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ILOPROST BINDING AND INHIBITION OF AGGREGATION IN PLATELET RICH PLASMA

DIFFERENCES BETWEEN NORMAL AND TYPE IIa HYPERCHOLESTEROLEMIC SUBJECTS

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Abstract—Platelets from type IIa hypercholesterolemic subjects have been previously shown to be less sensitive than normal platelets to the antiaggregatory effect of PGI_2. We demonstrate here that these platelets display a reduced response to iloprost, a chemically stable analogue of PGI_2, as well. In fact, the concentration of iloprost yielding 50% inhibition of PRP aggregation was higher in type IIa patients (0.77 ± 0.08 nM) than in controls (0.51 ± 0.06 nM, $P < 0.01$). In addition, an inverse relationship existed between the threshold aggregatory concentration for collagen and the concentration of iloprost yielding 50% inhibition of PRP aggregation, both in type IIa and normal individuals. In order to elucidate the mechanism of the different sensitivity of platelets to prostacyclin and its analogue, we characterized the binding of ^{3}H-iloprost to platelet rich plasma from single individuals. The binding was rapid, reversible, inhibited by iloprost, PGI_2 and PGE_1 (K_d = 50.7; 346.2 and 7500 nM, respectively); no heterogeneity of sites could be demonstrated in the PRP from a single individual. When binding studies were carried out in PRP of type IIa patients and controls, it appeared that the amount of ^{3}H-iloprost bound at a fixed (300 nM) concentration was significantly lower in platelets from type IIa individuals (0.94 ± 0.17 vs. 1.77 ± 0.27 fmol/10^6 platelets, for patients and controls, respectively). It is concluded that such difference in binding might represent the mechanism underlying the reduced response to PGI_2 and iloprost observed in platelets from type IIa patients.

Increasing evidence indicates that a link between hyperlipidemia and functional and biochemical alterations of platelets exists [1, 2]. In fact, platelets from patients with type IIa hypercholesterolemia, a pathological condition associated with high plasma levels of low density lipoproteins, are more sensitive to aggregating agents than platelets from normocholesterolemic subjects [3, 4]. In addition, arachidonic acid metabolism via both cyclo- and lipooxygenase pathways has been shown to be increased in platelets from type IIa patients [4, 5].

The greater sensitivity of platelets from type IIa patients to the effects of agonists of platelet aggregation is coupled to a reduced response of such platelets to the activity of inhibitors of the platelet aggregatory process [6]. In particular, platelets from type IIa patients have been shown to be less sensitive than platelets from a group of normal subjects, comparable for age and sex, to the antiaggregatory effects of exogenous prostacyclin (PGI_2)† [7]. The differences observed between patients and controls in terms of antiaggregatory potency of PGI_2 can be ascribed neither to a difference in PGI_2-sensitive adenylate cyclase of platelet membranes [7] nor to an effect of the various lipoproteins on such enzyme [8].

More recent data, however, suggest that many hormones or hormone-like substances, which have been shown to modulate adenylate cyclase activity, are indeed able to trigger other intracellular events, such as phosphatidylinositol turnover and increases in cytosolic calcium concentrations, possibly through a different set of receptors [9, 10]. This might apply also to prostacyclin in platelets.

Therefore, as a more general approach to the problem of molecular mechanisms underlying the above-mentioned differences in PGI_2 action, we investigated whether the binding of a chemically stable PGI_2 derivative, iloprost, to PGI_2 receptors was modified in platelets from type IIa hypercholesterolemic subjects in comparison to platelets from normocholesterolemic subjects, comparable for age and sex. Furthermore, at variance from previous studies [7, 8], the binding assay was performed in platelet rich plasma (PRP), an experimental approach which resembles the physiological situation more closely.

MATERIALS AND METHODS

Patients. Thirty-six subjects (16 males and 20 females, age range 35–63 years) with a diagnosis of type IIa hypercholesterolemia according to WHO

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† Abbreviation used: PGI_2, prostacyclin; PG prostaglandin; PRP, platelet rich plasma; WP, washed platelets; IC_{50}, concentration eliciting 50% inhibition of response.

