JACC Vol. 26, No. 3
September 1995:800–6

Platelet Receptor Desensitization Induced by Elevated Prostacyclin Levels Causes Platelet–Endothelial Cell Adhesion

HARALD DARIUS, MD, CHRISTIANE BINZ, MA, KERSTIN VEIT, BS, ANDREAS FISCH, PhD, JÜRGEN MEYER, MD, FACC

Mainz, Germany

Objectives. **The purpose of this study was to investigate the role of platelet prostacyclin receptor desensitization in platelet–endothelial cell adhesion.**

Background. **Platelet–endothelial cell adhesion is regulated by endothelial cell–derived mediators, such as prostacyclin and endothelium-derived relaxing factor. Prostacyclin activates platelet adenylate cyclase and augments cyclic adenosine monophosphate formation by way of specific membrane receptors. Platelet exposure to prostacyclin or chemically stable analogs results in a time- and dose-dependent prostacyclin receptor desensitization as it occurs during infusion therapy with prostacyclin analogs or in pathophysiologic situations such as acute myocardial infarction.**

Methods. **Adhesion of washed and radiolabeled human platelets stimulated with thrombin to cultured umbilical vein endothelial cells was measured under control conditions and under conditions of platelet prostacyclin receptor desensitization induced by incubation with the prostacyclin analog iloprost (10 to 100 nmol/liter) for 3 h.**

Results. **Thrombin (0.08 to 0.2 U/ml) increased platelet adhesion in a dose-dependent manner from 2.7 ± 0.3% to 6.4 ± 0.6% (mean value ± SEM). Preincubation of platelets resulted in a dose-dependent down-regulation of ^{3}H-iloprost binding up to 58.8 ± 6.7% of control platelets with 100 nmol/liter of iloprost. Co-incubation of prostacyclin receptor–desensitized platelets with endothelial cells resulted in a marked augmentation of thrombin-induced adhesion up to 28.6 ± 4.5%. Approximately the same increase in platelet adhesion was seen after complete abrogation of endothelial cell prostacyclin synthesis by pretreatment with aspirin. Comparison of iloprost-induced receptor desensitization and increased platelet–endothelial cell adhesion indicated a positive correlation.**

Conclusions. **Platelet prostacyclin receptor desensitization was observed in humans in vivo during acute myocardial infarction or during therapeutic administration of prostacyclin analogs. In vitro platelet prostacyclin receptor desensitization caused a marked augmentation of platelet–endothelial cell adhesion. This increase in adhesion might result in an enhanced tendency toward thrombus formation in humans.**

(J Am Coll Cardiol 1995;26:800–6)

During recent years, overwhelming evidence became available for the role of endothelial cell products in mediating vessel wall thromboresistence. Specifically, prostacyclin and endothelium-derived relaxing factor are held responsible for inhibiting platelet activity, that is, aggregation and adhesion in the immediate vicinity of the vessel wall. Both mediators are synthesized de novo on stimulation of endothelial cells and are released into the vessel lumen and abluminally. Abluminal secretion of prostacyclin is probably not involved in the physiologic regulation of vessel tone, but it may have important implications in situations of hemodynamic instability such as those of operative stress or sepsis (1). In contrast, convincing evidence from studies with endothelium-derived relaxing factor synthesis inhibitors like N^G-monomethyl-L-arginine (L-NMMA) or N^ω-nitro-L-arginine methyl ester (L-NAME) arose, indicating that endothelium-derived relaxing factor is primarily involved in the regulation of vessel tone and contributes to both systemic and pulmonary vascular resistance and to blood pressure (2).

Prostacyclin acts on platelets through a receptor-mediated activation of membrane-bound adenylate cyclase and a consecutive increase in intracellular cyclic adenosine monophosphate (AMP). An increase in cyclic AMP results in a diminished availability of cytoplasmic free calcium ions and an omnipresent inhibition of platelet functions. In contrast, endothelium-derived relaxing factor acts receptor-independently on platelets and causes an increase in cyclic guanosine monophosphate by activating soluble guanylate cyclase. In contrast to previous reports (3,4) emphasizing the role of prostacyclin in inhibiting the adhesion of activated platelets to endothelial cells, current work (5–8) has focused on the role of endothelium-derived relaxing factor. These reports have presented evidence that release of endothelium-derived relaxing factor is mainly responsible for the inhibition of platelet adhesion to endothelial cells (5–7) and that prostacyclin predominantly acts as an inhibitor of platelet-platelet interactions (i.e., aggregation) (8).

The actions of prostacyclin on platelets have been studied with radiolabeled prostacyclin or chemically stable prostacyclin

From the Department of Medicine II, Johannes Gutenberg University, Mainz, Germany. This study was supported financially by Grant Da 168/2-1 from the Deutsche Forschungsmeinschaft, Bonn, Germany.

Manuscript received May 12, 1994; revised manuscript received March 22, 1995, accepted May 2, 1995.

Address for correspondence: Privat Dozent Dr. Harald Darius, Department of Medicine II, Johannes Gutenberg University, 55101 Mainz, Germany.

