Reduction in prostacyclin platelet receptors in active spontaneous angina.

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REDUCTION IN PROSTACYCLIN PLATELET RECEPTORS IN ACTIVE SPONTANEOUS ANGINA

GIAN G. NERI SERNERI
ALBERTO FORTINI
ALESSANDRA LOMBARDI
PIETRO A. MODESTI
ROSANNA ABBATE
GIAN F. GENSINI

Clinica Medica I, University of Florence, Italy

Summary The number and affinity of binding sites for tritiated prostacyclin (PGI_2) in platelets were investigated in twenty-eight patients with spontaneous angina (fourteen in the active and fourteen in the inactive phase) and in nine healthy controls of similar age. Active-phase patients had significantly lower numbers of both classes of platelet PGI_2 receptors (high affinity and low affinity) than controls or inactive-phase patients. In contrast, the affinity for PGI_2 was not significantly different in the three groups. In six active-phase patients restudied in the inactive phase the previously low number of PGI_2 receptors had returned to normal. These results suggest that the instability of angina is associated with functional changes not confined to the coronary vasculature.

Introduction

SPONTANEOUS angina is characterised by fluctuations in the severity and frequency of ischaemic episodes:[1,2] periods of frequent ischaemic attacks (active phase of the disease) alternate with periods free from attacks (inactive phase). There are some differences between the active and inactive phases in platelets and blood clotting. Active-phase patients have significantly higher plasma fibrinopeptide A concentrations[3,4] and lower levels of prostacyclin (PGI_2)[5] than inactive-phase patients. Moreover, platelets from active-phase patients produce more thromboxane A_2 than those from inactive-phase patients.[3] Abnormally low platelet sensitivity to PGI_2 in patients with spontaneous angina has been reported.[6]

These functional alterations of platelets could be related to changes in platelet membrane receptors for PGI_2. We have investigated the number and affinity of PGI_2 receptors in patients with spontaneous angina.

TABLE I—CHARACTERISTICS OF PATIENTS AND CONTROL SUBJECTS*

	Controls	Patients	
		Active phase	Inactive phase
Sex (M:F)	5:4	8:6	9:5
Age (yr)	49·3±9·2	53·1±7·5	51·5±7·5
Plasma cholesterol (mg/l)	188·5±22·6	191·8±36·2	195·2±19·7
Blood pressure (mm. Hg)			
Systolic	135·5±19·7	141·6±18·4	139·2±15·7
Diastolic	84·4±9·1	83·2±10·1	83·8±10·7
Weight (kg)	75·4±9·1	67·3±9·5	68·7±9·2
Smoker	4	7	6

*Given as numbers of subjects or mean±SD

Patients and Methods

Fourteen patients with spontaneous angina (angina at rest or angina at rest superimposed on effort angina, showing S-T segment elevation or depression during the attacks) in the active phase, fourteen patients with spontaneous angina in the inactive phase, and nine healthy controls (table I) were investigated after giving informed consent. Platelet PGI_2 receptors were investigated 1 week after the withdrawal of all therapy except nitrates from the patients (observation week). No subjects had taken aspirin or other drugs interfering with platelets in the 2 weeks before the study. Patients taking β-blockers or calcium antagonists gradually stopped these drugs during a 4-day period before the observation week, and the drugs were substituted by nitrates.

