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Quantification of Sphingosine Derivatives in Human Platelets: Inducible Formation of Free Sphingosine[1]

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To elucidate the physiologic role of sphingolipid-derived products as signaling molecules, we analyzed the levels of endogenous sphingosine (Sph) derivatives in human platelets. When the platelets were stimulated with thrombin or 12-*O*-tetradecanoylphorbol 13-acetate, neither ceramide formation nor sphingomyelin hydrolysis was observed, which suggests that the sphingomyelin cycle may not be an essential part of the signaling pathway under these conditions. In contrast, Sph was found to increase in platelets upon stimulation. The level of Sph 1-phosphate, which is formed from Sph by the action of Sph kinase, was not affected under our conditions. Although it has been established that Sph inhibits protein kinase C, which regulates the functional responses of the platelets, Sph levels which exert an inhibitory effect on protein kinase C cannot be attained under physiological conditions (without exogenous Sph). Considering the stimulation of the synthesis of Sph by the physiological agonist thrombin, we speculate that Sph is a signaling molecule of physiological importance in platelets, but protein kinase C may not be its target.

Key words: ceramide, platelet, protein kinase C, sphingosine, sphingosine 1-phosphate.

The process of platelet activation in the circulation is highly regulated; these anucleate cells play a central role in hemostatic reactions, whereas overactive platelets have been implicated in the pathogenesis of harmful processes such as atherosclerosis and thrombosis. Signal transduction of platelets has been studied, and they are now considered to provide an excellent model for the study of transmembrane signaling (*1-3*). It has been established that the products of glycerolipids play second messenger functions in a variety of systems, including platelets. The best evidence for this is the phosphatidylinositol cycle, which involves the stimulus-dependent hydrolysis of phosphatidylinositol bisphosphate and yields diacylglycerol (DAG) and inositol trisphosphate (*1, 2*). The roles of DAG and inositol trisphosphate in the activation of protein kinase C and intracellular Ca^{2+} mobilization, respectively, have been clarified using platelets (*1, 2*).

Although signaling networks that use glycerolipid metabolites as second messengers have been well characterized in platelets, less is known of the second messengers derived from sphingolipids, another major class of membrane lipids. The original and general hypothesis on the role of the sphingolipids in signal transduction came from the observation that sphingosine (Sph), the fundamental backbone structure of all sphingolipids, inhibits protein kinase C *in vitro* and in intact cells, of which platelets are the best example (*4-6*). In platelets, Sph was also found to promote agonist-induced phospholipase C activation, thereby enhancing platelet aggregation (*7*), and to enhance phosphatidylinositol 4-kinase activity (*8*). However, the physiologic role of Sph in platelets remains obscure; current evidence for the involvement of Sph in signal transduction or cellular function consists of data on the effects of exogenous Sph on platelet activation (*5-8*). Only a few studies have characterized the quantitative changes of Sph in intact platelets. Recently, we reported that platelets store abundant sphingosine 1-phosphate (Sph-1-P), the initial product of catabolism of Sph by Sph kinase (*9, 10*). Considering that Sph-1-P is released extracellularly upon stimulation and that exogenous Sph-1-P activates platelets (possibly *via* an interaction with a plasma membrane receptor) (*9, 11*), we have proposed that Sph-1-P acts as an intercellular messenger following its discharge from activated platelets (*11*). This is a completely different hypothesis from the postulated role of Sph-1-P in proliferating cells, where this bioactive sphingolipid has been implicated as an intracellular signaling molecule (*12, 13*). Therefore, it is suggested that the sphingolipids play a unique role in the differentiated platelets.

In this study, we analyzed the levels of endogenous Sph derivatives to get an insight into the physiological role of

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Abbreviations: Sph, sphingosine; DAG, diacylglycerol; Sph-1-P, sphingosine 1-phosphate; Cer, ceramide; Cer-1-P, ceramide 1-phosphate; TPA, 12-*O*-tetradecanoylphorbol 13-acetate.

Sph, Sph-1-P, and other sphingolipid-derived signaling molecules.

MATERIALS AND METHODS

Materials—Sph-1-P (*14*) and C_2-ceramide (C_2-Cer) (*15*) were prepared as previously described. C_2-Ceramide 1-phosphate (C_2-Cer-1-P) was formed by the acylation of Sph-1-P with acetic anhydride, which was confirmed by FAB-MS analysis (*10*).

