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PROSTACYCLIN–LIPOPROTEIN INTERACTIONS

STUDIES ON HUMAN PLATELET AGGREGATION AND ADENYLATE CYCLASE

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Abstract—The *in vitro* effects of different lipoprotein fractions (VLDL, LDL and HDL) on human washed platelet aggregation, induced by collagen and thrombin, were evaluated in the presence and absence of PGI_2. Although VLDL and LDL increased the platelet aggregation while HDL showed an opposite effect, none of the tested lipoprotein fractions affected the potency of PGI_2 as inhibitor of platelet aggregation (IC_{50}). In addition, studies were performed to evaluate the effects of lipoproteins on adenylate cyclase activity in human platelet membranes. The three lipoprotein classes inhibited both basal and PGI_2-stimulated adenylate cyclase without affecting the EC_{50} for PGI_2. This inhibitory activity was not aspecifically elicited by any protein or lipid since neither bovine serum albumin nor a lipid emulsion (Intralipid®) displayed any inhibition. The effect on adenylate cyclase elicited by VLDL, LDL and HDL does not seem to be correlated with the activity on platelet aggregation. It is concluded that mediators other than cAMP might be involved in the control of platelet function by lipoproteins.

Prostacyclin (PGI_2)† is a potent physiological inhibitor of platelet aggregation and is a vasodilator [1]. PGI_2 formation by vascular tissue is involved in the regulation of platelet interactions with vessel walls and is considered to play a major role in the homeostatic balance.

The presence of atherosclerotic lesions has been shown to result in changes at the level of PGI_2 production by the endothelial layer. In particular, reduction of PGI_2 formation by aortic specimens from atherosclerotic animals and human atherosclerotic plaques has been described [2, 3]. This reduction at the site of vascular injury might contribute to an increased deposition of platelets and to the formation of platelet thrombi [4]. On the other hand a higher urinary excretion of PGI_2 metabolites, i.e. 2,3-dinor-6-keto-prostaglandin $F_{1\alpha}$, has been reported in atherosclerotic patients with documented platelet activation, suggesting that the rate of PGI_2 biosynthesis might be increased despite a reduced capacity of blood vessels to produce it at the site of the injury [5]. Synthetic PGI_2, however, has been proposed as a pharmacological treatment in patients with atherosclerotic disease such as peripheral vascular disease and in extracorporeal circulation [6, 7].

Increasing evidence exists in the literature indicating that, in pathological conditions associated with increased platelet aggregability, the sensitivity of platelets to the inhibitory effect of synthetic PGI_2 is reduced [8, 9]. Previous data from our group have shown that the aggregation of platelets from patients with type IIa hypercholesterolemia required more PGI_2 to be inhibited than that of platelets from normal subjects [10]. PGI_2 is supposed to inhibit platelet aggregation by stimulating adenylate cyclase activity, thus increasing intracellular cAMP levels [11–13]. No difference between type IIa hypercholesterolemic and normal subjects, however, was observed in the stimulatory effects of PGI_2 on platelet adenylate cyclase [10]. Therefore, the observed differences in platelet response to PGI_2 could not be ascribed to modifications of the receptor for this prostaglandin, coupled to adenylate cyclase.

It should be pointed out that platelet aggregation studies had been carried out in the presence of plasma, whereas adenylate cyclase activity was measured in isolated platelet membranes. It seemed therefore likely that the observed changes at the level of the platelet response to PGI_2 could be due to differences in plasma composition between normal and hypercholesterolemic subjects, i.e. an abnormal lipoprotein pattern in plasma of type IIa patients.

Separated lipoproteins have been shown to affect washed platelet aggregation *in vitro* directly. In fact, very low density and low density lipoproteins (VLDL and LDL) stimulate the response of platelets to aggregating agents, whereas high density lipoproteins (HDL) have been shown to inhibit it [14]. On the other hand, plasma lipoproteins can affect cAMP production *in vitro* in a number of systems [15–18], but no detailed investigation in platelets has been performed so far. It was therefore of interest to investigate the effect of the different lipoprotein fractions on washed platelet aggregation and adenyl-

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† Abbreviations used: PGI_2, prostacyclin; HDL, LDL, VLDL, high, low and very low density lipoproteins; WP, washed platelets; PRP, platelet rich plasma; Hepes, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid.

ate cyclase activity, and their possible interactions with PGI_2.

