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Integrin $\alpha_v\beta_3$ and phospholipase C regulate prostacyclin formation of endothelial cells caused by ancrod-generated fibrin

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Abstract

Ancrod-generated fibrin has been shown to stimulate prostacyclin synthesis of human umbilical vein endothelial cells (Chang et al., 1994, Biochem. Biophys. Res. Commun. 203, 1920). We further investigated its mechanism of action. The increment of 6-keto prostaglandin $F_{1\alpha}$ stimulated by ancrod-generated fibrin was almost completely inhibited when endothelial cells were either pretreated with 50 μM 8-(*N*,*N*′-diethylamino)octyl-3,4,5-trimethoxybenzoate (TMB-8) or preloaded with 15 μM 1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid (BAPTA). 6-Keto prostaglandin $F_{1\alpha}$ production during 2 and 10 h incubation period was also inhibited by 1.2 mM ethyleneglycol-bis-(β-aminoethylether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA) (41 ± 12 and 53 ± 17% inhibition, respectively). Further, ancrod-generated fibrin caused a rapid-onset increase in [^{3}H]inositol monophosphate (IP_1) formation in endothelial cells. This increase in IP_1 was significantly inhibited by 1 mM Gly-Pro-Arg-Pro, 1 mM neomycin or 100 ng/ml pertussis toxin. At the same time, neomycin and pertussis toxin also significantly inhibited 6-keto prostaglandin $F_{1\alpha}$ synthesis of endothelial cells stimulated by ancrod-generated fibrin. Additionally, the increment of IP_1 production as well as prostacyclin production were significantly inhibited by monoclonal antibodies directed against $\alpha_v\beta_3$. These results suggest that intra- and extra-cellular Ca^{2+} participate in prostacyclin synthesis stimulated by ancrod-generated fibrin. Ancrod-generated fibrin stimulates pertussis toxin-sensitive G-protein regulated phosphoinositide breakdown, which is responsible for prostacyclin synthesis. This augmentation in prostacyclin synthesis and phosphoinositide breakdown caused by ancrod-generated fibrin are, at least in part, mediated by fibrin binding to integrin $\alpha_v\beta_3$ on endothelial cells.

Keywords: Ancrod-generated fibrin; Integrin $\alpha_v\beta_3$; Prostacyclin; Pertussis toxin; Phospholipase C

1. Introduction

It is well known that the endothelial cells play important roles in the regulation of vascular tone (Henrich, 1991; Nabel, 1991), coagulation and fibrinolysis, cellular growth and inflammatory responses (Camussi et al., 1991; Cavender, 1991; Gottlieb et al., 1991). Various states of disease may lead to a deposition of fibrin on the endothelial cell surface by destroying the balance between pro- and anti-coagulant state (Tanaka and Sueishi, 1993). Formation of fibrin stabilizes the hemostatic plug and supports wound healing and revascularization at the site of injured blood vessels. The effect of fibrin on cultured endothelial cells has been studied (Tanaka and Sueishi, 1993). Short-term incubation of human umbilical vein endothelial cells with fibrin led to an increased release of von Willebrand factor (Ribes et al., 1987). Fibrin formed by the addition of $CaCl_2$ to citrated plasma also altered synthesis of prostacyclin and tissue-type plasminogen activator by human endothelial cells (Kaplan et al., 1989). The interaction of endothelial cell monolayers with fibrin caused a morphologic change of cells (Watanabe and Tanaka, 1983; Weimar and Delvos, 1986), and the degree of morphologic change correlated with the concentration of fibrin (Watanabe and Tanaka, 1983). However, the mechanisms of these actions are unclear.

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Ancrod is a thrombin-like enyzme purified from the venom of Malayan pit viper, *Calloselasma rhodostoma*. It selectively releases fibrinopeptide A from fibrinogen and causes fibrin polymerization without activating Factor XIII (Barlow et al., 1970; Ewart et al., 1970) and it has little effect on other coagulation factors (Martin et al., 1971). Ancrod-generated fibrin was rapidly removed from the circulation, either through the activation of the fibrinolytic system (Regoeczi et al., 1966) or through phagocytosis by the reticulo-endothelial system (Siberman et al., 1973), thus resulting in a rapid defibrinogenation (Mahir et al., 1987). Ancrod has been used in the prevention and treatment of a wide range of thrombotic disorders (Lattallo, 1983; Bell, 1988).