The following materials were obtained from the indicated suppliers: C_6-Cer (Biomol, Plymouth Meeting, PA); 12-*O*-tetradecanoylphorbol 13-acetate (TPA), Sph, Cer (type III), and sphingomyelinase (Sigma, St. Louis, MO); thrombin (Mochida Pharmaceutical, Tokyo); staurosporine (Kyowa Medex, Tokyo); [^{3}H]acetic anhydride (50 mCi/mmol), [γ-^{32}P]ATP (3,000 Ci/mmol), and [3-^{3}H]C_6-Cer (22.3 Ci/mmol) (Du Pont-New England Nuclear, Boston, MA).

Preparation of Platelets—The platelets were isolated from the blood of healthy adult volunteers. The blood was anticoagulated with 3.8% sodium citrate (9 volumes of blood to 1 volume of sodium citrate), then centrifuged at $120\times g$ for 10 min to obtain platelet-rich plasma. The washed platelets were then prepared and handled as described previously (*9*).

Quantitative Measurement of Cer—Cer was measured using the *sn*-1,2-DAG assay reagent system (Amersham, UK), following the protocol provided by the manufacturer. The level of Cer-1-P, formed from Cer by DAG kinase, was determined by use of a BAS2000 image analyzer (Fuji Film, Tokyo). The Cer level was quantified by comparison with a standard curve of Cer-1-P formed by the DAG kinase reaction of 250 pmol to 1 nmol of Cer.

Quantitative Measurement of Sph and Sph-1-P—Sph (*16*) and Sph-1-P (*10*) were quantitatively measured by their *N*-acylation with [^{3}H]acetic anhydride into [^{3}H]-C_2-Cer (*N*-[^{3}H]acetylated Sph) and [^{3}H]C_2-Cer-1-P (*N*-[^{3}H]acetylated Sph-1-P), respectively, as described previously.

[^{3}*H*]C_6*-Cer Metabolism in Platelets*—Platelet-rich plasma was labeled with 5 μM [^{3}H]C_6-Cer for 2 h at 37°C. The washed platelets were then prepared and incubated with or without the stimulus. At the indicated times, the reaction was terminated by the addition of 1.875 ml of ice-cold chloroform/methanol/concentrated HCl (100:200:1). The lipids were extracted from the cell suspensions, and the phases were separated by the method of Bligh and Dyer (*17*). The lower phase samples were dried and resuspended in small volumes of chloroform/methanol (2:1). Portions of the lipid extract and the appropriate standards were applied to silica gel 60 HPTLC plates (Merck, Darmstadt, Germany), which were then developed in butanol/acetic acid/water (3:1:1). The bands were identified by staining the control lipids with primulin, then visualized under UV light, followed by autoradiography with Kodak X-Omat film at −80°C for 1-3 days. [^{3}H]C_6-sphingomyelin was confirmed by its degradation by sphingomyelinase treatment.

Protein Kinase C Activation in Intact Platelets—Protein kinase C activation in intact platelets was evaluated by pleckstrin (47-kDa protein) (*18*) phosphorylation as previously described (*19*).

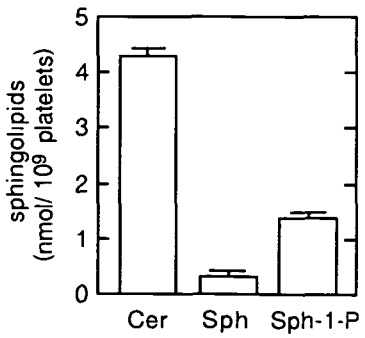

Fig. 1. **Quantification of Cer, Sph, and Sph-1-P in platelets.** The lipids extracted from human platelet suspensions were assayed for Cer, Sph, and Sph-1-P. The columns and error bars represent the mean±SD ($n=3$).

