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Short communication

Sphingomyelin inhibits platelet 12-lipoxygenase activity

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Abstract

The effect of sphingomyelin on the formation of 12-hydroxy-5,8,10,14-eicosatetraenoic acid (12-HETE), thromboxane B_2 and 12-hydroxy-5,8,10-heptadecatrienoic acid (HHT) in washed rabbit platelets was examined. Sphingomyelin had a powerful inhibitory effect on 12-HETE formation, while it produced only a small increase in thromboxane B_2 and HHT formation. The sphingomyelin metabolite ceramide did not affect the formation of 12-HETE, thromboxane B_2 and HHT. These results suggest that sphingomyelin is a selective inhibitor of platelet 12-lipoxygenase and may have functional effects in platelets.

Keywords: Sphingomyelin; Arachidonic acid; 12-Lipoxygenase; Cyclooxygenase; Platelet

1. Introduction

In platelets, arachidonic acid is converted into thromboxane A_2 and 12-hydroxy-5,8,10-heptadecatrienoic acid (HHT) by the cyclooxygenase pathway and into 12-hydroxy-5,8,10,14-eicosatetraenoic acid (12-HETE) by the 12-lipoxygenase pathway (Hamberg and Samuelsson, 1974). Thromboxane A_2 is a potent vasoconstrictor and inducer of platelet aggregation and rapidly breaks down to form the stable end-product thromboxane B_2. 12-HETE has been shown to play a central role in the regulation of platelet aggregation (Dutilh et al., 1978; Sekiya et al., 1991).

Sphingomyelin (*N*-acylsphingosine-1-phosphocholine or ceramide phosphocholine) is found in all tissues and lipoproteins (Merrill and Jones, 1990). Whether or not sphingomyelin has a specific function in cell membranes is unknown, although it is usually a significant component of the phospholipid bilayer and occurs in especially high concentrations in the plasma membrane. Previous studies have shown that sphingomyelin inhibits phosphatidylinositol phosphodiesterase in the brain and platelets (Dawson et al., 1985) and phospholipase Cδ in the liver (Pawelczyk and Lowenstein, 1992). However, little information is available concerning the influence of sphingomyelin on the metabolism of arachidonic acid via cyclooxygenase and 12-lipoxygenase in platelets.

In this paper, we describe our finding that sphingomyelin inhibits platelet 12-lipoxygenase activity.

2. Materials and methods

2.1. Materials

Thromboxane B_2, sodium salt of arachidonic acid, sphingomyelin from bovine erythrocytes (containing primarily lignoceric acid) or bovine brain (containing primarily stearic and nervonic acids), ceramide from bovine brain sphingomyelin and phosphocholine chloride were purchased from Sigma (St. Louis, MO, USA). HHT was purchased from Cayman Chemical (MI, USA) and 12-HETE was from Cascade Biochem (Berkshire, England). 9-Anthryldiazomethane was obtained from Funakoshi Pharmaceutical (Tokyo, Japan). All other reagents were analytical grade.

2.2. Preparation of platelets

Blood was withdrawn into a 3.8% solution of trisodium citrate (9:1, v/v) from the abdominal aorta of male rabbits (2–2.5 kg) under sodium pentobarbital anaesthesia. Platelets were then collected by differential centrifugation.

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Whole blood was centrifuged for 10 min at $200 \times g$ at room temperature and the platelet-rich plasma was withdrawn from above the pelleted erythrocytes. After addition of EDTA (to a final concentration of 1 mM), the platelet-rich plasma was cooled to 0°C and centrifuged at $2000 \times g$ for 10 min. The platelet pellet was washed twice with 134 mM NaCl, 5 mM glucose, 15 mM Tris–HCl buffer, pH 7.4 (buffer A) containing 1 mM EDTA, and then resuspended in buffer A.