We recently investigated the ex vivo antiplatelet effect of ancrod in rabbits and found that ancrod caused a significant increase in prostacyclin level upon administration to rabbits (Chang and Huang, 1995) and ancrod-generated fibrin stimulated prostacyclin synthesis of human endothelial cells via de novo synthesis of cyclooxygenase (Chang et al., 1994). In most tissues the rate-limiting step in prostacyclin biosynthesis is the release of arachidonic acid from phospholipids by endogenous phospholipase A_2 (Chang et al., 1987), and this enzyme activity is enhanced by the rise in cytosolic free Ca^{2+} (Hallam et al., 1988).

Due to this apparently physiological and pathological significance of the interaction of endothelial cells with fibrin, it is very important to investigate the mechanism involved. However, the use of thrombin to generate fibrin may not be a suitable approach to examine this issue because thrombin itself exerts directly many effects on endothelial cells (Demichele and Minner, 1992; Galdal et al., 1982; Gelehrter and Sznycer-Laszuk, 1986; Weksler et al., 1978). In contrast to thrombin, ancrod does not affect other coagulation factors, nor does it directly affect endothelial cell prostaglandin and inositol lipid metabolism when added in culture medium. Therefore, we examined the mechanism of prostacyclin synthesis of endothelial cells stimulated by fibrin generated by the thrombin-like enzyme, ancrod.

2. Materials and methods

2.1. Materials

Medium 199, fetal bovine serum, mixture of penicillin with streptomycin sulfate were obtained from Gibco, Grand Island, NY, USA. Endothelial cell growth supplement (ECGS) was obtained from Collaborative Research, Bedford, MA, USA. 1,2-Bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid/acetoxymethyl ester (BAPTA/AM) was purchased from Molecular Probes (Eugene, OR, USA). Collagenase, Gly-Pro-Arg-Pro (GPRP), neomycin, 8-(*N*,*N*′-diethylamino)octyl-3,4,5-trimethoxybenzoate (TMB-8), pertussis toxin, dodecapeptide of fibrinogen γ chain (γ 400–411), ethyleneglycol-bis(β-aminoethylether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), Dowex-1 (100–200 mesh: X8 chloride form) were purchased from Sigma Chemical Company, St. Louis, MO, USA. [^{3}H]Myoinositol was purchased from Amersham, England. An enzyme-linked immunosorbent assay (Elisa) kit for quantifying 6-keto prostaglandin $F_{1\alpha}$ was purchased from Cayman Chem Co., Ann Arbor, MI, USA. Monoclonal antibody to $\alpha_v\beta_3$ integrin (23C6) was purchased from Serotec, Oxford, UK. 7E3, a monoclonal antibody against platelet glycoprotein IIb-IIIa complex, which is cross-reactive with $\alpha_v\beta_3$ (Grossi et al., 1988; Martinez et al., 1989) was donated by Dr. Barry Coller, State University of New York, Stony Brook, NY, USA. Monoclonal antibody AP1, recognizing glycoprotein Ib-like protein on human endothelial cells (Montgomery et al., 1983; Asch et al., 1988; Sprandio et al., 1988) was generously provided by Dr. Robert Montgomery (Blood Center of Southeastern Wisconsin, Milwaukee, MI, USA).

Ancrod was purified from the venom of *Calloselasma rhodostoma* (Latoxan, France) through columns of Sephadex G-75, DEAE-Sephadex A-50 and heparin-Agarose. Subsequently, it was further purified by high performance liquid chromatography (HPLC) using gel filtration column (PROTEIN PAK 300 sw, Waters). This purified protein migrates as a single band as judged by sodium dodecyl sulphate/polyacrylamide gel electrophoresis (SDS/PAGE) and it contains only a single amino-terminal residue. Its NH_2-terminal sequence (Val-Ile-Gly-Gly-Asp-) was identical with that deduced from the cDNA sequence (Au et al., 1993). The specific activity of this ancrod preparation is around 1000 thrombin unit per mg in causing blood clot formation.

Fresh citrated platelet-poor plasma was prepared from human whole blood anticoagulated with 3.8% sodium citrate (9:1, v/v) by stepwise centrifugation. After centrifuging at $200 \times g$ for 10 min the supernatant obtained was subsequently centrifuged by an Eppendorf centrifuge (Model 5414) at 10000 rpm for 2 min. Aprotinin was added to this platelet-poor plasma for excluding a possible perturbation of fibrin degradation products on prostacyclin synthesis in this experiment (Chang et al., 1995a).

