The Mechanism of Cold-Induced Platelet Aggregation in the Presence of Heparin

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Akiyama, M., Takami, H. and Yoshida, Y. The Mechanism of Cold-Induced Platelet Aggregation in the Presence of Heparin. Tohoku J. Exp. Med., 1995, 177 (4), 365-374 — Low temperature induces platelet aggregation, but this phenomenon is slight and poorly reproduced. However, heparin potentiated the reaction in a dose dependent manner. The degree of aggregation increased as the temperature at which the platelet-rich plasma was chilled was lowered, and as the time of chilling lengthened. Acetylsalicylic acid, a cyclooxygenase inhibitor, and staurosporin, an inhibitor of protein kinase C, partially inhibited cold-induced platelet aggregation (CIPA), suggesting that at least part of the reaction mechanism involves production of thromboxane A₂ and activation of protein kinase C. Prostaglandin E₁ (PGE₁), which inhibits platelet responses through elevating platelet cyclic AMP, completely blocked CIPA, suggesting that PGE₁ dependent pathway in platelets plays an important role for CIPA. The inhibition of CIPA by these inhibitors suggests that CIPA is aggregation with platelet activation but not platelet agglutination. Extracellular Ca²⁺ is essential for CIPA because ethylene glycol-bis (β-aminoethyl ether) N,N,N',N'-tetraacetic acid (EGTA), extracellular Ca²⁺ chelating agent, completely inhibited CIPA. Monoclonal antibodies against glycoprotein (GP) IIb/IIIa (10E5, P2) and Ang-Gly-Asp-Ser-peptide (RGDS-peptide) which inhibit fibrinogen binding to GPIIb/IIIa completely blocked CIPA but monoclonal antibodies against GPIb (6D1, SZ2) partially. In addition, CIPA occurred only when fibrinogen was added to washed platelets suspension. These results indicate that CIPA is dependent on binding of fibrinogen to GPIIb/IIIa and GPIb is partly related with the reaction. ———

platelet; aggregation; temperature; heparin

Platelets are activated by various physiological or non-physiological stimuli and aggregate. The aggregation is usually measured turbidimetrically in an aggregometer after the addition of platelet aggregating agents such as adenosine diphosphate (ADP), collagen and thrombin, and has been mainly used for the diagnosis of qualitative platelet disorders. On the other hand, it is known that platelets incubated at low temperature spontaneously aggregate in the absence of aggregating agents, i.e., cold-induced platelet aggregation (CIPA) (Zucker and Borrelli 1960; Anstall and Hawkey 1962; Kattlove and Alexander 1971). But
little is known about the mechanism of this phenomenon, because the extent of aggregation is slight and reproducibility is poor. We have previously reported that heparin markedly potentiated CIPA. In the present study, we, therefore investigated the mechanism of the platelet aggregation induced by cold.

**MATERIALS AND METHODS**

Unfractionated sodium heparin was purchased from Novo (Copenhagen, Denmark). Ethylene glycol-bis (β-aminoethyl ether) N, N, N', N'-tetraacetic acid (EGTA), acetylsalicylic acid (ASA), prostaglandin E₁ (PGE₁), staurosporin, fibronectin, fibrinogen and N-2-hydroxyethylpiperazine-N'-2-ethane-sulfonic acid (HEPES) were purchased from Sigma Chemical Corp. (St. Louis, MO, USA). Arg-Gly-Asp-Ser peptide (RGDS-peptide) was obtained from Peptide Institute Inc. (Osaka) and von Willebrand Factor (vWF) from Calibicochem Novalicochem Corp. (San Diego, CA, USA). Murine monoclonal antibodies against human platelet membrane glycoprotein (GP) Ib (6D1) and glycoprotein IIb/IIIa (10E5) were kindly provided by Dr. Barry Coller (State University of New York, Stony Brook, NY, USA). Other monoclonal antibodies against GPIb (SZ2) and GPIIb/IIIa (P2) were purchased from Immunotech S.A. (Marseille, France).

The experiments were carried out by fresh human blood from healthy subjects who had not taken any drugs which affect platelet function for at least 10 days. The blood was drawn from an antecubital vein and immediately placed into plastic tubes containing heparin or 0.1 volume of 3.8% sodium citrate as anticoagulant. Platelet rich plasma (PRP) was obtained by centrifugation at 200 g for 10 min at room temperature. The platelet concentration in PRP was adjusted to approximately 30 x 10⁴/μl with autologous platelet poor plasma (PPP).

**Cold-induced platelet aggregation**

Twenty hundreds μl of heparinized or citrated PRP was pipetted in a cuvette with a magnetized stir bar and incubated at various temperatures. After the incubation a cuvette containing PRP was placed in a PAM-6 channels aggregometer (Mebanix Corp., Tokyo) and platelet aggregation was turbidimetrically measured with stirring at room temperature.