MATERIALS AND METHODS

Materials. [8-^{14}C]ATP and [8-^{3}H]cAMP were purchased from New England Nuclear (Boston, MA). ATP, cAMP, GTP, creatine phosphate, creatine phosphokinase and bovine serum albumin, fraction V, were from Sigma Chemical Co. (St. Louis, MO). Aggregating agents were collagen (Hormon Chemie) and thrombin (Topostasin®; Roche, Basel, Switzerland). PGI_2 sodium salt was from the Wellcome Foundation Laboratories (Beckenham, U.K.). 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 buffer pH 7.4. For adenylate cyclase studies a 10 mM Tris–HCl buffer pH 8 was used. Intralipid® (from Vitrum AB, Stockholm) is an emulsion containing 100 g/l soy lipids, 12 g/l egg yolk lecithin and 22.5 g/l glycerol.

Lipoprotein separation. Blood obtained from 12-hr-fasted healthy volunteers (20–35 years old) was collected in EDTA (1 mg/ml). The plasma lipoproteins VLDL ($d < 1.006$ g/ml); LDL ($d = 1.006$–1.0631 g/ml); HDL ($d = 1.063$–1.21 g/ml) were separated by sequential ultracentrifugation according to Havel *et al.* [19]. LDL and HDL were dialyzed against 0.15 M NaCl/30 mM Tris–HCl buffer pH 7.4 at 4° for 24 and 48 hr, respectively.

Platelet isolation. Blood from donors different from those selected for lipoprotein separation was collected in acid–citrate–dextrose (citric acid 0.8%, trisodium citrate 2.2%, dextrose 2.45%, pH 4.5), 9:1, v:v.

All the subjects had been off any pharmacological treatment known to interfere with platelet function for at least 10 days before blood collection. Platelet rich plasma (PRP) was prepared by centrifugation as previously described [20]. Platelet isolation was carried out according to Lagarde *et al.* [21]. The final platelet pellet was resuspended in Tyrode–Hepes buffer pH 7.35. Platelet concentration was adjusted to 3×10^8 platelets/ml.

Platelet aggregation studies. Washed platelet (WP) aggregation was carried out in an Elvi Logos aggregometer by the turbidimetric technique of Born [22]. For each subject, a concentration of collagen and thrombin yielding an aggregatory response of at least 70% decrease in optical density was selected. For all the subjects examined, this concentration ranged between 1 and 4 μg/ml and 0.02–0.04 U NIH/ml for collagen and thrombin respectively.

PGI_2 inhibition of platelet aggregation was studied in WP samples pre-incubated for 1 min with different concentrations of PGI_2 before the addition of the aggregating agents. Buffer or different lipoprotein fractions at a final concentration of 0.1 mg protein/ml were added to each sample immediately before the addition of PGI_2. To minimize any turbidity change, appropriate blanks were prepared with Tyrode–Hepes buffer with or without lipoproteins.

Preparation of platelet membranes. PRP from healthy individuals, non-smokers, off any pharmacological treatment, were centrifuged at 1000 *g* for 15 min at room temperature. The pellet was washed by resuspension with 10 mM Tris–HCl buffer, pH 7.4, then centrifuged and resuspended again under the same conditions. Platelets were disrupted by freezing and thawing (three times), and a crude membrane fraction was obtained by centrifugation at 14,000 *g* for 45 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 5–20 mg/ml. The membranes were stored at −80° until use.

Protein content of lipoproteins and membranes was quantified by the Lowry procedure [23] with bovine serum albumin as standard.

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; 33.7 U/ml creatine phosphokinase; 10^{-5} M GTP and PGI_2 (10^{-8}–2×10^{-5} M) dissolved in Tris–HCl buffer, pH 8. Where required, HDL and LDL were added suspended in 10 μl of dialysis buffer and VLDL were added in buffered saline. The same amount of buffer was added to the control samples. The samples had a final volume of 100 μl. The reaction, started with the addition of platelet membranes (diluted with Tris–HCl buffer, pH 8, to yield 40–70 μg protein/sample), was carried out at 30° for 8 min. Isolation and detection of [8-^{14}C, 8-^{3}H]cAMP was performed according to Salomon *et al.* [24]. Inclusion of [8-^{3}H]cAMP in the assay mixture allowed 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.

Statistical analysis. EC_{50} and IC_{50} were calculated by linear regression analysis and were considered to be not significantly different when the 95% confidence limits overlapped.