2.2. Endothelial cell culture

Human umbilical vein endothelial cells were prepared as described before (Jaffe et al., 1973). Endothelial cells were grown in medium 199 containing 2.2 mg/ml of sodium bicarbonate supplemented with 20% heat-inactivated fetal bovine serum, 150 μg/ml ECGS,

penicillin (100 IU/ml) and streptomycin (100 μg/ml). Cells were grown in 12-well plates (except as noted) at 37°C under 5% CO_2 and the medium was replaced at the first day after seeding and every 2 days thereafter. Cells were identified by their positive staining for von Willebrand factor antigen by indirect immunofluorescence. Confluent primary cultured cell monolayers were used 4–5 days after the cells were seeded.

2.3. Prostacyclin formation of endothelial cells

After the removal of the culture medium, the confluent primary cell monolayer cultured in 6-well plates was washed twice with phosphate-buffered saline (PBS) and the medium was replaced with 1 ml of Medium 199 or plasma. Ancrod was then added to the medium or plasma to detect any direct or indirect effect on endothelial cells. Fibrin formation was initiated upon the addition of ancrod to fresh citrated plasma. The supernatant of culture was taken at the indicated time intervals and mixed with indomethacin (50 μM) and EDTA (5 mM). The mixture was centrifuged to remove fibrin and then kept frozen at −20°C until tested. If there were inhibitors being tested, they were added to plasma 10 min at 37°C before the addition of ancrod except where indicated in the text.

2.4. Assay of 6-keto prostaglandin $F_{1\alpha}$

6-Keto prostaglandin $F_{1\alpha}$, the stable metabolite of prostacyclin, was measured using an Elisa kit according to the manufacturer's procedure.

2.5. Measurement of inositol phosphates

The procedure was carried out according to a previously described method (Brock and Capasso, 1988). In brief, confluent monolayer cells were incubated for 48 h in growth medium containing 5 μCi/ml of [^{3}H]myoinositol (specific activity, 16.2 Ci/mmol). Before experiments, cells were washed with PBS and treated with inhibitors as indicated. LiCl (10 mM) was added to plasma medium 20 min prior to the addition of ancrod. Incubation was terminated at 30 min by removing the fibrin clot and 500 μl of ice-cold trichloroacetic acid (100 mg/ml) were added. The mixture was centrifuged at 14000 rpm for 4 min. Trichloroacetic acid was removed by extracting the resultant supernatant with 10 ml of diethyl ether 3 times and the residual diethyl ether was finally evaporated off by heating samples at 70°C for 5 min. Samples were neutralized with 1 N NaOH to pH 7.2 and loaded onto Dowex-1 chromatography columns (bed volume, 1 ml) and inositol phosphates were eluted as described previously. In this experiment, only [^{3}H]inositol monophosphate (IP_1) was measured as an index of the total inositol phosphate formation, because the levels of inositol bisphosphate and inositol trisphosphate were very low.

2.6. Adhesion assay

The fibrin-coated plates were prepared as follows. Fibrinogen (50 μl of 2 mg/ml) in Medium 199 was incubated with ancrod (0.1 U/ml) in each well at 37°C for 2 h; firm clots resulted. Each well was then washed once with Medium 199 and further incubated with 1% bovine serum albumin for 30 min at 37°C. The plates were washed 3 times with Medium 199 and the adhesion assay was then carried out.

Endothelial cells were grown as monolayers in tissue culture plates and metabolically labeled for 72 h with [^{3}H]leucine (50 μCi/ml; 120 Ci/mol). The cells were harvested and suspended in serum-free Medium 199. Prior to the adhesion assay, cells were allowed to incubate with 23C6 or 7E3 for 20 min at 37°C. Endothelial cells (2×104 cells) were added to individual wells of 96-well plates that were previously coated with fibrin. Nonadherent cells were removed by aspiration following three washes. Finally, 200 μl of 2 N NaOH with 0.2% Triton X-100 were added to each well and the radioactivity counted. Cell adhesion was expressed in cpm. The radioactivity of the noncoated sample was found to be less than 200 cpm. All data (cpm) are expressed after subtracting the radioactivity of the noncoated well.

2.7. Data anaylsis

Statistical significance was determined by the Student's *t*-test, and a P value of < 0.05 was chosen to denote statistical significance between control and experimental groups. All experiments were repeated at least 3 times using seperate preparations.

