Biochemical Pharmacology, Vol. 32, No. 13, pp. 1989–1993, 1983.
Printed in Great Britain.

0006–2952/83 $03.00 + .00
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EFFECTS OF PGI_2 ON PLATELET AGGREGATION AND ADENYLATE CYCLASE ACTIVITY IN HUMAN TYPE IIa HYPERCHOLESTEROLEMIA

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(*Received* 5 *October* 1982; *accepted* 21 *January* 1983)

Abstract—The sensitivity to PGI_2 of platelets of 20 selected type IIa hypercholesterolemic patients was studied and compared to that of platelets of 14 normocholesterolemic subjects. Type IIa subjects required higher concentrations of PGI_2 to inhibit platelet aggregation elicited by 1 μM ADP, 1 μg/ml collagen and 1.4 μM epinephrine. Adenylate cyclase activity was also measured in washed platelet membranes from the two groups of subjects. Adenylate cyclase activity, both basal and PGI_2-stimulated, was not statistically different in the two groups examined. Therefore changes at the level of PGI_2 receptors coupled to adenylate cyclase are not likely to be responsible for the different platelet sensitivity to prostacyclin.

There is considerable evidence in the literature indicating that platelets from type IIa hypercholesterolemic subjects are more sensitive to aggregating agents than platelets from normal subjects [1–4]. Increased amounts of malondialdehyde and thromboxane B_2 are produced by platelets from these patients [4, 5]. In addition, higher levels of platelet factor 4 (PF4) and β-thromboglobulin are produced by platelets from type IIa patients [6], suggesting an increased release reaction.

Cholesterol *per se* may increase platelet response to aggregating agents [7] as well as arachidonic acid release from membrane phospholipids, and thromboxane B_2 formation following stimulation [8], as shown in experiments using normal platelets incubated with cholesterol-enriched liposomes. In platelets from type IIa subjects the cholesterol/phospholipid ratio was not found to be consistently elevated [9–11]. However, the platelet response not only to activators but also to inhibitors of platelet aggregation might be altered in type IIa hypercholesterolemic subjects.

Prostacyclin (PGI_2) is a potent anti-aggregatory agent which is considered to exert its activity by interacting with specific receptors [12, 13], and increasing cAMP levels [14] through activation of adenylate cyclase [13]. Reduced platelet sensitivity to the inhibitory effects of exogenous PGI_2 was reported in patients with coronary heart disease and angina pectoris [15]. In these pathological conditions, abnormalities of platelet function and prostaglandin formation were also reported. In addition, decreased sensitivity to PGI_2 was described after moderate exercise [16], and in patients with peripheral vascular disease. This effect was retained after long-term intra-arterial infusion of PGI_2 [17]. The mechanisms underlying these differences in sensitivity to PGI_2 have not been elucidated, even if changes in the response of the cAMP system to PGI_2 have been proposed.

In this study, the platelet sensitivity to the anti-aggregatory effects of exogenous prostacyclin was evaluated in a group of selected type IIa hypercholesterolemic subjects and compared to those of a group of normocholesterolemic subjects. In addition, PGI_2-sensitive adenylate cyclase activity was measured in order to assess whether any change existed in PGI_2 receptors coupled to adenylate cyclase.

MATERIALS AND METHODS

Patients. Twenty-eight type IIa hypercholesterolemic subjects (13 males, 15 females, age range 34–60 years) were selected in our Lipid Clinic according to WHO criteria (mean total serum cholesterol 310 ± 8.7, LDL cholesterol 239.4 ± 16.9, HDL cholesterol 45.5 ± 4 mg/dl, serum triglycerides 143.8 ± 9.5 mg/dl, serum glucose 93.9 ± 2 mg/dl). Twenty age and sex matched normocholesterolemic subjects were selected as controls (mean serum cholesterol 209.1 ± 7.8 mg/dl). All subjects with hypertension, peripheral vascular disease, diabetes or other clinical signs of atherosclerotic disease were excluded from the study. All the subjects had been off any pharmacological treatment for at least 1 month before blood collection.

Combined studies of platelet aggregation and platelet adenylate cyclase activity were carried out only in a limited number of subjects, due to the sizeable volume of blood required for both assays.

Materials. [8-^{14}C]ATP and [8-^{3}H]cAMP were pur-

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chased from Radiochemical Centre (Amersham, U.K.). ATP, cAMP, GTP, creatin phosphate and creatin phosphokinase were from Sigma Chemical Co. (St Louis, MO). Aggregating agents: ADP (Sigma), epinephrine (ISM), collagen (Hormon Chemie). PGI_2 sodium salt was a kind gift from Dr. C. Gandolfi, Farmitalia Carlo Erba, Italy. PGI_2 was dissolved in ethanol at 10^{-3} M and stored at $-20°$. For aggregation studies, PGI_2 was diluted in 50 mM Tris–HCl, pH 7.4. For adenylate cyclase activity a 50 mM Tris–HCl buffer, pH 8, was used.