0735-1097/95/$9.50
0735-1097(95)00264-5

analogs. After characterization of the supposed prostacyclin receptor on platelets (9), convincing evidence (10,11) showed a rapid decrease in the number of prostacyclin receptors on human platelets with exposure to nanomolar concentrations of prostacyclin over a period of 2 to 4 h. In clinical situations with administration of exogenous prostacyclin or analogs—for example, for the treatment of peripheral arterial occlusive disease or in pathophysiologic situations, such as acute myocardial infarction (12)—a significant down-regulation of platelet prostacyclin receptors has been reported.

Although the decrease in platelet prostacyclin receptor density during extended exposure of platelets to prostacyclin is known, the physiologic consequences of this desensitization process are unknown. Thus, we studied the decrease in ^{3}H-iloprost binding to human platelets after incubation with iloprost, the influence of receptor down-regulation on platelet cyclic AMP formation through adenylate cyclase activation and the effects of prostacyclin receptor desensitization on platelet adhesion to cultured endothelial cells. Because of the role of prostacyclin synthesis in endothelial thromboresistance, we assessed the effect of thrombin on endothelial prostacyclin release and, in some experiments, prevented prostaglandin production by cyclooxygenase inhibition.

Methods

Endothelial cell culture. Human umbilical vein endothelial cells were obtained from umbilical cord veins by digestion with 0.1% collagenase A dissolved in Hanks' buffered salt solution for 15 min at 37°C modified after the method described by Jaffe et al. (13). The harvested cells were cultured in T-25 flasks (precoated with 0.2% gelatin) in Medium 199 supplemented with 20% fetal calf serum, penicillin (50 IU/ml), streptomycin (50 μg/ml), and L-glutamine (2 mmol/liter) at 37°C under 5% carbon dioxide and constant humidity (95%). Cultured cells were identified as human umbilical vein endothelial cells by typical morphologic characteristics and positive indirect immunofluorescence staining for von Willebrand factor antigen (14). A monoclonal murine anti-von Willebrand factor antibody (IgG_1) was used as the primary antibody and a fluorescein thiocyanate (FITC)-labeled anti-gamma-globulin antibody from sheep as the secondary antibody (both purchased from Boehringer-Mannheim, Germany).

For subculture, human umbilical vein endothelial cells were harvested with a solution containing 0.01% ethylenediamine tetraacetic acid and 0.125% trypsin. All experiments were performed with endothelial cells in the first or second passage. The cells were seeded into 12- or 24-well plates with an initial density of 50,000 or 25,000 cells/well, respectively, and grown to confluence within 5 days.

Prostacyclin synthesis of human umbilical vein endothelial cells was measured under basal conditions in successive passages over a period of 24 h on 4 consecutive days within a passage and after stimulation with thrombin (0.02 to 2.0 U/ml) for 15 min. After the incubation period, supernatant was collected, supplemented with indomethacin (30 μmol/liter) and stored at −20°C until 6-keto-prostaglandin $F_{1\alpha}$ ($PGF_{1\alpha}$) determination by radioimmunoassay. Results were corrected for the number of endothelial cells in each well and expressed as 6-keto-$PGF_{1\alpha}$ release in ng/10^6 cells per 24 h or ng/10^6 cells per 15 min, respectively.

In some experiments human umbilical vein endothelial cells were pretreated with aspirin or N^G-monomethyl-L-arginine to prevent synthesis of the endothelial cell products prostacyclin or endothelium-derived relaxing factor. Prostacyclin synthesis was inhibited by preincubation with aspirin (1 mmol/liter, 30 min), endothelium-derived relaxing factor synthesis was reduced by preincubation with N^G-monomethyl-L-arginine (200 μmol/liter, 15 min) before coincubation experiments with endothelial cells and platelets were performed.

Preparation of washed platelets. Blood was drawn from the antecubital vein of healthy male volunteers and anticoagulated with acid citrate dextrose at a ratio of 5:1. Platelet-rich plasma was obtained by centrifugation (180*g*, 10 min), and platelets were radiolabeled by incubation with ^{14}C-arachidonic acid (55 mCi/mmol, Amersham, Braunschweig) at a final concentration of 0.9 μmol/liter for 3 h at 37°C. Total radioactivity was counted in an aliquot and tris-maleate buffer (200 mmol/liter; pH 5.3) was added to equal volumes of platelet-rich plasma. Platelets were centrifuged for 10 min at 1,600*g* and washed three times in citrate buffer (containing [in mmol/liter] sodium chloride 100, potassium chloride 5.0, calcium chloride 2.0, magnesium chloride 1.0, sodium citrate 30 and [in g/liter] D-glucose 0.991, bovine serum albumin 0.5; pH 6.5) to remove nonincorporated radioactivity. Radioactivity was determined in the buffer used for the last washing step to assure complete removal of nonincorporated ^{14}C-arachidonic acid and washed platelets were resuspended in Tyrode buffer (pH 7.4).