**Platelet function inhibitors**

The effect of platelet function inhibitors (ASA, PGE₁ and staurosporin) on CIPA was studied. Twenty μl of the inhibitors or control saline was added to 180 μl of heparinized PRP and then incubated at room temperature for 10 min (as for ASA, incubation at 37°C for 30 min). Then PRP was incubated at 4°C for 60 min and cold-induced platelet aggregation was measured in the aggregometer. The final concentration of inhibitors was 0.25 mM (ASA), 5 μM (PGE₁) and 5 μM (staurosporin), respectively.
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Effect of monoclonal antibodies and RGDS-peptide

Monoclonal antibodies against human platelet membrane GPIb (6D1, SZ2) and GPIIb/IIIa (10E5, P2), or RGDS-peptide were incubated with PRP. Twenty μl of each monoclonal antibody was added to 180 μl of heparinized PRP (final concentration of each antibody; 20 μg/ml, RGDS; 1 mM). After the incubation for 10 min at room temperature, PRP was placed for 60 min at 4°C and CIPA was measured in the aggregometer.

Effect of plasma proteins

The blood was drawn into 0.1 volume of 3.8% sodium citrate. PRP was obtained by centrifugation at 200 g for 10 min. The platelets were pelletted by centrifugation at 1,600 g for 15 min and washed three times in HEPES-Tyrode buffer (NaCl 137 mM, KCl 3 mM, NaHCO₃ 12 mM, NaH₂PO₄ 0.3 mM, MgCl₂ 1 mM and HEPES 5 mM, PH 7.4) containing 5 mM ethylenediamine tetraacetic acid (EDTA). Washed platelets were finally resuspended in HEPES-Tyrode buffer, PH 7.4 without EDTA and the platelet count was adjusted to 30×10⁴/μl. The washed platelet suspension was incubated at 4°C for 60 min in the presence of heparin (10 U/ml) and CaCl₂ (2 mM), and the platelet aggregation was measured in the aggregometer after the addition of fibrinogen (at a range of final concentration from 0.25 to 1.0 mg/ml), fibronectin (200 μg/ml), vWF (20 μg/ml) or control saline.

RESULTS

Cold-induced platelet aggregation

Chilled platelets spontaneously aggregated only when they were stirred. Heparin potentiatted the aggregation in a dose dependent manner, reaching the maximum at a heparin concentration of about 10 U/ml (Fig. 1), whereas the spontaneous aggregation did not occur in heparinized PRP which was incubated at room temperature. Furthermore, the spontaneous aggregation diminished when the chilled PRP was allowed to warm at 37°C without stirring and almost completely disappeared after 10 min of warming (Fig. 2). The degree of spontaneous aggregation of chilled platelets increased as the temperature for incubation lowered (Fig. 3). The aggregation also augmented with the incubation time of chilling and reached maximum at about 60 min (Fig. 4).

Effect of platelet function inhibitors

To study the mechanism of CIPA, heparinized PRP was pretreated with several platelet function inhibitors. PGE₁ (5 μM) completely inhibited CIPA. On the other hand, ASA (0.25 mM) and staurosporin (5 μM) partially blocked CIPA by approximately 30 to 40%. Chelating extracellular Ca²⁺ by 2 mM EGTA also completely abolished CIPA (Fig. 5).
Effect of monoclonal antibodies and RGDS-peptide

Monoclonal antibodies against GPIIb/IIIa (10E5 and P2) strongly inhibited CIPA by more than 94%, whereas monoclonal antibody against GPIb (6D1 and SZ2) slightly inhibited by approximately 24%. In the presence of RGDS-peptide, CIPA was nearly totally inhibited (Fig. 6).
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Effect of plasma proteins

CIPA required plasma proteins because chilled washed platelets in the buffer containing no plasma proteins did not aggregate spontaneously even in the presence of heparin. Therefore, in order to identify the plasma proteins required for CIPA, platelet aggregation was measured with the washed platelets in the presence of fibrinogen, fibronectin or vWF. Washed platelet aggregation after
Fig. 5. Effect of platelet aggregation inhibitors on CIPA
Heparinized PRP (5 U/ml) was pretreated with EGTA (2 mM), ASA (0.25 mM), PGE₁ (5 μM), staurosporin (5 μM) or saline (control) prior to the incubation for 60 min at 4°C and was immediately measured in the aggregometer (representative experiment).

Fig. 6. Effect of MAb against GP Ib (6D1, SZ2) and GP IIb/IIIa (10E, P2), and RGDS-peptide on CIPA.
Heparinized PRP (5 U/ml) was pretreated 6D1, SZ2, 10E5, P2 (each other 20 μg/ml), RGDS-peptide (1 mM) and saline (control) prior to the incubation for 60 min at 4°C and then was immediately measured in the aggregometer (representative experiment).
Cold incubation (4°C) was observed only in the presence of fibrinogen. The maximum aggregation was also increased in a dose-dependent manner with increasing concentration of fibrinogen (Fig. 7).