Table 1. Serum lipid profiles of normal and type IIa subjects

Subjects	Cholesterol (mg/dl)		Triglycerides (mg/dl)
	Total	HDL	
Controls (N = 40)	210 ± 3.9	44.5 ± 1.6	114 ± 5.0
Type IIa (N = 36)	315 ± 21.0	48.9 ± 3.3	133 ± 10.0

Values represent the mean ± SEM.

criteria [11] were selected in our Lipid Clinic. The results were compared with those obtained from a group of 40 age and sex matched normocholesterolemic subjects in terms of aggregation. Eleven patients and 12 controls out of this group, in addition to four extra controls, underwent platelet binding studies. The plasma lipid and lipoprotein levels of the studied subjects are reported in Table 1. Patients and controls did not take any drug known to affect platelet function for at least 10 days before the study and all the type IIa patients studied were on a standard low lipid diet [12] for at least 1 month.

Materials. Collagen was from Mascia Brunelli (Milan, Italy); ^{3}H-iloprost (14.7 Ci/mmol, in ethanol) was obtained from Amersham (Amersham, U.K.), the organic solvent was evaporated under nitrogen stream, and the residue was taken up in 10 mM Tris–HCl. Unlabelled iloprost was from Schering AG (Berlin, F.R.G.) and dissolved in sterile saline at 1.4 mM. Further dilutions were freshly prepared with 50 mM Tris–HCl buffer, pH 7.4. PGI_2 sodium salt was a gift from Dr. B. J. R. Whittle (Wellcome, Beckenham, U.K.), and the stock solution in ethanol (3 mM) was stored at −20°. Diluted solutions in 10 mM ice-cold Tris–HCl buffer, pH 8, were prepared immediately before use. Addition of these solutions to PRP for the binding assay (see below) did not alter the final pH.

Platelet aggregation studies. Blood was collected in plastic tubes containing 3.8% trisodium citrate (9:1). Platelet rich plasma (PRP) and platelet poor plasma (PPP) were obtained after centrifugations as previously described [13]. Platelet aggregation was determined using the Born turbidimetric technique [14] in an Elvi aggregometer (Elvi Logos, Milan, Italy). For each subject the minimal concentration of collagen giving a 60% decrease in optical density in 5 min was searched and defined as threshold aggregating concentration (TAC).

Iloprost inhibition of platelet aggregation. Different concentrations of iloprost were incubated in PRP samples at 37° for 1 min before the addition of a fixed concentration of collagen (1 μg/ml). This concentration was selected to obtain a maximal aggregatory response in all the subjects. The concentration of iloprost giving 50% inhibition (IC_{50}) of aggregation was calculated by plotting the percentages of inhibition of platelet aggregation versus the corresponding concentrations of the inhibitor. The percentage of inhibition was calculated on the basis of the amplitude of the aggregation curve at 5 min after the addition of collagen.

Binding assay. Assays were carried out in a total volume of 1030 μl. PRP (1 ml, 300–400 × 10^6 platelets/sample) was incubated at 30° for 10 min, unless otherwise indicated, with 10 nM ^{3}H-iloprost and increasing concentrations of unlabelled prostaglandins. Specific binding was considered to be that displaced by 100 μM iloprost. The incubation was terminated by centrifugation at 8140 *g* for 5 min in a Biofuge centrifuge (Heraeus) and the supernatant was rapidly removed by tube inversion. The pellet was carefully washed with 1 ml of saline, without resuspending it; the tubes were emptied and the walls wiped with a cotton swab. The pellet was completely dissolved by addition of 0.5 ml of 1 N NaOH. After 3 hr, 0.5 ml of water were added and the sample was transferred to the counting vial and counted in 9 ml of Filtercount (Packard).

Computer analysis of binding curves. The data were fitted by an iterative program (RECEPT) [15] for non-linear regression analysis in order to obtain the IC_{50} value, from which K_d was then obtained by the method of Cheng and Prusoff [16]. The amount of iloprost bound was calculated from the inhibition curves, taking into account the decrease in specific activity which derives from the dilution of ^{3}H-iloprost with unlabelled iloprost. The data in Fig. 5 were fitted to both a one-site and a two-site model; the one-site model was considered to be a better representation of the binding data when it yielded a higher correlation coefficient, or when the improvement of goodness-of-fit for the two-site model was not statistically significant according to the *F*-test on the sums of squared errors [15].