Platelet prostacyclin receptor desensitization. Platelet prostacyclin receptors were desensitized by incubation of platelet-rich plasma with the chemically stable prostacyclin analog iloprost (1, 10 or 100 nmol/liter) for 3 h at 37°C. In control experiments, platelets were treated identically in the absence of iloprost. The incubation period was terminated by adding equal volumes of tris-maleate buffer and washing three times with citrate buffer as described for the radiolabeling procedure. The complete removal of iloprost during the washing procedure was confirmed by using tritiated iloprost in control experiments performed simultaneously. Platelets to be used in the adhesion experiments were coincubated with iloprost (10, 30 or 100 nmol/liter) and ^{14}C-arachidonic acid. In preliminary experiments we had confirmed that the two processes did not interfere with each other (data not shown).

The degree of platelet prostacyclin receptor desensitization was determined in binding experiments using ^{3}H-iloprost as a radioligand. Washed platelets were resuspended in phosphate-buffered saline solution (pH 7.4) and the platelet count was adjusted to 5×10^8 cells/ml. Nonspecific binding was measured by incubating 1 ml of platelets with iloprost (10 μmol/liter) for 10 min at 30°C. Then ^{3}H-iloprost was added at a concentration adapted from the predetermined dissociation constant (K_D)

value (7.1 nmol/liter) and incubation was continued for 10 min at 30°C. The reaction was terminated by rapid centrifugation (12,000*g* for 12 s) and removal of the supernatant. The remaining pellet was solubilized overnight in sodium hydroxide (0.8 mol/liter) and radioactivity of the platelet homogenate was measured in a scintillation counter (LKB-Wallac 1410). Total binding was determined in a parallel sample without addition of nonlabeled iloprost. Specific binding was calculated by subtracting nonspecific binding from total binding. Results are presented as binding in fmol/10^9 platelets; receptor desensitization is expressed as percent diminution of ^{3}H-iloprost binding in comparison with that of control platelets incubated with saline solution for 3 h (= 100%).

Platelet cyclic AMP formation. Platelets were incubated for 3 h at 37°C in the absence or presence of iloprost (1 or 10 nmol/liter). Preparation of washed platelets and the assurance of complete iloprost removal were carried out by using ^{3}H-iloprost as previously described. After the washing procedure, adenylate cyclase of the control platelets and desensitized platelets were stimulated for 0.5 to 10 min by adding the maximal effective concentration of iloprost (100 nmol/liter) as determined in preliminary experiments (data not shown). Stimulation was terminated by adding trichloroacetic acid (final concentration 5%) to each sample followed by vortexing and centrifugation. The supernatants were extracted three times with water-saturated diethyl ether, lyophilized and solubilized in radioimmunoassay (RIA) buffer. Adenylate cyclase activity was assessed via measurement of cyclic AMP formation by cyclic AMP-RIA (Amersham-Buchler, Dreieich, Germany).

Platelet–endothelial cell adhesion. Platelet–endothelial cell adhesion was investigated with a method modified after Curwen et al. (15) using confluent human umbilical vein endothelial cells in 24-well plates and ^{14}C-arachidonic acid–labeled washed human platelets suspended in Tyrode buffer. Platelet count was adjusted to 2.5×10^8 cells/ml. All adhesion experiments were performed in duplicate with human umbilical vein endothelial cells of the first or second passage. Some endothelial monolayers were pretreated with aspirin (1 mmol/liter) to prevent prostacyclin synthesis. These monolayers were equilibrated with aspirin-Hanks' buffered salt solution for two times 10 min. The incubation medium was then renewed and incubation was continued for 30 min at 37°C followed by the removal of the supernatant and two washing steps with Hanks' buffered salt solution. Prostacyclin release into the medium was assayed by radioimmunoassay and the 6-keto-$PGF_{1\alpha}$ level was compared with that of control endothelial cells incubated with Hanks' buffered salt solution in the absence of aspirin.

In another series of experiments designed to study the role of endothelium-derived relaxing factor in thrombin-induced platelet adhesion, monolayers were pretreated with the amino acid analog N^G-monomethyl-L-arginine (200 μmol/liter) for 15 min at 37°C to inhibit endothelium-derived relaxing factor synthesis. N^G-monomethyl-L-arginine was washed from the monolayers two times with Hanks' buffered salt solution and then the platelet adhesion assay was performed. For measuring adhesion 400 μl of washed and labeled control or receptor desensitized platelets (10^8 platelets/well) were added to human umbilical vein endothelial cells monolayers, immediately followed by 100 μl of Tyrode buffer containing thrombin (0.08 to 0.2 U/ml). Cells were coincubated for 15 min at 37°C under gentle constant stirring.

The endothelial cell monolayer with adhering platelets was gently washed three times with Hanks' buffered salt solution under standardized conditions to remove nonadhering platelets. Hydrolysis of the monolayers and adherent platelets was achieved by an overnight incubation with 1 *N* sodium hydroxide. The solubilized cells were placed in counting tubes, mixed with 10 ml scintillation cocktail (Rotiszint eco plus, Roth, Karlsruhe, Germany) and radioactivity was determined in a LKB-Wallac 1410 scintillation counter (Pharmacia., Freiburg, Germany).

Platelet adherence is expressed as percent adhesion. It was calculated as the level of radioactivity of the solubilized sample divided by the total radioactivity of the platelets added to each well, multiplied by 100.

