaz, G.H., Ten-Hoor, F. & Nugteren, D.H. (1978) Lipids **14**, 241-246.
9. Brash, A.R. (1985) Circulation **72**, 702-707.
10. Sekiya, F., Takagi, J. & Saito, Y. (1989) Thrombos.Res. **56**, 407-415.
11. Sekiya, F., Takagi, J., Sasaki, K., Kawajiri, K., Kobayashi, Y., Sato, F. & Saito, Y. (1990) Biochim.Biophys.Acta **1044**, 165-168.
12. Shimazaki, T., Kobayashi, Y. & Sato, F. (1988) Chem.Lett. 1785-1788.
13. Nishimura, Y., Yamada, Y., Itoh, Y., Takenaka, O. & Inada, Y. (1975) FEBS Lett. **51**, 171-173.
14. Greco, N.J., Tenner, T.E.,Jr., Tandon, N.A. & Jamieson, G.A. (1990) Blood **75**, 1983-1990.
15. Kroll, M.H. & Schafer, A.I. (1989) Blood **74**, 1181-1195.
16. Lapetina, E.G. (1990) FEBS Lett. **268**, 400-404.
17. Aktories, K. & Jakobs, K.H. (1984) Eur.J.Biochem. **145**, 333-338.
18. Brass, L.F., Woolkalis, M.J. & Manning, D.R. (1988) J.Biol.Chem. **263**, 5348-5355.
19. Lester, H.A., Steer, M.L. & Levitzki, A. (1982) Proc.Natl.Acad.Sci.USA **79**, 719-723.

20. Schafer, A.I., Cooper, B., O'Hara, D. & Handin, R.I. (1979) J.Biol.Chem. **254**, 2914-2917.
21. Kitagawa, S., Kotani, K. & Kametani, F. (1990) Biochim.Biophys.Acta **1054**, 114-118.
22. Hamberg, M. & Samuelsson, B. (1974) Proc.Natl.Acad.Sci.USA **71**; 3400-3404.
23. Egan, R.W., Paxton, J. & Kuehl, F.A.,Jr. (1976) J.Biol.Chem. **251**, 7329-7335.
24. Jones, D.A. & Fitzpatrick, F.A. (1990) J.Biol.Chem. **265**, 20166-20171.
25. Hopkins, N.K., Oglesby, T.D., Bundy, G.L. & Gorman, R.R. (1984) J.Biol.Chem. **259**, 14048-14053.
26. Hammarström, S. (1983) Annu.Rev.Biochem. **52**, 355-377.