3. Results

3.1. Effect of BAPTA or TMB-8 on 6-keto prostaglandin $F_{1\alpha}$ formation of endothelial cells stimulated by ancrod-generated fibrin

As shown in Fig. 1, BAPTA/AM (15 μM loading for 30 min) or TMB-8 (50 μM, 10 min) pretreatment profoundly inhibited 6-keto prostaglandin $F_{1\alpha}$ formation of endothelial cells stimulated by ancrod-generated fibrin throughout all the experimental periods observed. BAPTA and TMB-8 showed 85.7% and 97.4% inhibition during a 24 h incubation of endothelial cells with ancrod in plasma medium. Additionally, this increase in 6-keto prostaglandin $F_{1\alpha}$ of endothelial cells during the 2–10 h incubation period was com-

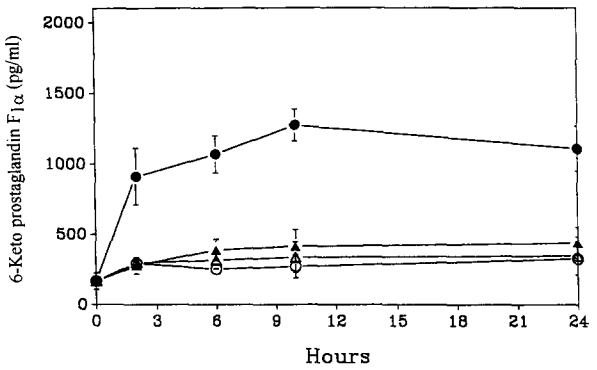

Fig. 1. Effect of BAPTA/AM and TMB-8 on 6-keto prostaglandin $F_{1\alpha}$ production induced by ancrod-generated fibrin. BAPTA/AM (15 μM, 30 min, ▲) or TMB-8 (50 μM, 10 min, △) was preincubated in 100 KIU/ml aprotinin-containing plasma at 37°C prior to the addition of ancrod (0.02 U/ml). 6-Keto prostaglandin $F_{1\alpha}$ levels in the medium of the cultured endothelial cells after the addition of ancrod (●, ▲, △) in the presence of plasma were determined at the indicated time intervals. Open circle curve represents the 6-keto prostaglandin $F_{1\alpha}$ levels in the absence of ancrod. Data are expressed as mean ± S.E.M. ($n = 3$).

pletely inhibited by Gly-Pro-Arg-Pro (5 mg/ml) pretreatment (data not shown).

3.2. Effect of extracellular Ca^{2+} on 6-keto prostaglandin $F_{1\alpha}$ production

EGTA (1.2 mM) inhibited the enhanced production of 6-keto prostaglandin $F_{1\alpha}$ of endothelial cells cultured in ancrod-treated plasma during the 2 and 10 h incubation by 41 ± 12 and 53 ± 17%, respectively (Fig. 2). However, both nifedipine (2 and 10 μM) and verapamil (25 and 50 μM) showed little effects on 6-keto prostaglandin $F_{1\alpha}$ formation.

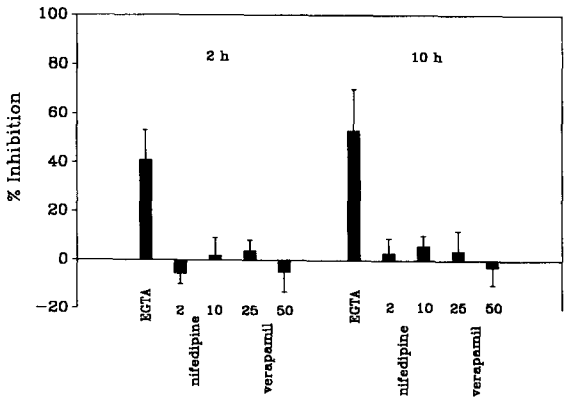

Fig. 2. Effect of Ca^{2+} antagonists on 6-keto prostaglandin $F_{1\alpha}$ formation of endothelial cells stimulated by ancrod-generated fibrin. Cells were incubated with ancrod (0.02 U/ml) in citrated plasma for 2 or 10 h at 37°C, and 6-keto prostaglandin $F_{1\alpha}$ levels were then determined. EGTA (1.2 mM), nifedipine (2, 10 μM) or verapamil (25, 50 μM) was added 10 min prior to the addition of ancrod. Data are expressed as precentage inhibition of control ($n = 3$).