Statistics. The data are expressed as mean value ± 1 SEM of n independent experiments, always performed in duplicate. Statistical analyses of dose-response relations was carried out by multiple regression analysis followed by an F test. An F value of 0.05 was regarded as indicating a statistically significant difference. If repeated measures were compared, the level of significance was adapted according to the rule given by Bonferroni. The correlation between the degree of receptor desensitization and platelet adhesion was calculated by the Spearman rank correlation coefficient.

Results

Endothelial cell prostacyclin formation. Human umbilical vein endothelial cells in the first and second passage were characterized according to morphologic and immunohistochemical criteria. Light phase contrast microscopy demonstrated that the endothelial cells grew as a homogeneous population of closely opposed, polygonal, large cells forming a monolayer with a cobblestone-like appearance. The endothelial origin of the cells was verified by the presence of von Willebrand factor located in cytoplasmic Weibel-Palade bodies. Cultures treated with an antibody to von Willebrand factor followed by an FITC-labeled secondary antibody developed an intense yellow fluorescence with >95% positive staining cells.

For further characterization endothelial prostacyclin synthesis was studied. Prostacyclin synthesis of human umbilical vein endothelial cells during passage 1 and 2 over a period of 24 h on 4 consecutive days from day 2 to day 5 was determined. Prostacyclin content of the culture medium collected over 24 h was assayed by radioimmunoassay for the degradation product 6-keto $PGF_{1\alpha}$. Prostacyclin synthesis decreased continuously with increasing cell density during passage 1 from 327 ± 51 ng/10^6 cells per 24 h on day 2 to 265 ± 48, 204 ± 42 and 155 ± 31 ng/10^6 cells per 24 h, respectively, on days 3, 4 and 5. During the second passage 6-keto-$PGF_{1\alpha}$ synthesis was substantially lower at a level of 143 ± 33 ng/10^6 cells per 24 h on

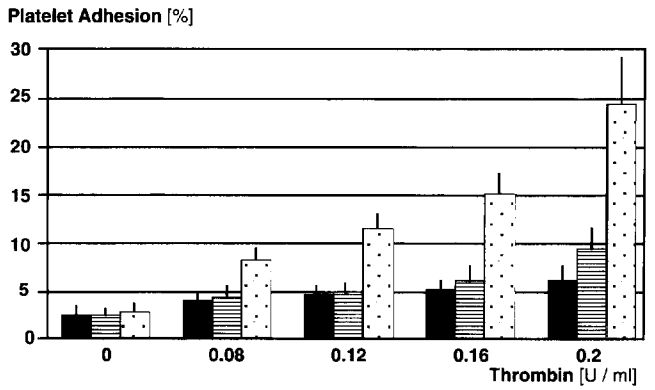


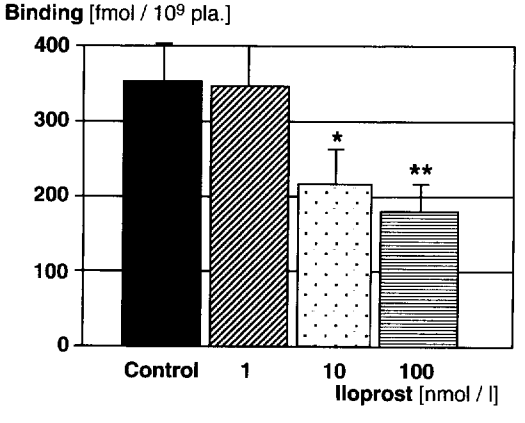
Figure 1. Alterations in thrombin-stimulated platelet adhesion (**solid columns**) after inhibition of endothelial cell nitric oxide synthase with N^G-monomethyl-L-arginine (20 μmol/liter, 15 min [**striped columns**]) or prostacyclin synthesis by pretreatment with aspirin (1 mmol/liter, 50 min [**dotted columns**]), respectively. Data are shown as mean value (**columns**) ± SEM (**vertical lines**) of 10 independent experiments performed in duplicate.

day 2, and 102 ± 27, 81 ± 15 and 67 ± 16 ng/10^6 cells per 24 h, respectively, on days 3, 4 and 5 (n = 6, triple determinations each).

Prostacyclin synthesis was stimulated in confluent endothelial cell monolayers in passage 2 by incubation with thrombin (0.02 to 2.0 U/ml) for 15 min at 37°C, and 6-keto-$PGF_{1\alpha}$ levels were measured in the incubation medium. Thrombin dose-dependently stimulated baseline prostacyclin production (11.5 ± 0.8 ng/10^6 cells per 15 min, resulting in a maximal 12.4-fold increase up to 140 ± 12 ng/10^6 cells per 15 min with 1.0 U/ml of thrombin, with 2 U/ml being equieffective.