Platelet aggregation studies. Blood was collected in 3.8% sodium citrate (9:1). Platelet-rich plasma (PRP) and platelet-poor plasma (PPP) were prepared as previously described [4]. Platelet aggregation was tested in PRP samples using an ELVI Logos aggregometer by the turbidimetric technique of Born [18]. For each subject the minimal concentration of each agent giving a 60% decrease in optical density in 5 min was selected. This concentration was defined as the threshold aggregating concentration. For studies using PGI_2, to obtain comparable aggregation tracings, a concentration of each agent giving a maximal aggregatory response in all subjects was selected (collagen 1 μg/ml, ADP 1 μM, epinephrine 1.4 μM).

PGI_2 inhibition of platelet aggregation. This was studied in PRP samples pre-incubated for 1 min with different concentrations of PGI_2 before the addition of the aggregating agents.

Preparation of platelet membranes. PRP was centrifuged at 1000 *g* for 15 min at 0°, the pellet was washed by resuspension with 10 mM Tris–HCl, pH 7.4, and the final pellet then resuspended in the same buffer. Platelets were disrupted by freezing and thawing (three times), and a crude membrane fraction was obtained by centrifugation at 27,000 *g* for 30 min. The resulting pellet was washed by recentrifugation under the same conditions, and then resuspended in the same buffer to yield a protein concentration of 10–30 mg/ml. The membranes were stored at −80° until use.

Adenylate cyclase assay. The standard assay mixture contained: 10 mM Tris–HCl buffer, pH 8; 0.1 mM [8-^{14}C]ATP (57 dpm/pmole); 0.5 mM [8-^{3}H]cAMP (360 dpm/nmole); 2 mM $MgCl_2$; 2 mM creatine phosphate; 17 U/ml creatine phosphokinase; 10^{-5} M GTP and PGI_2 (10^{-8}–2×10^{-5} M) dissolved in Tris–HCl buffer, pH 8.0, in a final volume of 100 μl. The reaction was initiated by the addition of platelet membranes (diluted with Tris–HCl to yield 70–130 μg protein/sample) and was carried out at 30° for 8 min. Isolation and detection of [8-^{14}C] and [8-^{3}H]cAMP was performed according to Salomon *et al.* [19]. Inclusion of [8-^{3}H]cAMP in the assay mixture permitted correction for the possible effect of phosphodiesterases, which in any case was almost negligible; pH 8 was chosen in order to minimize PGI_2 degradation during the incubation.

RESULTS

Platelet aggregation studies

Platelets from type IIa subjects required significantly lower amounts of ADP, collagen and epinephrine to aggregate, when compared to platelets from normal subjects (Table 1), confirming the results previously obtained [1–4].

PGI_2 inhibition of platelet aggregation

Figure 1 shows typical tracings describing the inhibitory effect of PGI_2 on platelet aggregation induced by collagen, epinephrine and ADP in a control subject. The IC_{50} was calculated on the basis of the regression curve obtained by plotting PGI_2 concentrations vs the per cent inhibition. For each subject, the IC_{50}s for PGI_2 were calculated using the different aggregatory stimuli.

The mean IC_{50}s for both normal and type IIa subjects are shown in Table 2. Platelets from type IIa patients required significantly higher concentrations of PGI_2 to inhibit 50% of the aggregation elicited by fixed concentrations of collagen, ADP and epinephrine. As shown in Table 3, however, no correlation was found between either total or HDL cholesterol levels and the IC_{50} values. Similarly, no significant correlation existed between the threshold concentrations of the three aggregating agents and the IC_{50}s for PGI_2.

Adenylate cyclase studies

The dose–response curves for the activation of adenylate cyclase by PGI_2 were determined in each type IIa and normal subject examined.

Shown in Fig. 2 are two representative examples of such curves obtained in platelet membranes from one hypercholesterolemic and one normocholesterolemic subject. From each curve, EC_{50} (related to the affinity of PGI_2 for its adenylate cyclase coupled receptors) and maximal fold-stimulation (related to the number of receptors) were evaluated. The results are shown in Table 4. No statistically significant difference between normal and type IIa subjects was apparent in either basal adenylate cyclase activity, its maximal fold-stimulation by PGI_2, or the amount of PGI_2 required to elicit half-maximal stimulation (EC_{50}).