3.3. Phosphoinositide breakdown of endothelial cells

Ancrod itself had no effect on phosphoinositide breakdown of endothelial cells when this preparation was cultured in Medium 199 (data not shown). However, when Medium 199 was replaced by plasma, ancrod increased IP_1 production of endothelial cells (Fig. 3). At 0.01 and 0.005 U/ml, ancrod in plasma medium caused a significant increase in IP_1 level, reaching the maximal level at the first 1 and 2 h, respectively (Fig. 3A). Ancrod at 0.02 U/ml induced fibrin formation and IP_1 production at a faster fashion than did ancrod at 0.01 or 0.005 U/ml. Ancrod (0.02 U/ml) increased IP_1 level to the maximal level at 30 min.

The IP_1 increment caused by ancrod (0.02 U/ml)-generated fibrin during the first 30 min incubation

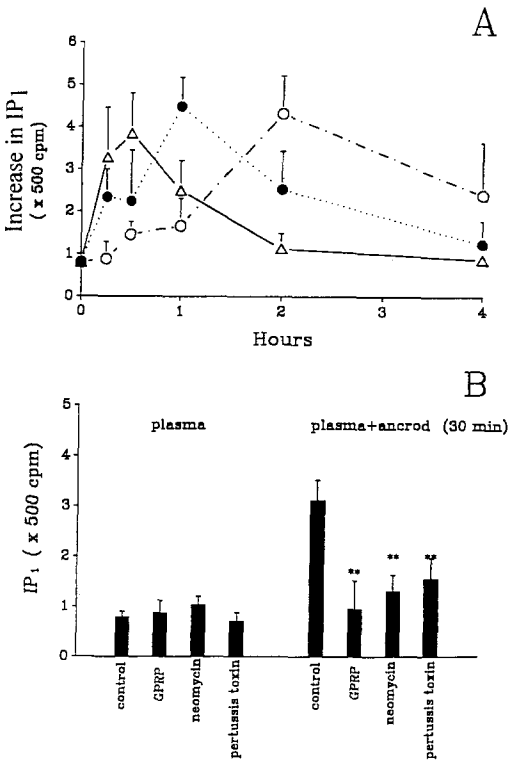

Fig. 3. Inositol monophosphate formation of [^{3}H]inositol-labeled endothelial cells caused by ancrod in plasma medium. (A) Endothelial cells were pretreated with 10 mM LiCl and incubated in platelet-poor plasma medium, and ancrod (○, 0.005 U/ml; ●, 0.01 U/ml; △, 0.02 U/ml) was then added to the cells and incubated at 37°C for various time intervals. At the indicated time intervals, reactions were quenched by the addition of trichloroacetic acid. IP_1 level was then determined as described in Methods. (B) Endothelial cells were incubated in plasma in the absence (control) or presence of GPRP (1 mM) or neomycin (1 mM) for 10 min, or cells were pretreated with pertussis toxin (100 ng/ml) at 37°C for 16 h prior to the addition of ancrod (0.02 U/ml). After 30 min of incubation, IP_1 levels of endothelial cells were determined. IP_1 levels were very low and did not change throughout experimental time course in the absence of ancrod. Values are expressed as means ± S.E.M. ($n = 6$).

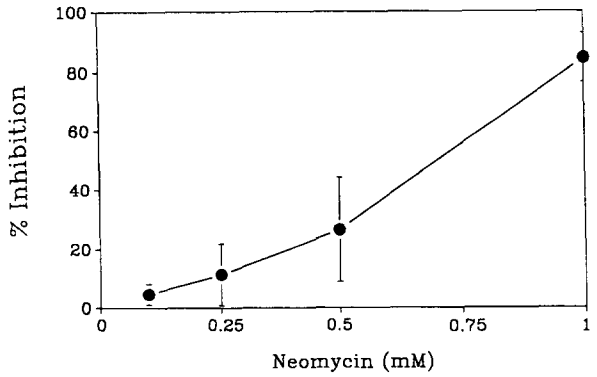

Fig. 4. Neomycin inhibited 6-keto prostaglandin $F_{1\alpha}$ production in a concentration-dependent manner. Percentage inhibition was obtained by comparing the 6-keto prostaglandin $F_{1\alpha}$ level in the presence of neomycin (at various concentrations) to that of control (without neomycin). Ancrod at 0.02 U/ml was added to aprotinin-containing platelet-poor plasma medium of cultured confluent endothelial cells which were further incubated for 10 h at 37°C. Data are expressed as means ± S.E.M. ($n = 4$).