To study the effects of cyclooxygenase inhibition on prostacyclin synthesis human umbilical vein endothelial cells were preincubated with aspirin at a final concentration of 1 mmol/liter for 20 min, and incubation was continued with aspirin for another 30 min after exchange of the medium. The concentration of 6-keto-$PGF_{1\alpha}$ decreased from 2.04 ± 0.16 ng/500 μl per 30 min under control conditions to 0.04 ± 0.01 ng/500 μl per 30 min with aspirin (n = 8), indicating that prostacyclin synthesis was almost completely prevented (by 98.3 ± 0.9%). Comparable results were obtained using indomethacin (30 μmol/liter) as a cyclooxygenase inhibitor, which reduced 6-keto-$PGF_{1\alpha}$ in the medium by 97.9 ± 2.0% (n = 3).

Figure 2. Dose-dependent decrease in ^{3}H-iloprost binding after incubation of human platelets with iloprost (1, 10 and 100 nmol/liter) for 3 h. *p < 0.01 versus control. **p < 0.001 versus control. Data represent mean value (**columns**) ± SEM (**vertical lines**). l = liter; pla. = platelets.



Platelet–endothelial cell adhesion. Adhesion experiments were performed by coincubating washed human platelets with human umbilical vein endothelial cells grown to confluency in 24-well plates. Therefore, platelets were radiolabeled with ^{14}C-arachidonic acid for 3 h and washed three times. After the washing procedure 26% of the total radioactivity had been incorporated into the platelets with no radioactivity detectable in the washing buffer. To quantify the amount of arachidonic acid that was metabolized by platelets during thrombin stimulation, labeled platelets were activated by addition of thrombin (3 U/ml). Measurement of the radioactivity released from these platelets amounted to <4% of the radioactivity incorporated, demonstrating that a decrease in counts during the adhesion experiments did not result from ^{14}C-arachidonic acid metabolism.

Co-incubation of washed platelets (10^8 platelets/well) and confluent human umbilical vein endothelial cells during passage 2 for 15 min resulted in platelet adhesion of 2.7 ± 0.3% to the endothelial cells. Addition of thrombin in concentrations ranging from 0.08 to 0.2 U/ml caused a dose-dependent increase in platelet adhesion to a maximum of 6.4 ± 0.6% with 0.2 U/ml of thrombin (n = 10) (Fig. 1).

Endothelial thromboresistance in vivo probably is mediated through the endothelial cell autacoids prostacyclin and endothelium-derived relaxing factor. Therefore, the influence of these endothelial cell products on thrombin-stimulated platelet adhesion was measured in our model in another series of experiments using the respective synthesis inhibitors. Reduction of endothelial prostacyclin formation by 98% during preincubation of human umbilical vein endothelial cells with aspirin did not significantly change adhesion of nonstimulated platelets from 2.7 ± 0.3 to 3.1 ± 0.3% (p > 0.05). However, thrombin-stimulated platelet adhesion was significantly enhanced, resulting in adhesion of 24.2 ± 4.3% with 0.2 U/ml of thrombin (n = 10) (Fig. 1). In contrast to the marked effects of cyclooxygenase inhibition by aspirin pretreatment, inhibition of endothelium-derived relaxing factor synthesis by preincubation of the endothelial cells with the nitric oxide synthase inhibitor N^G-monomethyl-L-arginine exerted only minor alterations in platelet adhesion (Fig. 1).

Platelet prostacyclin receptor desensitization. According to Scatchard plot analysis, the chemically stable prostacyclin analog iloprost dose-dependently binds to platelets with a maximal binding (B_{max}) of 1.29 pmol/mg protein and a K_D of 39.5 nmol/liter. Long-term exposure of platelet prostacyclin receptors to high agonist concentrations resulted in a time- and dose-dependent decrease in prostacyclin binding (Fig. 2). This is indicative of a decrease in binding sites (10,11). In this study, platelet prostacyclin receptors were desensitized by incubation with iloprost at concentrations of 1, 10, or 100 nmol/liter for

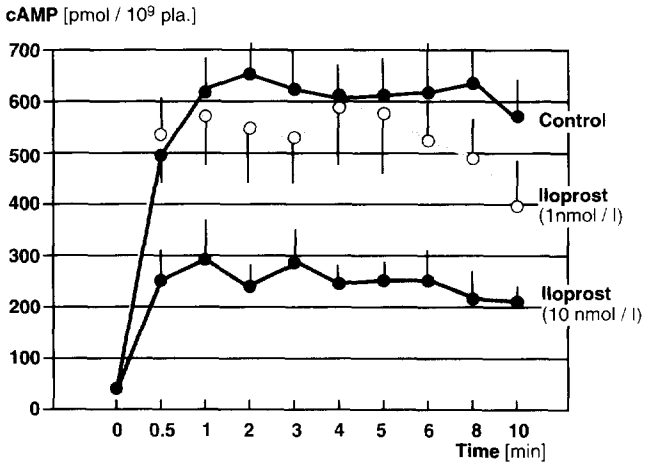


Figure 3. Time-dependent increase in platelet cyclic adenosine monophosphate (cAMP) after stimulation with iloprost (100 nmol/liter). Platelets had previously been incubated with solvent (Control) or 1 or 10 nmol/liter of iloprost for 3 h at 37°C. Data represent mean value (**circles**) ± SEM (**vertical lines**) of seven independent experiments performed in duplicate. Abbreviations as in Figure 2.