2.3. Incubation conditions and measurement of metabolites from arachidonic acid

The washed platelet suspension (3×10^8 platelets) was preincubated for 5 min at 37°C in 1 ml of buffer A with or without the indicated concentrations of sphingomyelin, ceramide or phosphocholine chloride. Sphingomyelin and ceramide were dissolved in ethanol. The final concentration of ethanol in the platelet suspension was held constant at 2.5% (v/v) in all experiments. At this concentration, ethanol had no effect on arachidonic acid metabolism in platelets. Arachidonic acid (40 μM) was subsequently added to the platelet suspension, and the mixture was incubated at 37°C for 5 min. The reaction was terminated by quickly adding 20 μl of 0.25 M HCl to bring the pH of the reaction mixture to 3.0. The reaction mixture was then extracted with 3 ml of ethyl acetate. 12-HETE, thromboxane B_2 and HHT in the extracted lipid were simultaneously determined by a high-performance liquid chromatographic method as described in our previous studies (Fujimoto et al., 1990; Tsunomori et al., 1996). Briefly, 12-HETE and HHT were separated by normal-phase chromatography and simultaneously quantitated by using a UV spectrophotometric detector. Thromboxane B_2 was measured after esterification with 9-anthryldiazomethane. Thromboxane B_2 esterified with 9-anthryldiazomethane was separated by reverse-phase chromatography and simultaneously quantitated by using a fluorescence spectrofluorometer. Our previous study with indomethacin, an inhibitor of cyclooxygenase (Flower, 1974), and quercetin, an inhibitor of lipoxygenase (Hope et al., 1983; Nakadate et al., 1985), demonstrated the capacity of the present in vitro system to simultaneously detect changes in the activities of platelet cyclooxygenase and 12-lipoxygenase (Fujimoto et al., 1992).

2.4. Statistics

Results are means ± S.E.M. Statistical significance was determined by Student's *t*-test.

3. Results

Washed rabbit platelets (3×10^8/ml) metabolized exogenously added arachidonic acid (40 μM) to 12-HETE, thromboxane B_2 and HHT (Fig. 1). Under the experimental conditions described under Section 2, without the addition of sphingomyelin, rabbit platelets produced 4–5 times more 12-HETE than thromboxane B_2 or HHT. A similar tendency has been described for human platelets (Vanderhoek et al., 1980). The high generation of 12-HETE probably reflects the special importance of this metabolite to the regulation of platelet function. As shown in Fig. 1, preincubation of platelets with sphingomyelin from bovine erythrocytes for 5 min before addition of arachidonic acid inhibited the production of 12-lipoxygenase metabolite (12-HETE); in the range of 100 to 500 μg/ml (from 120 to 610 μM), the effect of sphingomyelin was concentration dependent. The formation of products of cyclooxygenase (thromboxane B_2 and HHT) was slightly enhanced by sphingomyelin. At concentrations between 100 and 500 μg/ml (about 130–650 μM) sphingomyelin from bovine brain inhibited 12-HETE formation and stimulated thromboxane B_2 and HHT formation to the same extent as sphingomyelin from bovine erythrocytes.

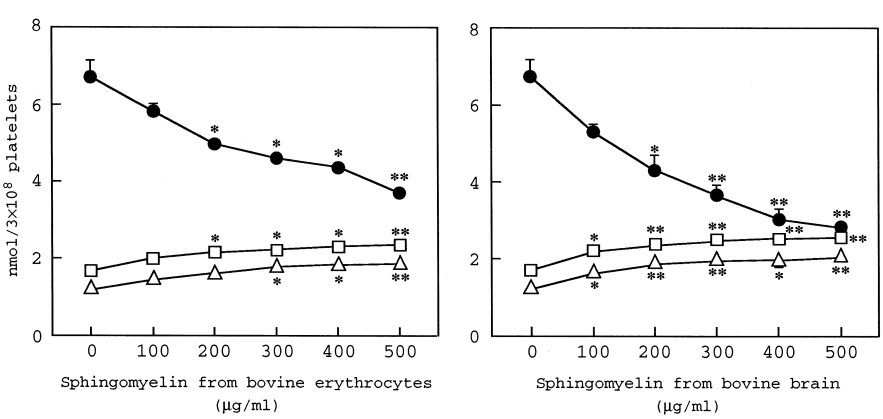

Fig. 1. Effects of sphingomyelin from bovine erythrocytes or bovine brain on the formation of 12-HETE (●), thromboxane B_2 (□) and HHT (△) in washed rabbit platelets. Platelets (3×10^8/ml) were preincubated with or without various concentrations of sphingomyelin for 5 min at 37°C prior to incubation with arachidonic acid (40 μM) for 5 min at 37°. Each point indicates the mean of five experiments; vertical lines show S.E.M. $^*P < 0.05$, $^{**}P < 0.01$; significantly different from the corresponding value in the absence of sphingomyelin.