RESULTS

Failure of the Platelet Agonists to Elicit the Sphingomyelin Cycle—Cer, which is derived from the stimulus-dependent hydrolysis of sphingomyelin, is a potent biomolecule with effects in multiple signaling pathways (*20, 21*). We first measured the level of Cer in platelets by its phosphorylation into Cer-1-P. Resting platelets were found to contain about 4 nmol of Cer/10^9 platelets (Fig. 1). This level was not affected by the incubation of the platelets with thrombin or TPA (Fig. 2, upper panel), or their vehicles (data not shown). We next labeled the platelets with [^{3}H]-C_6-Cer to assess the sphingomyelinase activity in intact

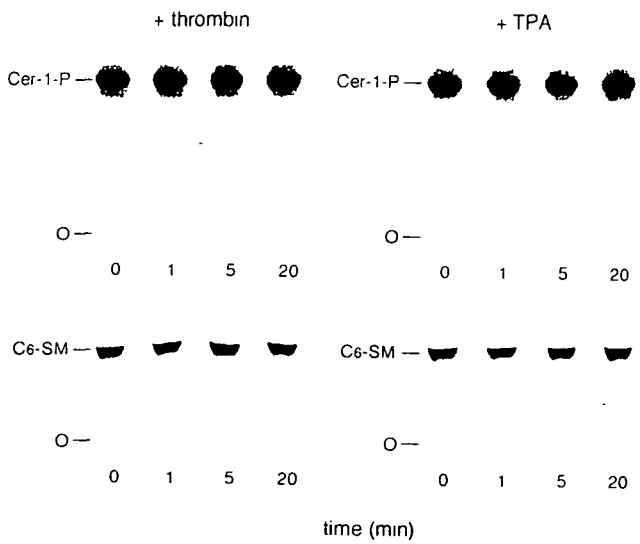

Fig. 2. **Failure of thrombin and TPA to activate the sphingomyelin cycle in platelets.** (Upper panel) The platelets were stimulated with 0.1 U/ml of thrombin or 1 μM TPA for the periods indicated below the lanes. Lipids were extracted, and Cer was assayed by its conversion into Cer-1-P with DAG kinase. A representative autoradiogram from one of three experiments is shown. (Lower panel) The platelets labeled with [^{3}H]C_6-Cer were challenged with 0.1 U/ml of thrombin or 1 μM TPA for the periods indicated below the lanes. The extracted lipids were analyzed with TLC autoradiography, as described in "MATERIALS AND METHODS." The location of C_6-sphingomyelin (SM) is indicated on the left. A representative autoradiogram from one of three experiments is shown. O, origin.

platelets. Under the conditions employed, [^{3}H]C_6-sphingomyelin was efficiently formed and the label was unchanged when there was no stimulation (data not shown). When the platelets were stimulated with thrombin or TPA, the [^{3}H]C_6-sphingomyelin radioactivities were not affected (Fig. 2, lower panel). Collagen, U46619 (the thromboxane A_2 analog), and epinephrine also did not affect the C_6-sphingomyelin levels (data not shown). These results indicate that stimulus-dependent sphingomyelin hydrolysis does not occur, at least when the platelets are challenged with common agonists.

Sph Formation in Activated Platelets—Recently, not only Cer but also Sph has emerged as a signaling molecule (*22, 23*). In platelets, the effect of exogenous Sph has been extensively studied (*5–8*). However, only a few studies have reported on the changes in the endogenous Sph levels in platelets, and the physiologic role of Sph in platelets is unknown. We previously quantitated Sph by its acylation into [^{3}H]C_2-Cer with [^{3}H]acetic anhydride and reported that resting platelets contain about 0.4 nmol of Sph/10^9 platelets (*10*). Hence, the amount of Sph present in platelets is much lower than that of Cer (Fig. 1), the substrate of ceramidase for Sph formation. When the platelets were incubated at 37°C under our conditions, a significant increase in Sph was detected even without stimulation (Fig. 3). The reason for this result is unknown. The Sph increase was significantly augmented 3 min after the platelets were stimulated with TPA, which can substitute for DAG and directly activate protein kinase C (*1, 2*), or thrombin, which produces DAG and activates protein kinase C as a result of phosphatidylinositol-4,5-bisphosphate hydrolysis (*1, 2*) (data not shown). The Sph formation induced by these protein kinase C activators was time-dependent (Fig. 3) and inhibited by staurosporine (Fig. 4), which is an inhibitor of protein kinases, including protein kinase C (*24*). Under our conditions, a marked increase in the phosphorylation of pleckstrin, an established protein kinase C substrate in platelets (*18*), was induced by TPA or thrombin, and this phosphorylation was completely inhibited by staurosporine (data not shown). These results suggest that protein kinase C is involved in stimulus-dependent Sph formation, although partial involvement of intracellular Ca^{2+} mobilization is also pos-