RESULTS

PGI_2 inhibition of washed platelet aggregation

Synthetic PGI_2 dose-dependently inhibited the WP aggregation induced by threshold concentrations of either thrombin or collagen (data not shown). The IC_{50}s obtained by plotting the concentration of PGI_2 vs the per cent inhibition of platelet aggregation, stimulated by either collagen or thrombin, are shown in Table 1.

Effects of incubation of washed platelets with VLDL, LDL and HDL

Separated lipoprotein fractions, or correspondent buffers, were added at fixed concentrations (0.1 mg protein/ml) to WP samples. LDL and VLDL slightly increased the response of platelets to aggregating agents, in the absence of PGI_2. On the contrary, HDL exerted an opposite effect (Figs 1A, 2A, 3A).

When PGI_2 was added immediately after the lipoproteins, a lower inhibitory effect of PGI_2 on platelet aggregation was recorded in the presence of 0.1 mg protein/ml LDL (Fig. 1A). This effect, however, was mainly due to the stimulatory action that LDL had on platelet aggregation, since the IC_{50} for PGI_2

Table 1. PGI_2 inhibition of platelet aggregation (IC_{50}) in the presence of lipoprotein fractions

	Collagen		Thrombin	
	Control buffer	Lipoprotein	Control buffer	Lipoprotein
VLDL (0.1 mg/ml)	1.64 (0.72–3.70)	1.61 (0.76–3.42)	0.60 (0.09–3.87)	0.84 (0.49–1.44)
LDL (0.1 mg/ml)	1.45 (0.59–3.56)	1.65 (1.05–2.59)	0.96 (0.43–2.12)	0.93 (0.35–2.46)
HDL (0.1 (mg/ml)	1.45 (0.59–3.56)	1.03 (0.44–2.42)	0.96 (0.43–2.12)	0.79 (0.05–7.02)

IC_{50}s are means expressed as nM of at least five single experiments, and the 95% confidence limits are indicated in parentheses. Control buffer was saline for studies with VLDL, and 0.15 M NaCl/30 mM Tris–HCl (pH 7.4) for LDL and HDL.

(Fig. 1B and Table 1) was not modified by the presence of LDL.

The action of HDL on the platelet–PGI_2 interaction was opposite to that of LDL. In fact 0.1 mg protein/ml HDL added to WP resulted in an apparent increase of the PGI_2 inhibitory effect, which, however, was related mainly to the direct inhibitory effect of this lipoprotein fraction on WP aggregation (Fig. 2A). Similarly to LDL, the differences in the IC_{50}s for PGI_2 in the absence and presence of HDL did not reach the statistical significance, even if a trend toward lower values was observed with HDL (Fig. 2B and Table 1).

VLDL (0.1 mg protein/ml) added to the system did not apparently affect the platelet antiaggregatory activity of PGI_2 (Fig. 3A), as well as the IC_{50} for PGI_2 (Fig. 3B and Table 1).

Lipoproteins and adenylate cyclase

The effect of the various lipoprotein fractions on adenylate cyclase activity in membrane preparations from human platelets was investigated (Fig. 4).

VLDL, LDL and HDL dose-dependently inhibited both PGI_2-sensitive (Fig. 4A) and basal adenylate cyclase (Fig. 4B), the rank order of potency being: VLDL > HLD > LDL. For each lipoprotein fraction, the IC_{50} was approximately the same when calculated on basal and PGI_2-sensitive activity (Table 2). Also, despite the higher enzyme activity observed in the presence of PGI_2, the per cent inhibitory effect of each lipoprotein fraction was similar whether the prostaglandin was present or not, being 67 and 70% for VLDL (0.11 mg protein/ml); 67 and 67% for HDL (1 mg protein/ml); 22 and 31% for LDL (0.8 mg protein/ml), in the absence and presence of 10^{-5} M PGI_2, respectively.

Figure 5 shows the dose–response curves for PGI_2 in the absence and presence of a single concentration of lipoproteins. As already mentioned, the per cent inhibition of the different lipoproteins on adenylate