Patients were evaluated for the activity of the disease during the week when they were receiving only nitrates, by recording of typical anginal pain and by 24 h Holter monitoring, done every 2 days. Patients who had no ischaemic attacks at rest or on minimum effort, symptomatic or symptomless, during this week were considered to be in the inactive phase, the others in the active phase. All the patients were maintained on six 10 mg isosorbide dinitrate tablets daily. Supplementary sublingual isosorbide dinitrate tablets were administered if necessary. In preliminary investigations platelet PGI_2 receptors were found not to be significantly affected (Student's t test) by the short-term administration of nitrates (table II). Angina was diagnosed on the basis of typical chest pain, electrocardiogram at rest and on exercise, Holter monitoring (Del Mar Avionics, Irvine, California, USA), and coronary angiography. None of the patients had diabetes. Six of the fourteen active-phase patients were investigated again 4–9 weeks later, when, on nitrates only, they had become free of ischaemic attacks at rest. The control subjects were selected so that age, sex distribution, body weight, blood pressure, plasma cholesterol levels, and smoking habits did not significantly differ from those of the patient groups (table I). The control subjects had normal ergometric effort tests and Holter monitoring (two 24 h periods), and did not have cardiovascular

TABLE II—EFFECT OF NITRATE ADMINISTRATION ON PGI_2 RECEPTORS OF PLATELETS FROM FOUR HEALTHY SUBJECTS AGED 38–56

—	Before nitrates	After nitrates*
High-affinity receptors		
Receptors/platelet	98±3	1[illegible]±4[illegible]
Kd (nmol/l)	[illegible]	[illegible]
Low-affinity receptors		
Receptors/platelet	2[illegible]	[illegible]
Kd (nmol/l)	55[illegible]	[illegible]8±26

*Isosorbide dinitrate, 10 mg four times daily for 3 days

Kd = dissociation constant

disease, diabetes, hyperthyroidism, hypertension, or other serious disease.

Blood samples were drawn by clean venepuncture without stasis into polypropylene syringes from fasting subjects between 0800 and 0900 h, with 19-gauge needles. Blood was anticoagulated with 15% acid-citrate dextrose (National Institutes of Health, formula A) and platelet-rich plasma was obtained by centrifugation at 160 *g* at room temperature (20–22°C). The pH was adjusted to 6·5 with acid-citrate dextrose and a platelet pellet was prepared by centrifugation at 1800 *g* for 30 min at room temperature. The pellet was resuspended in 10 ml EDTA-phosphate buffer (8 mmol/l Na_2HPO_4, 2 mmol/l NaH_2PO_4, 10 mmol/l EDTA, 5 mmol/l potassium chloride, 135 mmol/l sodium chloride, pH 7·2), and recentrifuged at 1800 *g* for 30 min at room temperature. The supernatant was discarded and the platelet pellet resuspended in 3·5 ml assay buffer (138 mmol/l sodium chloride, 5 mmol/l magnesium chloride, 1 mmol/l EGTA, and 25 mmol/l "tris"-HCl, pH 7·5). If necessary, assay buffer was added to obtain a platelet concentration of 10^6 platelets/µl. Platelet counts were done with an automatic platelet counter (TOA PL-100, Kobe, Japan).

The radioligand was used in three types of experiments to prove that platelet-bound radioactivity represented radioligand bound to receptors and not to other sites that degrade, transport, or non-specifically bind it. The three types of experiments were binding isotherms (saturation curves), kinetic studies (time course), and competitive binding experiments.

Saturation analysis.—The platelet suspension (0·1 ml) was incubated in a final volume of 0·2 ml with 10 nmol/l tritiated PGI_2 (PGI_2 methyl ester, 9-^3H(N); specific activity 10 Ci/mmol, NEN, Dreieich, West Germany) plus 0–1000 nmol/l unlabelled PGI_2 (Wellcome, Beckenham, UK) for 10 min at room temperature.[7] These tubes were considered "total binding tubes". In "non-specific tubes" 10 µmol/l (final concentration) unlabelled PGI_2 was added in the same final volume to evaluate non-specific binding. The different concentrations of PGI_2 were obtained by adding to a fixed amount of ^3H-PGI_2 increasing amounts of