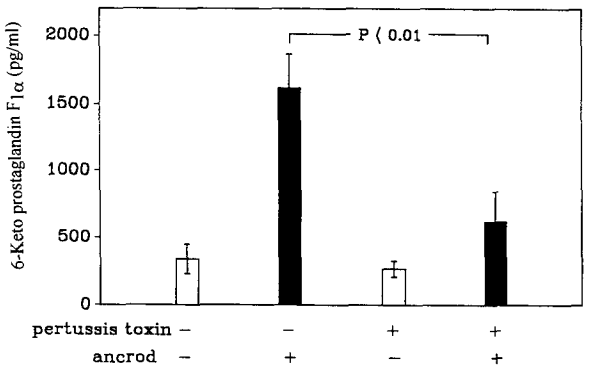

Fig. 5. Effect of pertussis toxin on ancrod-generated fibrin-stimulated 6-keto prostaglandin $F_{1\alpha}$ synthesis. Endothelial cell monolyers were preincubated with pertussis toxin (100 ng/ml) for 16 h, followed by the addition of ancrod (0.02 U/ml, black bar) to citrated plasma and incubated for 10 h. Values are expressed as means ± S.E.M. ($n = 4$).

period was completely inhibited by Gly-Pro-Arg-Pro (1 mg/ml), which completely prevented fibrin formation through the experimental period, and was significantly inhibited by 1 mM neomycin and pertussis toxin (100 ng/ml, 16 h) (Fig. 3B).

3.4. The effects of neomycin or pertussis toxin on 6-keto prostaglandin $F_{1\alpha}$ formation of endothelial cells

Neomycin inhibited 6-keto prostaglandin $F_{1\alpha}$ generation of endothelial cells in a concentration-dependent manner (Fig. 4). Neomycin at 1 mM inhibited 6-keto prostaglandin $F_{1\alpha}$ production by 84.6 ± 8.1% during a 10 h incubation after ancrod was added to the plasma medium.

When endothelial cells were pretreated with 100 ng/ml of pertussis toxin for 16 h in culture medium, this pretreatment caused a significant inhibition (77.7 ± 3.6%) on the enhanced 6-keto prostaglandin $F_{1\alpha}$ production of endothelial cells caused by ancrod-generated fibrin (Fig. 5).

3.5. The role of integrin $\alpha_v\beta_3$ on the phosphoinositide breakdown and 6-keto prostaglandin $F_{1\alpha}$ formation

The increment of IP_1 stimulated by ancrod in plasma medium was significantly inhibited by 7E3 and 23C6 (Fig. 6). 23C6 (1:200 and 1:100) or 7E3 (25 mg/ml) treatment reduced IP_1 level from 4.0 ± 0.7-fold to 2.0 ± 0.5, 1.7 ± 0.4 and 1.9 ± 0.6-fold, respectively. On the other hand, we also examined the effects of these compounds on 6-keto prostaglandin $F_{1\alpha}$ formation of endothelial cells. 7E3 (25 μg/ml) and 23C6 (1:100) significantly inhibited the increment of 6-keto prostaglandin $F_{1\alpha}$ stimulated by ancrod-generated fibrin to a similar extent (53–57% inhibition) (Fig. 7). Monoclonal antibody AP1 (80 μg/ml) and C-terminal peptide of fibrinogen γ chain, γ 400–411 (500 μM) showed little effect either on IP_1 or on 6-keto prostaglandin $F_{1\alpha}$ increment.

3.6. Effect of monoclonal antibodies 23C6 and 7E3 on endothelial cell attachment to fibrin

Cell attachment to ancrod-generated fibrin was inhibited by 23C6 (1:50) and 7E3 (30 μg/ml) by approx-