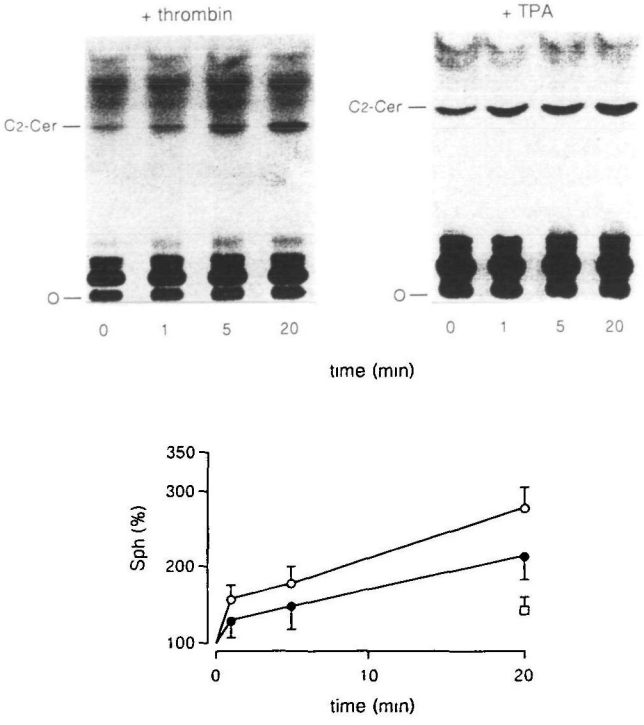

Fig. 3. **Sph increase in platelets stimulated with thrombin or TPA.** (Upper panel) The platelets were stimulated with 0.1 U/ml of thrombin or 1 μM TPA for the times indicated below the lanes, then assayed for the amount of Sph by its *N*-acylation into *N*-[^{3}H]acetylated Sph ([^{3}H]C_2-Cer) with [^{3}H]acetic anhydride. A representative autoradiogram shows the increase of the amount of Sph in stimulated platelets, as shown by the enhancement of the [^{3}H]C_2-Cer formed. (Lower panel) The Sph levels in unstimulated platelets (□) or those stimulated with thrombin (●) or TPA (○) were calculated by extrapolation from Sph standards and expressed as a percentage of the control (0 min without stimulation). The results are presented as the mean±SD (n=4).

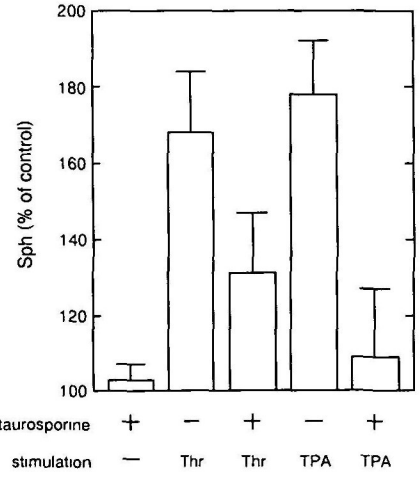

Fig. 4. **Inhibition by staurosporine of the Sph increase in platelets stimulated with thrombin or TPA.** The platelets were pretreated without or with 1 μM staurosporine for 5 min, then stimulated with 0.1 U/ml of thrombin (Thr) or 1 μM TPA for 20 min, as shown below the columns. The extracted lipids were assayed for Sph. Sph levels were expressed as a percentage of the control (parallel incubations without treatment). Columns and error bars represent the mean±SD (n=3).