unlabelled PGI_2 giving labelled PGI_2 with decreasing specific activities. After 10 min incubation, 4 ml ice-cold buffer was added to each tube to stop the reaction and the contents were rapidly filtered under reduced pressure through Whatman GF/C glass microfibre filters. Time-course experiments showed that under these conditions equilibrium had been reached. Each assay tube and the filters were then washed with four 5 ml volumes of ice-cold buffer. The entire washing procedure was completed within about 20 s. Filters were then dried under air flow and suspended in 7 ml 'Aquasol 2' (NEN, Dreieich). Counting was done in a Beckman liquid scintillation spectrometer Type LS 1800, Irvine, California). Total binding for each concentration was determined by dividing counts/min (cpm) by the corresponding calculated specific activity. Specific binding was taken to be that displaced by 10 μmol/l unlabelled PGI_2 in parallel incubation. The experiments were carried out in triplicate and a saturation curve was carried out for each subject.

Kinetics and specificity studies.—To assess the kinetics of the reaction, single experiments were carried out incubating tubes containing 2 ml of the same concentrations of reagents as described above. At selected times (0·5, 1, 2, 3, 5, 8, 10, 15, 20, and 30 min), 0·2 ml reaction mixture was pipetted into a clean tube, the reaction terminated as described above, and filtration performed. To assess the reversibility of the binding, 10 μmol/l (final concentration) PGI_2 was added to the reaction mixture after 10 min of incubation and samples processed in the same way. To check the specificity of ^{3}H-PGI_2 binding, prostaglandins D_2 and E_1 at two different concentrations (10 μmol/l and 50 μmol/l) were added to the reaction mixture at equilibrium. The incubation was continued for 10 min and the binding evaluated at defined times.

Evaluation of binding data.—Specific binding was calculated as the difference between total and non-specific binding. The interaction of ^{3}H-PGI_2 with platelets was analysed according to Scatchard.[8] Further analysis was done by the curve peeling method, according to Rosenthal,[9,10] and by Hill plot.[11]

The number of megathrombocytes was determined according to Garg et al.[12] Platelet protein content was measured by the method of Lowry et al.[13]

Results are expressed as mean±standard deviation (SD). The data were analysed by the Student *t* test for paired or unpaired data. The tests and other calculations were carried out by a T 20 'General Processor' computer (Italy).

Results

Kinetics and Specificity of ^{3}H-PGI_2 Binding

Experiments on platelets from four controls showed that ^{3}H-PGI_2 rapidly bound and dissociated from its binding sites with kinetics of the same order as the biological response to the unlabelled ligand. 50% of maximum binding was reached in about 1 min and the equilibrium of the binding reaction

within 6 min. Binding was stable for at least 10 min and the addition of 10 μmol/l unlabelled PGI_2 at equilibrium resulted in rapid displacement of ^{3}H-PGI_2 (50% after 1 min, 85% after 4 min, and 90% after 20 min). 60% ^{3}H-PGI_2 displacement was also obtained by adding 50 μmol/l unlabelled PGE_1, whereas the addition of 10 μmol/l PGD_2 did not affect ^{3}H-PGI_2 binding to platelets.

Saturability and Affinity of ^{3}H-PGI_2 Binding

The concentration-dependent binding experiments showed that ^{3}H-PGI_2 binding was saturable (fig 1). The data obtained from these binding experiments yielded Scatchard plots with upward concavity (fig 2). Hill plots of the same binding experiments constantly gave slopes below 1 (0·5–0·7 at the point of flexus), indicating the presence of heterogeneous binding sites. The profile of the Scatchard curve is consistent with the presence of two different classes of binding sites, one high affinity and one low affinity. In the control group the mean number of binding sites was 90±17 high-affinity receptors/platelet and 2804±952 low-affinity receptors/platelet (fig 3). The dissociation constants (Kd) were 10·2±4·1 nmol/l and 899±467 nmol/l respectively (fig 4).