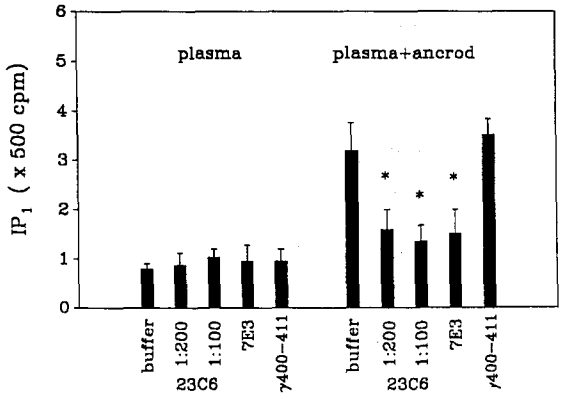

Fig. 6. Effects of monoclonal antibodies against $\alpha_v\beta_3$, and γ 400–411 on IP_1 production stimulated by ancrod-generated fibrin. 7E3 (25 μg/ml) or 23C6 (1:200, 1:100) was added to plasma of endothelial cell monolayers at 37°C for 10 min and followed by aspiration and readdition with fresh plasma prior to the addition of 0.02 U/ml ancrod. γ 400–411 (500 μM) was added to plasma for 10 min at 37°C; 0.02 U/ml ancrod was then added to cause fibrin formation on endothelial cell monolayers. The IP_1 level was determined after a 30 min incubation. Data are expressed as means ± S.E.M. ($n = 5$). * $P < 0.05$.

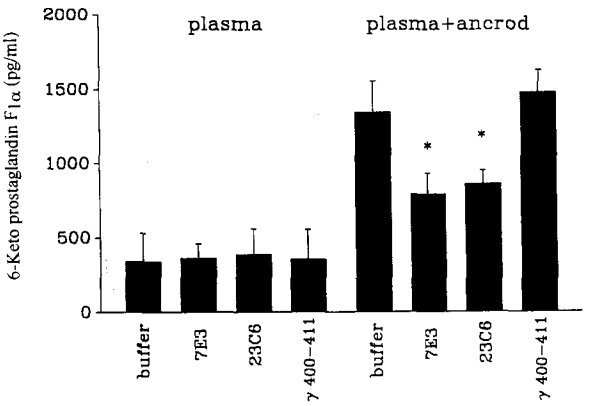

Fig. 7. Inhibition of monoclonal antibodies against $\alpha_v\beta_3$ on 6-keto prostaglandin $F_{1\alpha}$ level of endothelial cells stimulated by ancrod-generated fibrin. Endothelial cells were pretreated with 7E3 (25 μg/ml) or 23C6 (1:100) for 20 min followed by aspiration and readdion with fresh platelet-poor plasma, or treated with γ 400–411 (500 μM), and then ancrod (0.02 U/ml) was added to form fibrin. After a 10 h incubation, 6-keto prostaglandin $F_{1\alpha}$ levels were determined. Each column represents mean ± S.E.M. ($n = 5$). * $P < 0.05$ as compared with control.

imately 65% (control, 50280 ± 2550 cpm; data not shown).

4. Discussion

Prostacyclin synthesis is regulated by the activity of two key enzymes, phospholipase A_2 and cyclooxygenase. Mepacrine and indomethacin or aspirin, which are phospholipase A_2 and cyclooxygenase inhibitors, respectively, completely inhibited the 6-keto prostaglandin $F_{1\alpha}$ generation stimulated by ancrod-generated fibrin (unpublished data), indicating that the stimulatory effect on prostacyclin synthesis is mediated by activation of endogenous phospholipase A_2. As the free intracellular level of arachidonic acid is low, its release from membrane lipids by phospholipase A_2 is the key step in the regulation of prostacyclin synthesis (Chang et al., 1987).

Many reports indicate that prostacyclin production is related to an increase in intracellular Ca^{2+} (Jaffe et al., 1987; Hallam et al., 1988). In this study, TMB-8, an inhibitor of intracellular Ca^{2+} mobilization (Chiou and Malagodi, 1975), almost completely inhibited prostacyclin production of endothelial cells stimulated by ancrod-generated fibrin. Endothelial cells loaded with BAPTA for chelating intracellular Ca^{2+} also showed lesser capacity in enhancing prostacyclin synthesis by ancrod-generated fibrin. Overall, the intracellular Ca^{2+} is crucial for prostacyclin synthesis. In our experiments, extracellular chelation with EGTA (1.2 mM) pronouncedly inhibited the 6-keto prostaglandin $F_{1\alpha}$ synthesis. This may either result from inhibition of the binding of fibrin to $\alpha_v\beta_3$, which is dependent on a divalent cation, or result from the inhibition of Ca^{2+} influx into the cells. However, nifedipine and verapamil, the specific voltage-gated Ca^{2+} channel blocker (Schwartz and Triggle, 1984), had no effect on prostacyclin production. Further, it has been reported that voltage-activated Ca^{2+} channels may be absent in endothelial cells (Criscuolo et al., 1989; Himmel et al., 1993). Therefore, this Ca^{2+} influx induced by ancrod-generated fibrin is independent of voltage-gated Ca^{2+} channels.