**DISCUSSION**

CIPA slightly occurred in citrated PRP and heparin remarkably enhanced this aggregation in a dose dependent manner. On the other hand, the aggregation did not occur in heparinized PRP that was incubated at room temperature. These results indicate that the increase of CIPA in heparinized PRP results from the proaggregatory effect of heparin (Thomson et al. 1973; Chen and Sylven 1992) and not from platelet aggregation induced by heparin itself (Eika 1972; Saba et al. 1984; Brace and Fareed 1985). Chilled platelets spontaneously aggregated only when they were stirred and the spontaneous aggregation diminished when the chilled PRP was allowed to warm at 37°C. Furthermore, the degree of aggregation of chilled platelets increased as the temperature was lowered and as the time of chilling was lengthened. These observations are consistent with those of Kattlove and Alexander (1971), who studied CIPA in citrated PRP.

In order to elucidate the mechanism, several inhibitors of platelet function were studied for their effect on CIPA. ASA which inhibits platelet cyclooxygenase and thromboxane A₂ production partially inhibited CIPA, indicating that at least part of the mechanism is associated with the production of thromboxane A₂. Staurosporin (Oka et al. 1986; Watson et al. 1988), a potent inhibitor
of protein kinase C (PKC) in platelets, partially blocked CIPA. Although the inhibitory effect of staurosporin on PKC is not specific, the activation of PKC in chilled platelets may be partly involved in CIPA. PGE$_1$ interferes with platelet activation through elevating platelet cyclic AMP (Feinstein et al. 1983; Pannocchia and Hardisty 1985). This result suggests that PGE$_1$ dependent pathway in chilled platelets plays an important role for CIPA. EGTA completely inhibited CIPA, suggesting that CIPA requires extracellular Ca$^{2+}$. The inhibition of CIPA by these inhibitors in chilled platelets suggests that CIPA is aggregation with platelet activation but not platelet agglutination.

Agonist-induced platelet aggregation is dependent on the binding of fibrinogen to GPIIb/IIIa on platelet surface membrane (Gogstad et al. 1982; Nachman and Leung 1982) and ristocetin-induced platelet agglutination depends on the interaction GPIb and vWF (Gralnick and Coller 1977). However, it is uncertain whether the major membrane glycoproteins such as GPIb and GPIIb/IIIa are involved in CIPA. To investigate this point, we treated heparinized PRP with 6D1 and SZ2 (monoclonal antibodies against GPIb), 10E5 and P2 (monoclonal antibodies against GPIIb/IIIa) or synthetic RGDS-peptide which antagonizes at GPIIb/IIIa (Bennett and Gartner 1985; Haverstick et al. 1985), and found that 10E5, P2 and RGDS-peptide completely inhibited CIPA and 6D1, SZ2 only partially. These results indicate that GPIIb/IIIa is essential for CIPA, and GPIb is partly involved in this aggregation.

CIPA requires plasma proteins since chilled platelets suspended in plasma protein free medium did not aggregate spontaneously. We identified this plasma protein as fibrinogen, because CIPA was observed only when fibrinogen was added to chilled platelets suspension. Addition of fibronectin and vWF showed no effect on CIPA. With the result that monoclonal antibody against GPIIb/IIIa strongly inhibited CIPA, it appears that the binding of fibrinogen to platelet GPIIb/IIIa is essential for the aggregation. The molecular mechanism by which cold may induce platelet aggregation remains unknown at present. Kattlove and Alexander (1971) have proposed that cold in citrated PRP may induce platelet aggregation by causing conformational changes of platelet membrane proteins, because protein conformation is affected by changes of temperature. With respect to the mechanism of shear-induced platelet aggregation which does not require any agonists like CIPA, Peterson et al. (1987) have proposed that shear stress affects the conformation of GPIb and GPIIb/IIIa, inducing the binding of vWF in plasma and the glycoproteins on platelets. Therefore, the possible mechanism of CIPA is that cold may alter some characteristic of platelet glycoproteins, especially GPIIb/IIIa, so that GPIIb/IIIa is capable of binding fibrinogen. The mechanism of proaggregatory effect of heparin in CIPA is unclear, however, heparin may potentiate this phenomenon.

From the results above, it is suggested that CIPA is aggregation with platelet activation and is dependent on binding of fibrinogen to GPIIb/IIIa and extracel-
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CIP A is a unique platelet aggregation because it does not require any platelet agents and occurs only under low temperature. Studies on the platelet activation have been mainly on agonist-induced platelet aggregation. Therefore, further study of CIPA should provide a clue to the molecular mechanism of platelet activation.

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References