Scatchard analysis and Hill plots indicated that platelets from angina patients also had two distinct classes of binding

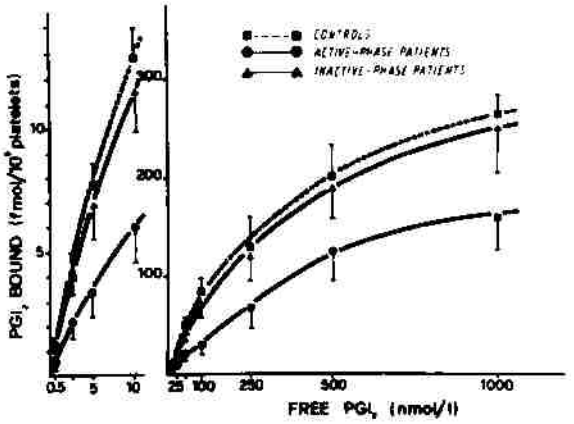

Fig 1—Concentration-dependent specific binding of ^{3}H-PGI_2 to platelets in vitro (after 10 min incubation at 20–22°C).

Points represent means of triplicate determinations. Bars indicate standard deviation (SD).

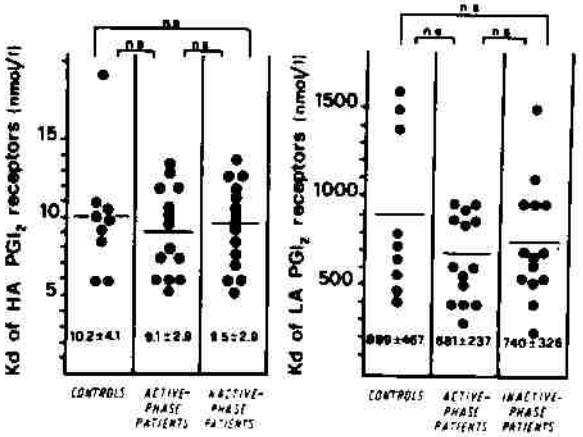

Fig 4—Dissociation constant (Kd) of PGI_2 high-affinity (HA) and low-affinity (LA) receptors of platelets.

The platelet count, routinely assessed in all subjects, was similar in the three groups investigated, whereas the percentage of megathrombocytes was higher in active-phase than in inactive-phase patients or controls (table III). Moreover, platelet mass was similar in all three groups (table III).

The pattern in the six patients studied in both the active and the inactive phase differed greatly between the two investigations. There were significantly higher numbers of both high-affinity and low-affinity receptors in the inactive than in the active phase ($p<0·02$ for high-affinity receptors and $p<0·025$ for low-affinity receptors). The values found in inactive phase were in the range of the control group (fig 5).

TABLE III—PLATELET COUNT, MEGATHROMBOCYTES, AND PLATELET PROTEIN MASS

	Controls	Patients	
		Active phase	Inactive phase
Platelet count ($\times 10^9$/l)	240±36	233±36	229±39
Megathrombocytes (%)	6·21±1·22	10·01±3·27*	7·03±1·61
mg protein per 10^9 platelets	3·77±0·30	3·91±0·35	3·82±0·30

*Significantly greater than in inactive-phase patients ($p<0·01$) and controls ($p<0·005$).

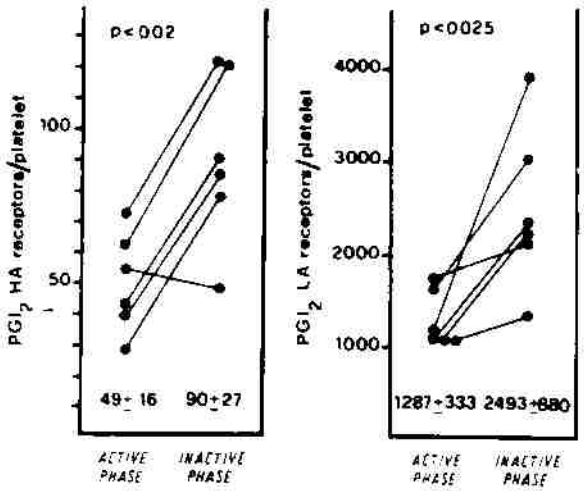

Fig 5—Numbers of PGI high-affinity (HA) and low-affinity (LA) receptors per platelet from patients studied in the active and in the inactive phase.