RESULTS

Comparison of the inhibitory effects of iloprost on PRP aggregation in patients and controls

As shown in Fig. 1, the concentrations of iloprost required to inhibit by 50% the aggregation of platelets from type IIa patients, elicited by 1 μg/ml collagen, were significantly greater ($P < 0.01$) than those necessary to achieve the same inhibition in PRP of normocholesterolemic subjects ($IC_{50} = 0.77 \pm 0.08$ and 0.51 ± 0.06 nM, respectively). No significant correlation, however, was found between the IC_{50}s for iloprost and the levels of total and low density lipoprotein cholesterol in plasma (data not shown). To assess whether the sensitivity of platelets to the inhibitory effects of iloprost was related to the response of platelets to the action of aggregating agents, for each subject the threshold concentration of collagen (TAC), required to elicit 60% decrease in optical density in the absence of iloprost, was searched and subjects were grouped according to their TAC values. For each group the mean IC_{50}s, assessed in PRP stimulated with 1 μg/ml collagen, for both patients and controls are represented versus

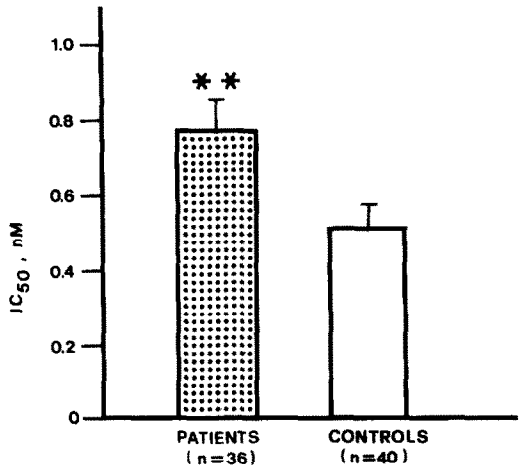

Fig. 1. Effect of iloprost on platelet aggregation induced by 1 μg/ml collagen in control and type IIa subjects. Data are expressed as IC_{50}s for iloprost (means ± SEM).

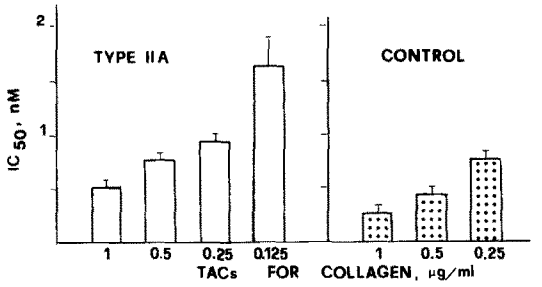

Fig. 2. Relationship between threshold aggregating concentrations of collagen (TACs) and IC_{50}s for iloprost. TACs for collagen were determined in PRP in the absence of iloprost; IC_{50} for iloprost was determined in PRP stimulated by 1 μg/ml of collagen in control and type IIa subjects.

the different TAC values (Fig. 2). Indeed, the IC_{50}s for iloprost were inversely related to the TACs for collagen in both patients and controls. In other words, platelets which were more sensitive to the effects of collagen (lower TAC value) required greater amounts of iloprost to achieve 50% inhibition of aggregation induced by a fixed amount of collagen.

Characteristics of ^{3}H-iloprost binding

The binding of ^{3}H-iloprost to PRP was rapid, equilibrium being attained in approximately 5 min or less (Fig. 3). Investigation of a shorter incubation time was not possible because the separation of bound from free ligand by centrifugation required 5 min itself. The binding did not decline up to at least 17 min, indicating that both the binding sites and the ligand were stable under the assay conditions selected. The binding was rapidly reversible (Fig. 4), since approximately 70% dissociated in 2 min upon addition of an excess of unlabelled iloprost. The data on the semi-log plot of the dissociation curve (inset to Fig. 4) are well fitted by a straight line ($P < 0.01$), suggesting a simple first-order kinetic, which would be in agreement with the existence of a single class of binding sites.

Figure 5A shows the inhibition of ^{3}H-iloprost specific binding by unlabelled iloprost and PGI_2. The two curves are approximately parallel, as one would expect if the two ligands interacted in a similar manner with the same sites. Iloprost displayed an affinity 7-fold higher than PGI_2. The results in Fig. 5B indicate that PGE_1, as well, dose-dependently inhibited ^{3}H-iloprost specific binding, albeit with an affinity lower than PGI_2 ($K_d = 7500$ nM), while PGD_2 displayed a very low affinity.