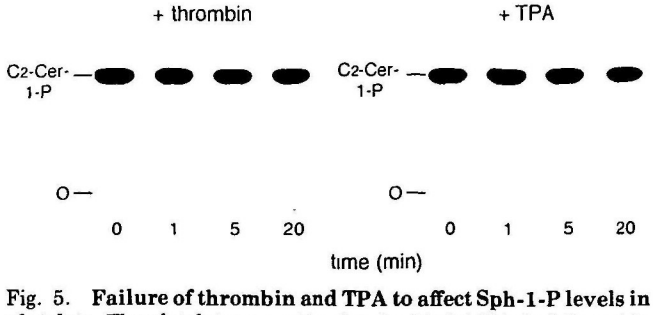

Fig. 5. **Failure of thrombin and TPA to affect Sph-1-P levels in platelets.** The platelets were stimulated with 0.1 U/ml of thrombin or 1 μM TPA for the periods of time indicated below the lanes. Lipids were extracted, and Sph-1-P was assayed by its *N*-acylation into *N*-[^{3}H]acetylated Sph-1-P ([^{3}H]C_2-Cer-1-P) with [^{3}H]acetic anhydride. A representative autoradiogram from one of three experiments is shown.

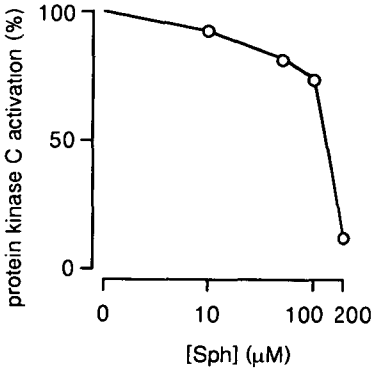

Fig. 6. **Effect of Sph on protein kinase C activation in intact platelets stimulated with TPA.** $^{32}P_i$-loaded platelets (3×10^8/ml) were pretreated with various concentrations of Sph for 1 min, then stimulated with 100 nM TPA for 2 min. The protein kinase C activation in intact platelets was evaluated by the phosphorylation of pleckstrin, a protein kinase C substrate in platelets. The results were expressed as a percentage of the control (without Sph pretreatment). Similar results were obtained in three independent experiments.

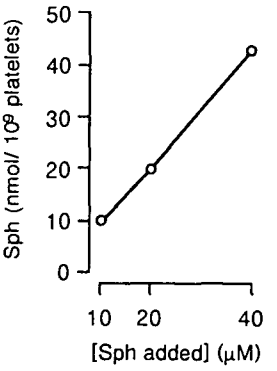

Fig. 7. **Sph levels in platelets incubated with exogenous Sph.** Platelet suspensions (3×10^8/ml) were incubated with 10, 20, or 40 μM Sph for 1 min. The lipids were then extracted from the cell pellet after separation by centrifugation and assayed for Sph.

sible; staurosporine, which abolishes thrombin-induced pleckstrin phosphorylation but not Ca^{2+} mobilization (*25*), only partially inhibits the Sph formation induced by this platelet agonist (Fig. 4).

Failure of the Platelet Agonists to Affect the Level of Sph-1-P—We recently developed a method to quantitatively measure Sph-1-P by its acylation with [^{3}H]acetic anhydride into [^{3}H]C_2-Cer-1-P and found that the unstimulated platelets contain about 1.4 nmol of Sph-1-P/10^9 platelets (*10*). The amount of Sph-1-P in the platelets was much less than that of Cer but about four times more than that of Sph (Fig. 1). The amount of Sph-1-P in the platelet suspensions was not affected by incubation with thrombin or TPA (Fig. 5), or their vehicles (data not shown). This is consistent with our previous finding that the activity of Sph kinase, which converts Sph into Sph-1-P, was not affected by thrombin or TPA (*11*).

Protein Kinase C Inhibition and Sph Levels in Platelets Incubated with Exogenous Sph—It has been established that Sph can inhibit protein kinase C and block the platelet processes dependent on this enzyme (*5, 6*), although the effects that have been reported may not be physiological but pharmacological. As described above, stimulus-dependent Sph formation was observed in platelets (Fig. 3). Accordingly, we examined the correlation between protein kinase C inhibition and cellular Sph levels in platelets incubated with (exogenous) Sph to gain an insight into the physiological role of Sph. When the washed platelets (3×10^8/ml) were incubated with Sph for 1 min, at least 20 μM Sph was needed to inhibit protein kinase C, as assessed by pleckstrin phosphorylation (Fig. 6). Under the same conditions, the cellular concentration of Sph in platelets exposed to 10 to 40 μM Sph was approximately 10 to 40 nmol/10^9 platelets (Fig. 7), which is much more than the concentration of endogenous Sph elicited by thrombin or TPA (Figs. 1 and 3). Therefore, it is unlikely that endogenous Sph is related to the inhibition of protein kinase C in platelets under physiological conditions.