Discussion

The reduction in PGI_2 receptor density in active-phase spontaneous angina shown in this study is temporary; it seems to disappear in the inactive phase.

Several investigators have reported the presence on the platelet membrane of a common receptor for PGI_2 and PGE_1,[14,15] which is distinct from the PGD_2 receptor.[7,16] Under our experimental conditions, the binding of ^{3}H-PGI_2 to intact platelets is rapid, reversible, and saturable. The binding dissociation constant lies within the range of biologically active concentrations. Thus, ^{3}H-PGI_2 binding met the criteria for a specific receptor. The Scatchard plot of the data obtained from saturation analysis was curvilinear and the Hill plot of the same data gave a slope of 0·5–0·7 at the point of flexus, indicating the presence of two classes of specific PGI_2 receptors.[11] The platelet alpha$_2$-adrenergic receptor also exists in two affinity states.[17] The affinity of the binding sites for PGI_2 does not change in the active phase of spontaneous angina.

The reduced ability of platelets from active-phase patients to bind PGI_2 seems to be due to a decrease in the number of the binding sites. It is not related to a fall in platelet volume; the mean protein content of the platelets was the same in the three groups investigated. The high number of

megathrombocytes found in active-phase patients could be responsible for the reduced density of PGI_2 binding sites if megathrombocytes had a lower capacity for binding ^3H-PGI_2 than other platelets. However, this hypothesis can be ruled out because even if megathrombocytes were absolutely unable to bind PGI_2, they would have to make up at least 50% of the circulating platelets, instead of the 10% found in our study, in order to account for the reduction in PGI_2 binding sites seen in active-phase patients.

The reduction in PGI_2 receptors might be related to reduced PGI_2 synthesis. It is well known that, in addition to the heterospecific regulation (ie, the receptor for the hormone is regulated by a second hormone), cell surface receptors can have homospecific up or down regulation.[16] Certain hormones (eg, prolactin[19]), or more generally some agonists, can modulate the cell density of their own receptors. PGI_2 might cause an up-regulation of its own receptors because PGI_2 production is lower in patients with active-phase spontaneous angina than in patients with inactive disease and in controls.[5] FitzGerald et al's findings,[20] however, might suggest a down-regulation for prostacyclin receptors.

Patients with active disease studied again in the inactive phase had a similar number of receptors to controls. We do not know whether the remission of the anginal attacks was spontaneous or caused by the drug regimen. Even if spontaneous angina undergoes cyclical remission,[1,2] we cannot rule out the possibility that the normalisation of the number of platelet binding sites in these patients was induced by the nitrates, although short-term nitrate administration did not affect platelet PGI_2 binding sites. Thus, our findings do not answer the most important question about the relation between platelet receptor changes and the occurrence of myocardial ischaemic attacks—is the reduction in platelet membrane receptors for PGI_2 a consequence or a parallel event of the ischaemic attacks or do the platelet changes precede and favour the occurrence of anginal attacks? The reduction in platelet receptors for PGI_2 seems not to be the only unusual feature of patients with active spontaneous angina. These patients show increased formation of thromboxane A_2 by platelets,[3] raised thromboxane B_2 concentrations in peripheral venous[21] and sinus blood,[22] reduced PGI_2 production,[5] and elevation of fibrinopeptide A levels in plasma.[3,4] These functional changes, as a whole, seem to suggest that the reduction in platelet receptor density for PGI_2 might not be only a consequence of myocardial ischaemic attacks but rather a part of a generalised disorder, characteristic of the instability of ischaemic heart disease.

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Correspondence should be addressed to G. G. N. S., Clinica Medica I, University of Florence, Viale Morgagni 85, 50134 Florence, Italy.

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