Non-linear fitting of the inhibition curves yielded IC_{50} values of 60.9 ± 9.4 and 415.7 ± 61.8 nM, from which K_ds of 50.7 and 346.2 nM for iloprost and

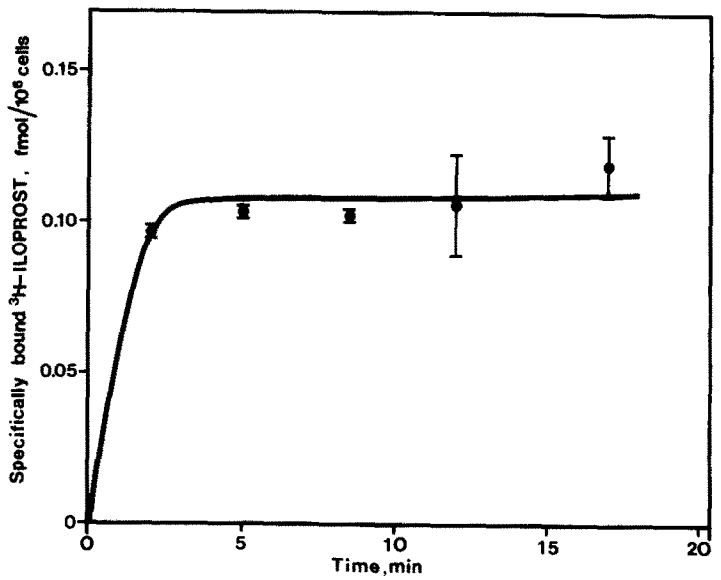

Fig. 3. Time-course of ^{3}H-iloprost association to PRP from a normal individual. ^{3}H-iloprost was 12.1 nM, and platelet concentration was 452 × 10^6/ml.

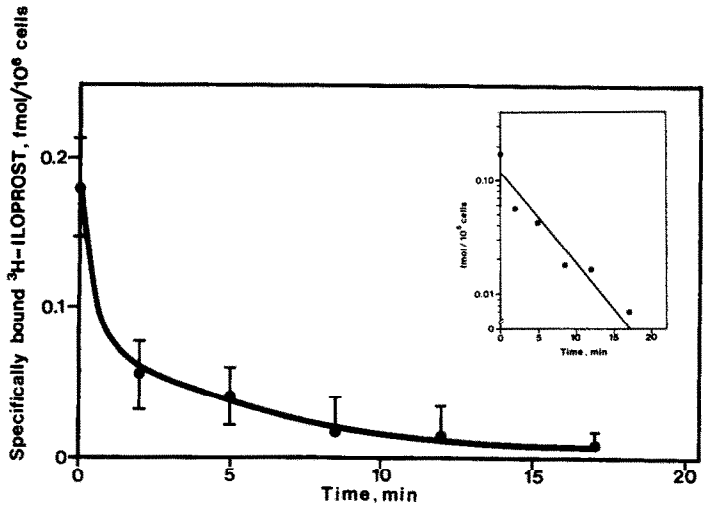

Fig. 4. Time-course of ^{3}H-iloprost dissociation from PRP of a normal individual. ^{3}H-iloprost was 15 nM, and platelet concentration was 360×10^6/ml. The inset represents the semi-log plot of the same data.

PGI_2, respectively, were calculated. Although in some cases the correlation coefficient for the fitting based on a two-site model was slightly better than that for the one-site model, the improvement in goodness-of-fitting was never statistically significant ($P > 0.05$), whether single curves or the mean of three curves were considered. The modified Scatchard plot (Eadie–Hofstee plot) for the inhibition of ^{3}H-iloprost binding by unlabelled iloprost in PRP from a single normal individual is shown in Fig. 6. The data can be fitted by a single straight line (correlation coefficient: 0.84, $P < 0.01$). These latter findings are in agreement with our suggestion that it is not required to postulate the existence of heterogeneous sites in order to interpret the binding data.

Therefore, the existence of more than one class of binding sites cannot be considered proven in this system.

The influence of plasma on the binding of ^{3}H-iloprost to human platelets was also investigated by comparing the binding to PRP or to washed platelets (adjusted to the same concentration as in PRP) obtained at the same time from the same individual. As shown in Fig. 7, the IC_{50} for iloprost was approximately 5-fold higher in the presence of plasma than in its absence.