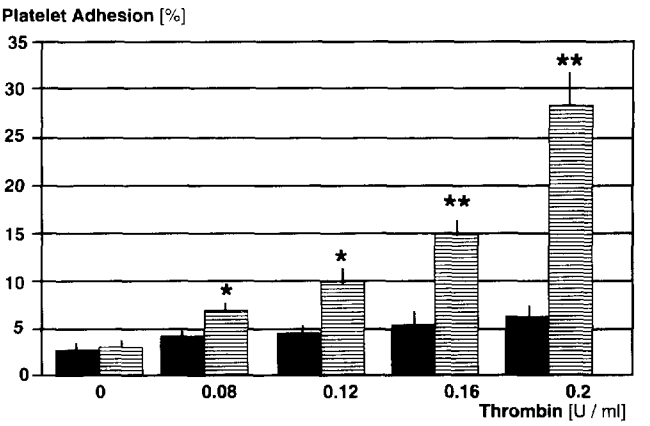


Figure 4. Significant augmentation of thrombin-stimulated platelet adhesion (**solid columns**) to human endothelial cells after platelet prostacyclin receptor desensitization (**striped columns**). The data represent mean value (**columns**) ± SEM (**vertical lines**) of 10 independent experiments performed in duplicate. *$p < 0.05$. **$p < 0.001$.

3 h. This caused a dose-dependent decrease in prostacyclin binding from a control value of 361 ± 17 to 352 ± 29 ($p > 0.05$), 220 ± 15 ($p < 0.01$) and 181 ± 19 ($p < 0.001$) fmol/10^9 platelets with 1, 10 and 100 nmol/liter of iloprost, respectively.

The influence of receptor desensitization on stimulation of adenylate cyclase by iloprost was measured. Platelets were incubated for 3 h with iloprost (1 or 10 nmol/liter) followed by triple washout. Desensitized and control platelets were then stimulated with iloprost (100 nmol/liter) for 0.5 to 10 min. Figure 3 shows that cyclic AMP formation was unaltered by incubation with low concentrations of iloprost (1 nmol/liter) which had not resulted in a significant decrease in binding. In contrast, incubation with desensitizing concentrations of iloprost (10 nmol/liter) resulted in a significant attenuation of the adenylate cyclase–stimulating efficacy of 100 nmol/liter of iloprost when compared with measurements in control platelets.

Adhesion of receptor-desensitized platelets. Co-incubation of prostacyclin receptor–desensitized platelets with human umbilical vein endothelial cells in passage 2 resulted in a marked augmentation of thrombin-stimulated platelet adhesion depending on the degree of receptor desensitization. Whereas nonstimulated adhesion remained unaltered by receptor desensitization (2.7 ± 0.3% vs. 3.2 ± 0.4%), adhesion was already enhanced in the presence of the lowest concentration of thrombin (0.08 U/ml) from 4.0 ± 0.5% to 7.0 ± 0.5% ($p < 0.05$). Platelet adhesion was enhanced by a factor of 6.7 in the presence of the highest concentration of thrombin (0.2 U/ml) used for stimulation ($p < 0.001$; $n = 6$). These dose-dependent effects of thrombin on the adhesion of receptor-desensitized platelets are depicted in Figure 4.

Another series of adhesion assays concerning receptor-desensitized platelets was performed with human umbilical vein endothelial cells during passage 1, which previously had been shown to exert the highest level of prostacyclin synthesis. This synthetic activity may be the reason why adhesion of nonstimulated platelets to endothelial cells from passage 1 (0.7 ± 0.1%) was substantially lower than that of endothelial cells during passage 2 (2.7 ± 0.3%). When platelets were preincubated with three different concentrations of iloprost (10, 30 or 100 nmol/liter) for 3 h, a dose-dependent receptor desensitization was detectable. When the data for the iloprost-induced receptor desensitization and increased platelet–endothelial cell adhesion were plotted, a positive correlation for these two variables was detected for iloprost concentrations between 10 and 100 nmol/liter. By using a Spearman rank test the correlation coefficient r_s was 0.71, indicating a significant correlation when tested two-sided ($p < 0.001$; Fig. 5).

Discussion

The decrease in prostacyclin binding on platelets depends on the incubation time and the prostacyclin dose used for incubating platelets in vitro. This phenomenon is similarly observed when chemically stable prostacyclin analogs like iloprost are used. In our experiments, iloprost caused a dose-dependent decrease in binding that was significantly lower than the control level. This decrease, observed at a dose of 10 nmol/liter and unaltered with 1 nmol/liter of iloprost (data not shown), might be due either to a true receptor desensitization or to an alteration in the number of functionally incompetent spare receptors. Therefore, we studied the alterations in the receptor-linked second-messenger system and estimated adenylate cyclase activity by the amount of cyclic AMP synthesized as a result of maximal receptor-mediated stimulation. The decrease in prostacyclin binding correlates very well with the diminished adenylate cyclase activity. Platelets incubated with 1 nmol/liter of iloprost exerted an unaltered binding, and maximal stimulation of cyclic AMP synthesis by iloprost was not different from that of control platelets. However, diminu-