DISCUSSION

The sphingolipids are a family of lipids that are found ubiquitously in eukaryotic plasma membranes, and they have emerged as active signaling molecules involved in the cell regulation process (*4, 20-23*). The putative second messenger Cer is generated by the sphingomyelin cycle, in which membrane sphingomyelin is hydrolyzed in response to extracellular stimuli (*20, 21*). Cer is degraded into Sph by the action of ceramidase (*22, 23*), and Sph is phosphorylated into Sph-1-P by Sph kinase (*22, 23*). All of these sphingolipid breakdown products are now considered to play roles in the regulation of growth, differentiation, and apoptosis (*12, 13, 20-23*). However, less is known of their physiologic roles in anucleate and highly differentiated platelets. Therefore, we analyzed the level of the endogenous sphingolipid derivatives.

Thrombin is a potent agonist that is able to activate almost all of the signaling pathways in platelets (*1, 2*). TPA potently and selectively activates protein kinase C, which plays an important role in platelets (*1, 2*). When the platelets were stimulated with various stimuli, including TPA and thrombin, neither Cer formation nor sphingomyelin hydrolysis was observed. In view of the idea that sphingomyelin breakdown products mainly participate in the regulation of cell growth, differentiation, and apoptosis in nucleate cells (*12, 13, 20-23*), the sphingomyelin cycle may not constitute an essential part of the signaling pathways of anucleate platelets.

In contrast, Sph, which is usually generated from Cer through the action of ceramidase (*22, 23*), was found to increase in platelets in a stimulus-dependent manner. We tried to identify the source of the Sph increase. Cer is not only the substrate of ceramidase for Sph formation, but also the product of Sph reacylation (*26*). Sph-1-P is formed from Sph by the action of Sph kinase and can be a source of Sph by the action of its phosphatase (*27*). However, we could not detect changes in the amounts of Cer and Sph-1-P under our present conditions, when the Sph level was increased. It may be difficult to measure accurately small changes in the amount of endogenous Cer or Sph-1-P in the platelets, because the amount of Cer or Sph-1-P is much greater than that of Sph. On the other hand, the large

changes in the amount of Sph may be caused by the low levels of Cer or Sph-1-P degradation and hence easily detected.

Sph is now considered to play a second messenger role in diverse cellular functions, including apoptosis and mitogenesis. This hypothesis originated from the observation that Sph production is enhanced in TNFα-treated human neutrophils (*28*) and TPA-treated HL60 cells (*29*), resulting in apoptosis. Recently, involvement of Sph in TNFα-induced apoptosis (*30*) and immediate negative inotropic effects (*31*) in cardiac myocytes have been reported. In rat glomerular mesangial cells, growth factors mediate proliferation through Sph formation (*32*). Furthermore, the level of Sph has been observed to change following the simple act of changing cell-culture medium (*33*), and this has been suggested to modulate protein kinase C (*34*).

Our present study is the first demonstration of inducible formation of free Sph in anucleate and highly differentiated platelets. Considering that Sph can be formed by the stimulation caused by the physiological agonist thrombin (0.1 U/ml) and that, in fact, over 0.15 U/ml of thrombin can be formed in clotting blood (*35*), it is possible that Sph is a signaling molecule that has physiological importance in platelets. Furthermore, it may be also possible to speculate that Sph is a negative feedback regulator of protein kinase C in platelets, because Sph formation correlates well with protein kinase C activation. However, we have found that the Sph levels that exert an inhibitory effect on protein kinase C cannot be attained under physiological conditions (without exogenous Sph). It is unlikely that the Sph formation in activated platelets, observed in this study, is related to the physiological regulation of protein kinase C; multiple biochemical targets and biological activities of Sph, besides its effect on protein kinase C, have been identified recently (*22, 23, 36*). Unlike most of the platelet signaling pathways (including intracellular Ca^{2+} mobilization and protein kinase C activation), which respond rapidly to agonist stimulation, Sph elevation observed in this study is a relatively slow reaction. The stimulated Sph formation may be involved not in prompt platelet responses such as aggregation and release but in a slow process like clot retraction, which, at least *in vitro*, reaches its maximum at 1.5-2 h after stimulation (*37*).

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