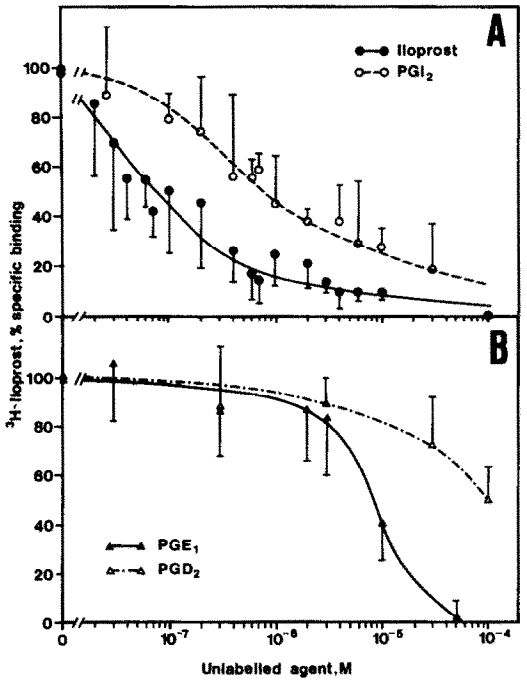

Fig. 5. Inhibition of ^{3}H-iloprost specific binding by unlabelled iloprost, PGI_2 (panel A), PGE_1 and PGD_2 (panel B). Each curve is the mean of 3 (panel A) or 2 (panel B) experiments, each performed in triplicate, on PRP from a single normal individual. 100% binding was 0.172 ± 0.013 (panel A) and 0.106 ± 0.021 (panel B) fmol/10^6 platelets.

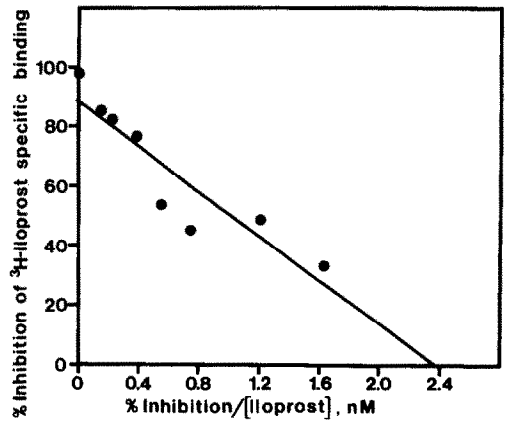

Fig. 6. Modified Scatchard plot (Eadie–Hofstee plot) for the inhibition of ^{3}H-iloprost specific binding by unlabelled iloprost.

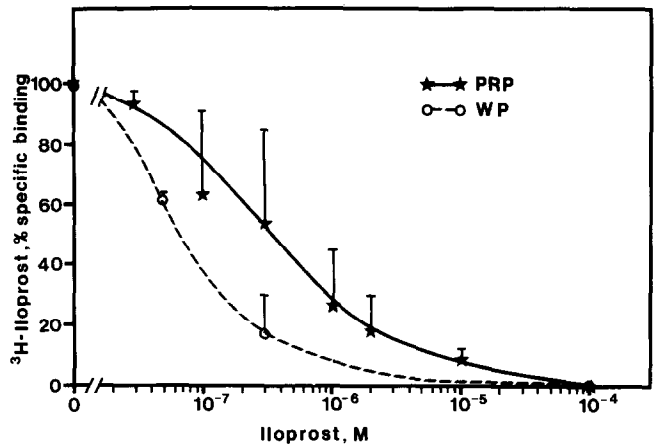

Fig. 7. Inhibition of ^{3}H-iloprost specific binding by unlabelled iloprost in washed platelets (WP) and platelet rich plasma (PRP) from the same normal individual. 100% binding was 0.352 ± 0.0025 and 0.106 ± 0.021 fmol/10^6 platelets in WP and in PRP, respectively.