DISCUSSION

An imbalance between the generation of prostacyclin by vascular tissue and thromboxane formation by platelets may be involved in the initiation of the

Table 1. Threshold concentrations of aggregating agents in normal and type IIa subjects

Subjects	ADP (μM)	Collagen (μg/ml)	Epinephrine (μM)
Normal (N = 14)	0.9 ± 0.1	0.4 ± 0.02	0.41 ± 0.07
Type IIa (N = 20)	0.6 ± 0.09*	0.15 ± 0.03**	0.21 ± 0.05*

* $P < 0.05$; ** $P < 0.001$ vs normal subjects.

Table 2. Inhibition of platelet aggregation by exogenous PGI_2 in normal and type IIa subjects

Subjects	ADP	Collagen	Epinephrine
Normal (N = 14)	$0.46 \pm 0.1 \times 10^{-8}$	$0.70 \pm 0.1 \times 10^{-8}$*	$1.31 \pm 0.4 \times 10^{-8}$**
Type IIa (N = 20)	$1.51 \pm 0.4 \times 10^{-8}$*	$1.50 \pm 0.3 \times 10^{-8}$**	$2.71 \pm 0.3 \times 10^{-8}$**

The results are IC_{50} (mean ± S.E.M.) for PGI_2, expressed as moles/l. ADP, collagen and epinephrine concentrations were 1 μM, 1 μg/ml and 1.4 μM, respectively.
* $P < 0.05$; ** $P < 0.01$ vs normal subjects.

Table 3. Correlation between different parameters and IC_{50}s for PGI_2 in normal and type IIa subjects

Subjects	Correlation between PGI_2 IC_{50} and	Correlation coefficients for the aggregating agents		
		ADP	Collagen	Epinephrine
Normal (N = 14)	Total cholesterol	0.406 (n.s.)	0.510 (n.s.)	0.380 (n.s.)
	HDL cholesterol	0.458 (n.s.)	0.509 (n.s.)	0.333 (n.s.)
	ADP threshold	0.449 (n.s.)	—	—
	Collagen threshold	—	0.505 (n.s.)	—
	Epinephrine threshold	—	—	0.482 (n.s.)
Type IIa (N = 20)	Total cholesterol	0.313 (n.s.)	0.276 (n.s.)	0.267 (n.s.)
	HDL cholesterol	0.157 (n.s.)	0.087 (n.s.)	0.025 (n.s.)
	ADP threshold	0.375 (n.s.)	—	—
	Collagen threshold	—	0.278 (n.s.)	—
	Epinephrine threshold	—	—	0.259 (n.s.)

The PGI_2 IC_{50} corresponding to each aggregating agent was considered for the correlation.

Table 4. Stimulation of adenylate cyclase activity by PGI_2 in normal and type IIa subjects

Subjects	Basal activity cAMP (pmoles/mg protein)	Max-fold stimulation by PGI_2	EC_{50} for PGI_2 (M)
Normal (N = 11)	86.4 ± 25.8	7.06 ± 1.03	$2.06 \pm 0.21 \times 10^{-7}$
Type IIa (N = 12)	106.6 ± 21.8	7.10 ± 0.73	$1.57 \pm 0.10 \times 10^{-7}$

The results are means ± S.E. Maximal stimulation was obtained at 10^{-5}–2×10^{-5} M PGI_2.

atherosclerotic processes [20, 21]. The factors which may modulate the interactions between platelets and vessel walls, however, have not yet been completely identified. In particular, the sensitivity of circulating platelets to the inhibitory effects of prostacyclin might be determinant for the regulation of platelet-vessel wall interactions.

Reduced sensitivity to prostacyclin has been reported in patients with coronary heart disease, angina pectoris and peripheral vascular disease [15–17].

Our data in type IIa hypercholesterolemic subjects indicate that patients with high cholesterol levels have a reduced sensitivity to the effects of exogenous prostacyclin, even if no statistical correlation was found between serum total or HDL cholesterol levels and the platelet sensitivity to prostacyclin.

The decreased inhibitory effect of prostacyclin in type IIa subjects might be related to the increased *in vitro* sensitivity to aggregation of their platelets. However, the lack of correlation between threshold concentrations of aggregating agents and the IC_{50}s for PGI_2 suggests that this is not the case.

An alternative mechanism, possibly underlying the reduced sensitivity to PGI_2 of platelets from the hyperlipidemic subjects, is a difference in the activation of the adenylate cyclase system. Our results, however, did not show evidence of differences between the two groups. In fact, the maximal stimulation of adenylate cyclase by PGI_2 and the PGI_2 concentration eliciting half-maximal activation were not statistically different in patients and normal subjects.