In this study, we have demonstrated that ancrod in plasma medium increased prostacyclin formation and IP_1 production of endothelial cells due to polymerized fibrin, since Gly-Pro-Arg-Pro, an inhibitor of fibrin polymerization (Laudano and Doolittle, 1980), completely inhibited IP_1 production and prostacyclin increment. Neomycin, an inhibitor of phosphoinositide metabolism (Carney et al., 1985; Silvka and Insel, 1988), not only significantly inhibited IP_1 formation, but also inhibited the production of prostacyclin stimulated by ancrod-generated fibrin in a dose-dependent manner. Therefore, this prostacyclin increment of endothelial cells caused by ancrod-generated fibrin is mainly mediated through the activation of endogenous phospholipase C.

Most of the extracellular stimuli require G-proteins for signal transduction in endothelial cells. The transduction mechanism for receptors presented on endothelial cells probably involves pertussis toxin-sensitive as well as pertussis toxin-insensitive G-proteins (Himmel et al., 1993). Our previous study showed that ancrod-generated fibrin caused an enhancement of inducible cyclooxygenase resulting in an increment of prostacyclin during a 2 h incubation period (Chang et al., 1994). Instead, in this study, we examined effects of neomycin or pertussis toxin on prostacyclin production during a 10 h incubation. Pertussis toxin pretreatment inhibited not only IP_1 increase (67.2% inhibition) but also 6-keto prostaglandin $F_{1\alpha}$ production (77.7% inhibition) stimulated by ancrod-generated fibrin. Whether pertussis toxin or neomycin affect the prostacyclin level through a blockade of the induction of cyclooxygenase awaits further investigation. The exact mode of coupling between G-protein and phospholipase A_2 or phospholipase C remains to be elucidated.

Human endothelial cell attachment to ancrod-generated fibrin was markedly inhibited by monoclonal antibodies raised against $\alpha_v\beta_3$ integrin (this study) and by disintegrins and Gly-Arg-Gly-Asp peptide (Chang et al., 1995b). We examined the effect of these antibodies on phosphoinositide metabolism and prostacyclin synthesis in the present study. 7E3 and 23C6 significantly inhibited IP_1 increase as well as 6-keto prostaglandin $F_{1\alpha}$ synthesis, whereas AP1 did not. Therefore, it appears that $\alpha_v\beta_3$ integrin is the recognition site for

ancrod-generated fibrin in transducing the signal for prostacyclin synthesis. However, we did not exclude the possibility that the binding of anti-$\alpha_v\beta_3$ antibodies to endothelial cells may inhibit the binding of fibrin to its receptor by steric hindrance. The effect of dodecapeptide γ 400–411 on the interaction of fibrin(ogen) and endothelial cells has shown conflicting results. One report showed that γ 400–411 had a lower affinity than Arg-Gly-Asp-Phe for human endothelial cells, and partially inhibited the binding of fibrinogen to endothelial cells (Tranqui et al., 1989). However, Smith et al. (1990) reported that this peptide effectively blocked Arg-Gly-Asp and fibrinogen binding to glycoprotein IIb-IIIa of platelets, but had no effect on the ability of integrin $\alpha_v\beta_3$ on endothelial cells to bind fibrinogen. In our experiments, dodecapeptide γ 400-411 did not show significant effects on the IP_1 increase and prostacyclin synthesis of endothelial cells stimulated by ancrod-generated fibrin.

In conclusion, elevated cytosolic Ca^{2+} is the primary cause in enhancing prostacyclin synthesis. However, both Ca^{2+} influx and intracellular stores are essential. Ancrod-generated fibrin causes phosphoinositide breakdown of human endothelial cells, which is related to a pertussis toxin-sensitive G-protein, and the activation of endogenous phospholipase C is closely related to prostacyclin synthesis of endothelial cells in response to ancrod-generated fibrin. This cascade involving the binding of ancrod-generated fibrin to $\alpha_v\beta_3$ integrin, which may be coupled to a pertussis toxin-sensitive G-protein in transducing the activation of phospholipase C, results in a phosphoinositide breakdown. The resultant IPs mobilize Ca^{2+} from the intracellular Ca^{2+} stores and cause the subsequent activation of phospholipase A_2 and the enhancement of prostacyclin production. However, the exact sequence of this cascade needs to be further elucidated. Although our experimental data favor the involvement of a G-protein-coupled phospholipase C in this cascade, it is also possible that different entities of G-proteins are coupled to different phospholipase A_2 or phospholipase C isoenzymes.

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