Plasma membranes contain a neutral sphingomyelinase which hydrolyses sphingomyelin to ceramide and phosphocholine (Merrill and Jones, 1990). The sphingomyelin metabolite ceramide has been proposed to play a pivotal role in a variety of cellular processes (Hannun, 1994). Therefore, we examined the effects of ceramide and phosphocholine on the formation of 12-HETE, thromboxane B_2 and HHT in washed rabbit platelets (data not shown). Preincubation of the platelets with ceramide from bovine brain sphingomyelin or phosphocholine failed to show any effect on the formation of 12-HETE, thromboxane B_2 and HHT at concentrations of up to 500 μg/ml (about 830 μM, ceramide) or 1000 μM (phosphocholine).

4. Discussion

Sphingomyelin has been shown to affect the activity of a number of cellular phospholipases (Dawson et al., 1985; Pawelczyk and Lowenstein, 1992). However, few reports indicate an effect on the activity of platelet 12-lipoxygenase and cyclooxygenase. In the present study, we obtained the first evidence that sphingomyelin had an inhibitory effect on 12-HETE formation by platelets. The concentration for 50% inhibition by sphingomyelin was approximately 600 μM. Simultaneously, sphingomyelin stimulated slightly thromboxane B_2 generation. The net increased amount of thromboxane B_2 was much smaller than the net decreased amount of 12-HETE (22%) (Fig. 1).

Lignoceroyl sphingomyelin (from erythrocytes) and stearoyl and nervonoyl sphingomyelin (from brain) were similarly potent in causing inhibition of platelet 12-lipoxygenase, suggesting that the difference in the fatty acid moiety is not important for the inhibition by sphingomyelin. In addition, the inhibitory effect of sphingomyelin on platelet 12-lipoxygenase was not due to an intracellular degradation product of sphingomyelin, because neither ceramide nor phosphocholine was found to have an effect on the activity of this enzyme. Therefore, it seems that sphingomyelin itself is a putative naturally occurring regulator of 12-lipoxygenase activity in platelets.

Sphingomyelin is asymmetrically distributed in the lipid bilayer of cellular membranes (Rawyler et al., 1983). The average sphingomyelin content of liver is about 7 μmol/g fresh weight, of which about 20% is found in the plasma membrane (Pawelczyk and Lowenstein, 1992). A total of 2.2 μmol of sphingomyelin/ml (2.2 mM) are on the cytosolic side. In erythrocytes, 2.1–2.8 μmol of sphingomyelin/ml (2.1–2.8 mM) are on the cytosolic side. Comparison of the physiological concentrations of sphingomyelin with the concentration yielding 50% inhibition of platelet 12-lipoxygenase indicates that sphingomyelin is likely to be a major factor in regulating the activity of 12-lipoxygenase in platelets.

When platelets are activated, vigorous metabolism of arachidonic acid is initiated. Thromboxane A_2 is immediately formed but readily disappears due to its lability. In addition, the production of thromboxane A_2 is restricted by the suicide inactivation of cyclooxygenase (Egan et al., 1976) and thromboxane A_2 synthase (Jones and Fitzpatrick, 1990). The production of 12-HETE by 12-lipoxygenase, however, proceeds linearly (Dutilh et al., 1978), and leads to the accumulation of a considerable amount of the product. 12-HETE is essential for irreversible blood platelet aggregation and accelerates thrombus formation (Dutilh et al., 1978; Sekiya et al., 1991). Thus, it seems likely that sphingomyelin, by inhibiting 12-HETE formation, could play a regulatory role on platelet function.

The mechanism of inhibition remains to be investigated; however, we have provided the first direct evidence that sphingomyelin inhibits platelet 12-lipoxygenase activity. It is suggested that some physiological and pharmacological actions of sphingomyelin may be related to its ability to modulate platelet 12-lipoxygenase activity.

References

Dawson, R.M.C., Hemington, N., Irvine, R.F., 1985. The inhibition of diacylglycerol-stimulated intracellular phospholipases by phospholipids with a phosphocholine-containing polar group. A possible physiological role for sphingomyelin. Biochem. J. 230, 61–68.