Jakubowski *et al.* [11] reported that platelets from

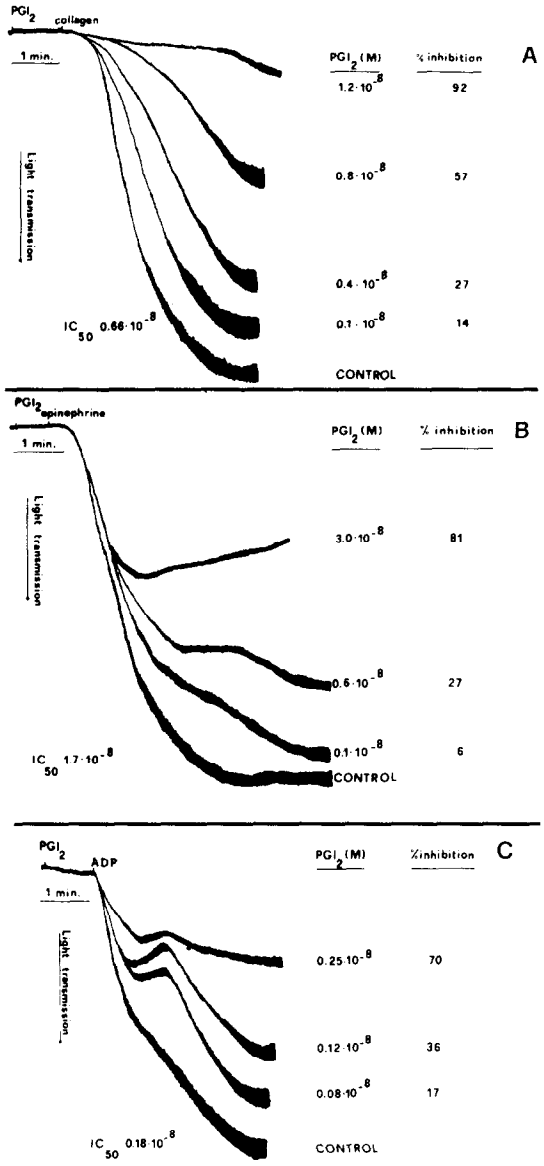

Fig. 1. An example of PGI_2 inhibition of platelet aggregation induced by 1 μg/ml collagen (panel A), 1.4 μM epinephrine (panel B) or 1 μM ADP (panel C). For IC_{50} calculation, see text.

hypercholesterolemic subjects have an impaired ability to accumulate cAMP as compared with controls when challenged with PGI_2. Our results demonstrate that the effects of PGI_2 on cAMP levels observed in intact platelets cannot be attributed to changes in either affinity or number of PGI_2 receptors coupled to adenylate cyclase. The effect of PGI_2 on phosphodiesterase activity [22] might be responsible for the changes in cAMP levels observed by these authors.

The reduced platelet response to PGI_2 in type IIa patients might be due to a different interaction between PGI_2 receptors and the receptors for either ADP or epinephrine, which have been shown to inhibit platelet adenylate cyclase activity [23, 24]. However, our data do not support this hypothesis,

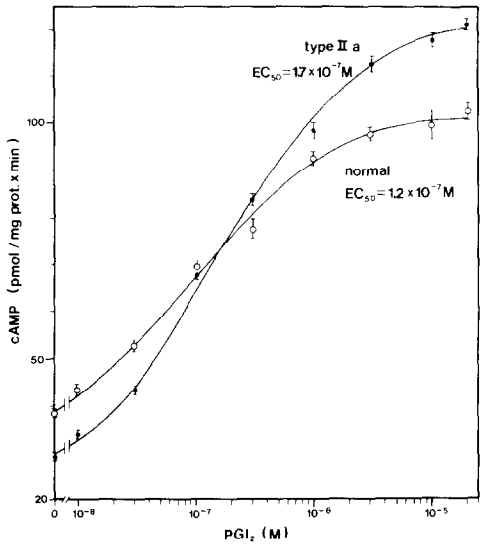

Fig. 2. Dose–response curves for the activation of adenylate cyclase by PGI_2 in a normal and in a type IIa subject.

since higher amounts of PGI_2 were required to inhibit platelet aggregation induced not only by ADP and epinephrine, but also by collagen, which is supposed to act through indirect mechanisms [25].

Other hypotheses could be put forward in order to explain the observed difference in platelet sensitivity. For instance, more PGI_2 might be required *in vitro* to counteract the effect of thromboxane A_2, which is produced in higher amounts by platelets of hypercholesterolemic subjects [5]. Alternatively, substances present in the plasma of type IIa patients in higher concentrations (such as cholesterol or some lipoproteins) could influence the interaction of PGI_2 with its receptors.

Acknowledgements—We thank Gabriele Morazzoni for his helpful technical assistance. This work was partially supported by a grant from the Italy–USA programme of the National Research Council, No. 81.00296.04, to Dr. Simonetta Nicosia.

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