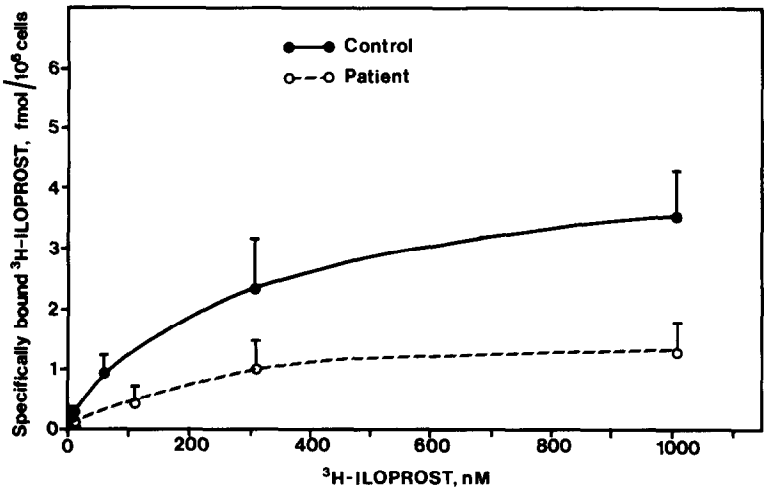

Fig. 8. Representative examples of saturation curves for ^{3}H-iloprost binding to PRP from a normal (●—●) and a type IIa (○—○) individual. ^{3}H-iloprost was 8.6 nM, and platelet concentration was 360 and 340 × 10^6/ml in control and patient, respectively.

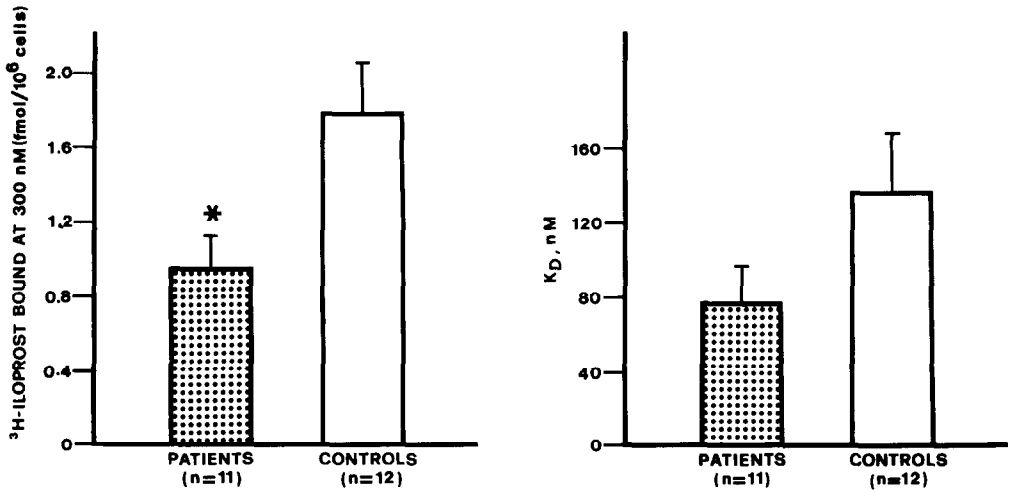

Fig. 9. ^{3}H-iloprost binding characteristics in controls and type IIa patients. Left panel: amount bound at 300 nM iloprost; right panel: K_d values.

Comparison of binding parameters in platelets from type IIa patients and controls

The binding of increasing concentrations of iloprost to PRP was investigated in 11 hypercholesterolemic subjects (type IIa) and in 12 sex- and age-matched controls.

The amount of blood that we could draw from each individual limited the concentration range that we could explore. While we were able to measure the IC_{50} in platelets from each individual, and from that to calculate the K_d, saturation was not always attained at 300 nM, which was the highest concentration tested in all the subjects.

Representative saturation curves in the case of one control and one type IIa subject are shown in Fig. 8, while Fig. 9 summarizes the K_d values obtained in controls and patients, as well as the values of the amount bound at 300 nM ^{3}H-iloprost. The K_d values were not significantly different in type IIa patients as compared to controls (75 ± 20 and 135 ± 31 nM, respectively); on the contrary, the amount bound was significantly lower in patients (0.94 ± 0.17 fmol/10^6 platelets) than in control (1.77 ± 0.27 fmol/10^6 platelets) subjects ($P < 0.02$).

DISCUSSION

The data on the inhibitory activity of iloprost, a chemically stable analogue of prostacyclin, on the aggregation of PRP from type IIa patients and normocholesterolemic subjects confirm and extend previous findings from our laboratory, indicating that platelets from hypercholesterolemic individuals display a reduced sensitivity to the antiaggregatory effects of PGI_2 [7]. Furthermore, in this study we could demonstrate that a relationship exists between the sensitivity of platelets to the effects of agonists of platelet aggregation and the amounts of iloprost required to inhibit the aggregatory process.