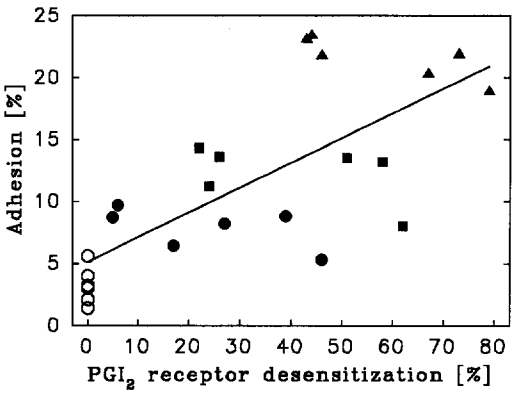


Figure 5. Correlation between platelet prostacyclin (PGI_2) receptor desensitization (%) and platelet–endothelial cell adhesion (%). Platelets were incubated with saline solution (**open circles**) or 10 (**solid circles**), 30 (**squares**) or 100 nmol/liter of iloprost (**triangles**). Each point represents the mean of individual platelet adhesion and receptor desensitization experiments performed in duplicate. A Spearman rank test revealed a correlation coefficient r_s of 0.71 and indicated a significant correlation between the two variables ($p < 0.001$).

tion of binding after long-term incubation with iloprost (10 nmol/liter), was accompanied by a significant reduction in adenylate cyclase activity from that of control platelets (Fig. 3). These data confirm that extended exposure of platelets to iloprost results in a true desensitization of the prostacyclin receptor with a decrease in binding and a diminished activity of the receptor-linked second-messenger system.

In previous studies, a decrease in prostacyclin binding on human platelets was discovered in vitro, and its physiologic relevance in humans was evaluated by administering infusions with prostacyclin and iloprost (11,16). MacDermot (11) succeeded in explaining the obvious contradiction between very low plasma levels of prostacyclin and the occurrence of receptor desensitization when the infusion was administered through a peripheral vein. In contrast, receptor density remained unaltered when prostacyclin was infused through a central venous catheter, indicating the physiologic importance of short-term platelet exposure to high prostacyclin concentrations in its immediate vicinity.

If prostacyclin receptor desensitization occurs in vivo during infusion therapy, two questions arise. 1) Are there physiologic or pathophysiologic situations in which a receptor desensitization is provoked and becomes detectable ex vivo, and 2) is the observed receptor desensitization of any physiologic importance? The first question was previously studied in vivo in patients with atherosclerotic disease and acute myocardial ischemia. FitzGerald et al. (17) reported in 1984 that the excretion of prostacyclin metabolites in urine was markedly elevated in patients with severe atherosclerotic vascular disease. This is due to a precursor transfer on the level of arachidonic acid or prostaglandin endoperoxides PGG_2 or PGH_2 from activated platelets to endothelial cells (18). A decreased number of platelet-binding sites for prostacyclin from patients with spontaneous angina or acute myocardial ischemia was observed by Neri-Serneri et al. (19), Jaschonek et al. (20) and Kahn et al. (12). Although it has not yet been conclusively demonstrated that prostacyclin receptors are diminished in patients who have increased prostacyclin metabolite excretion in urine, the evidence for this hypothesis is increasing.

With respect to prostacyclin receptor desensitization either during infusion therapy with prostacyclin or its analogs or in pathophysiologic situations, the question of its physiologic importance is unresolved. The main purpose of this study was to investigate the effect of prostacyclin receptor desensitization on the adhesion of human platelets to human endothelial cells in culture. Therefore, human umbilical vein endothelial cells in culture were identified by light microscopy and indirect immunofluorescence for von Willebrand factor. Endothelial prostacyclin synthesis was characterized depending on cell density and number of passages. Because prostacyclin release into the incubation medium decreased markedly with successive passages, only confluent monolayers of passages 1 and 2 were used for the adhesion experiments. Addition of thrombin resulted in a dose-dependent increase in endothelial prostacyclin.

Co-incubation of washed and radiolabeled platelets with human umbilical vein endothelial cells resulted in very little platelet adhesion in the absence of thrombin. Despite the augmentation in prostacyclin release with increasing doses of thrombin added to the medium, platelet adhesion increased in a dose-dependent manner, indicating the strong platelet-activating effect of thrombin in this model. Complete inhibition of endogenous prostacyclin formation by pretreating the endothelial cells with aspirin markedly enhanced stimulated platelet adhesion by a factor of 4. These results confirm published reports (4,8) stating that prostacyclin is an important mediator of endothelial thromboresistance under conditions of low shear rates. The minor effects of the nitric oxide synthase inhibitor N^G-monomethyl-L-arginine on platelet adhesion may result from the fact that the adhesion experiments were performed during gentle stirring and not in a flow chamber model. In addition, other reports (5–7) indicate that endothelial cells in culture rapidly lose their capability of synthesizing endothelium-derived relaxing factor.