Dutilh, C.E., Haddeman, E., Jouvenaz, G.H., Ten Hoor, F., Nugteren, D.H., 1978. Study of the two pathways for arachidonate oxygenation in blood platelets. Lipids 14, 241–246.

Egan, R.W., Paxton, J., Kuehl, F.A. Jr., 1976. Mechanism for irreversible self-deactivation of prostaglandin synthetase. J. Biol. Chem. 251, 7329–7335.

Flower, R.J., 1974. Drugs which inhibit prostaglandin biosynthesis. Pharmacol. Rev. 26, 33–67.

Fujimoto, Y., Nakatani, E., Minamino, H., Takahashi, M., Sakuma, S., Fujita, T., 1990. Effective use of rabbit gastric antral mucosal slices in prostaglandin synthesis and metabolism studies. Biochim. Biophys. Acta 1044, 65–69.

Fujimoto, Y., Takai, S., Matsuno, K., Sumiya, T., Nishida, H., Sakuma, S., Fujita, T., 1992. Effect of *tert*-butyl hydroperoxide on cyclooxygenase and lipoxygenase metabolism of arachidonic acid in rabbit platelets. Prostaglandins, Leukotrienes Essent. Fatty Acids 47, 259–264.

Hamberg, M., Samuelsson, B., 1974. Prostaglandin endoperoxides. Novel transformations of arachidonic acid in human platelets. Proc. Natl. Acad. Sci. U.S.A. 71, 3400–3404.

Hannun, Y.A., 1994. The sphingomyelin cycle and the second messenger function of ceramide. J. Biol. Chem. 269, 3125–3128.

Hope, W.C., Welton, A.F., Fiedler-Nagy, C., Batula-Bernardo, C., Coffey, J.W., 1983. In vitro inhibition of the biosynthesis of slow reacting substance of anaphylaxis (SRS-A) and lipoxygenase activity by quercetin. Biochem. Pharmacol. 32, 367–371.

Jones, D.A., Fitzpatrick, F.A., 1990. ''Suicide'' inactivation of thromboxane A_2 synthase. Characteristics of mechanism-based inactivation with isolated enzyme and intact platelets. J. Biol. Chem. 265, 20166–20171.

Merrill, A.H. Jr., Jones, D.D., 1990. An update of the enzymology and regulation of sphingomyelin metabolism. Biochim. Biophys. Acta 1044, 1–12.

Nakadate, T., Yamamoto, S., Aizu, E., Kato, R., 1985. Inhibition of 12-*O*-tetradecanoylphorbol-13-acetate-induced increase in vascular permeability in mouse skin by lipoxygenase inhibitors. Jpn. J. Pharmacol. 38, 161–168.

Pawelczyk, T., Lowenstein, J.M., 1992. Regulation of phospholipase Cδ activity by sphingomyelin and sphingosine. Arch. Biochem. Biophys. 297, 328–333.

Rawyler, A.J., Roelofsen, B., Op den Kamp, J.A.F., Van Deenen, L.L.M., 1983. Isolation and characterization of plasma membranes from Friend erythroleukaemic cells. A study with sphingomyelinase C. Biochim. Biophys. Acta 730, 130–138.

Sekiya, F., Takagi, J., Usui, T., Kawajiri, K., Kobayashi, Y., Sato, F., Saito, Y., 1991. 12*S*-Hydroxyeicosatetraenoic acid plays a central role in the regulation of platelet activation. Biochem. Biophys. Res. Commun. 179, 345–351.

Tsunomori, M., Fujimoto, Y., Muta, E., Nishida, H., Sakuma, S., Fujita, T., 1996. 15-Hydroperoxyeicosapentaenoic acid inhibits arachidonic acid metabolism in rabbit platelets more potently than eicosapentaenoic acid. Biochim. Biophys. Acta 1300, 171–176.

Vanderhoek, J.Y., Bryant, R.W., Bailey, J.M., 1980. 15-Hydroxy-5,8,11,13-eicosatetraenoic acid. A potent and selective inhibitor of platelet lipoxygenase. J. Biol. Chem. 255, 5996–5998.