In order to elucidate the mechanisms underlying the observed modification of platelet response to PGI_2 and iloprost, we have characterized the binding sites for ^{3}H-iloprost in human PRP. These sites have many of the characteristics expected from receptors: the interaction with the ligand is rapid, reversible, saturable and specific. Iloprost itself displays a higher affinity (7-fold) for the binding sites than does PGI_2, and this finding lends further support to the hypothesis that the sites might coincide with the receptors involved in the inhibition of platelet aggregation by the natural prostaglandin. In fact, iloprost proved to be more potent than PGI_2 as an inhibitor of collagen-induced platelet aggregation [17–19].

Furthermore, PGI_2 inhibited ^{3}H-iloprost binding with a concentration–effect curve parallel to that obtained with the stable analogue, suggesting that the two prostaglandins interact with the same sites. PGE_1 also inhibited ^{3}H-iloprost binding, while PGD_2 was much less effective. This is expected, if the sites labelled by ^{3}H-iloprost coincide with PGI_2 binding sites; in fact, PGE_1 has been shown to compete with ^{3}H-PGI_2 for its specific sites [20, 21], while PGD_2 is supposed to interact with different sites [22].

By investigating the binding of ^{3}H-iloprost to PRP from single individuals, by means of equilibrium as well as kinetic studies, we have not been able to evidence the existence of more than one class of sites, at variance with the findings of our [20] and other laboratories [21, 23], which indicate that ^{3}H-PGI_2 interacts with at least two classes of binding sites. This difference might be explained by a number of reasons: (a) it is possible that the sites are indeed heterogeneous, and that PGI_2, but not iloprost, displays an affinity different enough for the two classes and therefore discriminates between them; (b) alternatively, the heterogeneity revealed in the previous studies might have been due to the use of platelets pooled from different individuals, while in the present paper the binding experiments have been performed on cells prepared from a single individual; (c) finally, one might hypothesize that factors from plasma, which was obviously absent in membrane studies, can influence the interaction of ligands with PGI_2 receptors. It is interesting to note, however, that other authors as well, using the same criterion we use to discriminate between one- and two-site model [24, 25], or working with gel-filtered platelets [26, 27], have detected only one binding site for PGI_2 in human platelets. In addition, experiments performed with ^{3}H-iloprost in platelet membranes again revealed a single homogeneous class of sites [28].

The binding sites identified here display an affinity for both iloprost and the natural prostaglandins lower than previously reported. This, however, is not surprising because the present work has been performed in PRP, that is in the presence of plasma proteins, instead of washed platelets or plasma membranes. While the use of PRP in our case was advisable, in order to maintain the different environment to which platelets were exposed in normal and hypercholesterolemic subjects, plasma proteins, by binding to iloprost and PGI_2 [29, 30], reduce the apparent affinity of the prostaglandins for the platelets. This is indeed apparent from the data in Fig. 7.

Investigation of iloprost binding to PRP from normal and type IIa subjects revealed that the amount of ^{3}H-iloprost bound at a fixed ligand concentration was lower in platelets from patients than controls. Since there is no significant variation in the K_d values between the two groups, the difference in the amount of iloprost bound is very likely to represent a difference in the number of binding sites. A lower number of sites, possibly receptors, in the platelets of hypercholesterolemic subjects might be the cause of their decreased response to both prostacyclin [7] and iloprost. It is interesting to note that platelets of type IIa hypercholesterolemic individuals also possess a number of binding sites for PGD_2 lower than normal [31]. This finding correlates well with the diminished response to this antiaggregating prostaglandin found by the same authors. It is possible that such differences in binding capacity are related to the different lipid composition of the platelet membranes in hypercholesterolemic versus normal individuals [32], as other receptors have been shown to be affected by the lipid environment. However, at present this is merely a hypothesis.

In a previous study we had demonstrated [7] that PGI_2-sensitive adenylate cyclase in platelet membranes from type IIa subjects is not significantly

different from that of normal ones. In light of the present results, demonstrating reduced binding in the platelets from patients vs controls, one should conclude that: (a) either activation of adenylate cyclase is not the only mechanism through which PGI_2 exerts its effects on platelets; or (b) some modulating factor(s) exist(s) in PRP, which are lost upon platelet washing or membrane preparation.

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