Platelet preincubation with iloprost caused a dose-dependent prostacyclin receptor desensitization and, in accordance with the decreased number of binding sites, we observed a marked increase in platelet adhesion to endothelial cells (Fig. 4) comparable to the enhanced adhesion to aspirin-pretreated endothelial cells (Fig. 1) (i.e., after complete abrogation of endothelial prostacyclin synthesis). Thus, desensitized platelets are less sensitive toward endogenous prostacyclin released from thrombin-stimulated endothelial cells. The data for the decrease in prostacyclin binding and the increase in platelet adhesion is shown in Figure 5 and with a p value < 0.001 in the Spearman rank correlation coefficient. Thus, the adhesion-preventing effect of endogenous prostacyclin in our model is completely lost if the platelet prostacyclin receptor is desensitized.

Conclusions. Our findings may have implications for the use of prostacyclin or iloprost, or both, in the treatment of patients with vascular disease, especially peripheral vascular

disease. Prostacyclin receptor desensitization is determined by the duration of treatment, dose of drug administered and mode of drug administration. Additionally, these effects may play an important pathophysiologic role if the enhanced prostacyclin release reaches receptor-desensitizing concentrations in the vicinity of the endothelium.

References

1. Terragno NA, Terragno T, McGiff JF. Contribution of prostaglandins to the renal circulation in conscious, anaesthetized and laparatomized dogs. Circ Res 1977;40:590–4.
2. Murad F, Ishii K. Hormonal regulation of the different isoforms of guanylate cyclase: EDRF is a ubiquitous activator of soluble guanylate cyclase. In: Rubanyi GM, Vanhoutte PM, editors. Endothelium-derived relaxing factors. Basel: Karger, 1990:151–65.
3. Fry GL, Czervionke RL, Hoak JC, Smith JB, Haycraft DL. Platelet adherence to cultured vascular cells: influence of prostacyclin (PGI_2). Blood 1980;55:271–6.
4. Buchanan MR, Dejana E, Gent M, Mustrad JF, Hirsh J. Enhanced platelet accumulation into carotidi arteries in rabbits following aspirin treatment. J Clin Invest 1981;67:503–8.
5. de Graaf JC, Banga JD, Moncada S, Palmer RMJ, de Groot PG, Sixma JJ. Nitric oxide functions as an inhibitor of platelet adhesion under flow conditions. Circulation 1992;85:2284–90.
6. Radomski MW, Palmer RMJ, Moncada S. Endogenous nitric oxide inhibits human platelet adhesion to vascular endothelium. Lancet 1987;2:1057–8.
7. Sneddon JM, Vane JR. Endothelium-derived relaxing factor reduces platelet adhesion to bovine endothelial cells. Proc Natl Acad Sci U S A 1988;85:2800–4.
8. Curwen KD, Gimbrone MA, Handin RI. In vitro studies of thromboresistence: the role of prostacyclin (PGI_2) in platelet adhesion to cultured normal and virally transformed human vascular endothelial cells. Lab Invest 1980;42:366–74.
9. Siegl AM. Receptors for PGI_2 and PGD_2 on human platelets. Methods Enzymol 1992;86:179–92.
10. Jaschonek K, Faul C, Schmidt H, Renn W. Desensitization of platelets to iloprost. Loss of specific binding sites and heterologous desensitization of adenylate cyclase. Eur J Pharmacol 1988;147:187–96.
11. MacDermot J. Desensitization of prostacyclin responsiveness in platelets. Apparent differences in the mechanism in vitro and in vivo. Biochem Pharmacol 1986;35:2645–749.
12. Kahn NN, Mueller H, Sinha AK. Impaired prostaglandin E_1/I_2 receptor activity of human blood platelets in acute ischemia heart disease. Circ Res 1991;66:932–40.
13. Jaffe EA, Nachman RL, Becker CG, Mimick CR. Culture of human endothelial cells derived from umbilical veins. Identification by morphologic and immunologic criteria. J Clin Invest 1973;52:2745–56.
14. Hoyer LW, De Los Santos RP, Hoyer JR. Antihemophilic factor antigen. Localisation in endothelial cells by immunofluorescent microscopy. J Clin Invest 1973;52:2737–44.
15. Curwen KD, Kim H-Y, Vazquez M, Handin RI, Gimbrone MA. Platelet adhesion to cultured vascular endothelial cells. A quantitative monolayer adhesion assay. J Lab Clin Med 1982;100:425–31.
16. Modesti PA, Fortini A, Poggesi L, Boddi M, Abbate R, Gensini GF. Acute reversible reduction of PGI_2 platelet receptors after iloprost infusion in man. Thromb Res 1987;48:663–9.
17. FitzGerald GA, Smith B, Pedersen AK, Brash AR. Increased prostacyclin biosynthesis in patients with severe atherosclerosis and platelet activation. N Engl J Med 1984;310:1065–8.
18. Schafer AI, Crawford DD, Gimbrone MA. Unidirectional transfer of prostaglandin endoperoxides between platelets and endothelial cells. J Clin Invest 1984;73:1105–12.
19. Neri-Serneri GG, Modesti PA, Fortini A, Abbate R, Lombardi A, Gensini GF. Reduction in prostacyclin platelet receptors in active spontaneous angina. Lancet 1984;2:838–41.
20. Jaschonek K, Weisenberger H, Karsch KR, et al. Impaired platelet prostacyclin binding in acute myocardial infarction. Lancet 1984;2:1341.