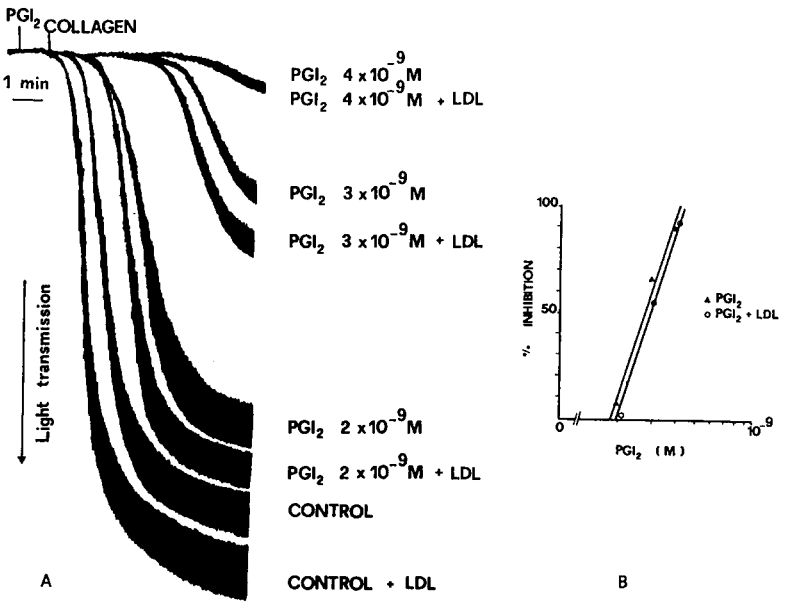

Fig. 1. Effects of LDL (0.1 mg protein/ml) on WP aggregation in the presence and absence of PGI_2. Panel A: an example of aggregation tracings induced by collagen. Panel B: percent inhibition of platelet aggregation exerted by PGI_2 vs PGI_2 concentrations in the absence and presence of the lipoprotein.

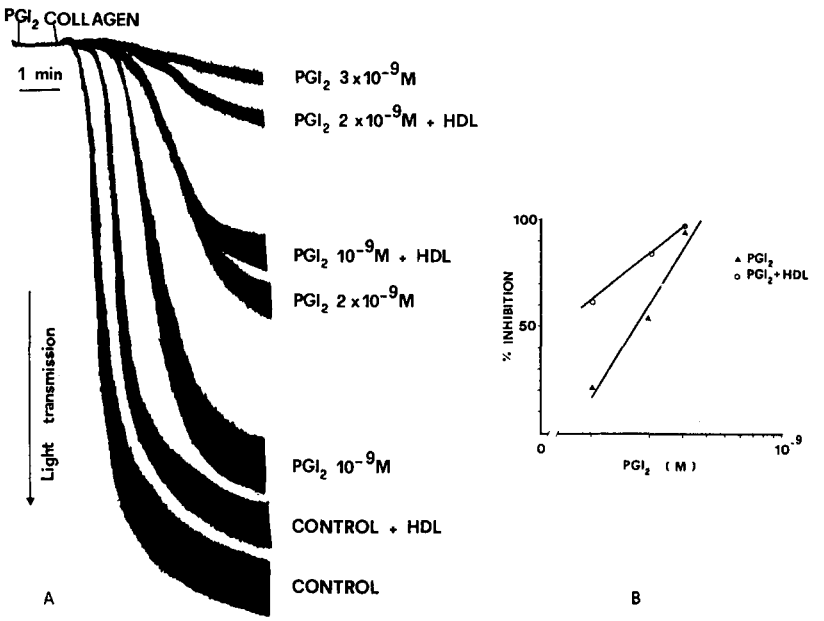

Fig. 2. Effects of HDL (0.1 mg protein/ml) on platelet aggregation induced by collagen and on its inhibition by PGI_2. For details see legend to Fig. 1.

cyclase activity was similar in the presence and absence of PGI_2. Interestingly, the EC_{50} for PGI_2 was not significantly affected by lipoproteins, being 1.9×10^{-7}M in the absence and 1.4, 2.2 and 1.5×10^{-7}M in the presence of 0.8 mg/ml LDL, 0.11 mg/ml VLDL and 1 mg/ml HDL, respectively.

In order to rule out the possibility that the observed inhibitory effect of lipoproteins was aspecifically elicitable by any protein or lipid, the action on adenylate cyclase activity of bovine serum albumin (BSA) and of Intralipid® (a triacylglycerol/phospholipid/glycerol emulsion) was evaluated. As shown in Table 3, neither BSA nor Intralipid®, at different concentrations, significantly inhibited basal or PGI_2-sensitive adenylate cyclase activity. Actually, BSA at the lower concentration tested seemed to stimulate the enzyme activity. The concentrations of Intralipid® used provide an amount of total lipids comparable with that added with the lipoproteins at the used concentrations, on the basis of the composition reported by Nichols [25].

DISCUSSION

The plasmatic environment and, in particular, the lipoprotein pattern and the plasma cholesterol levels,

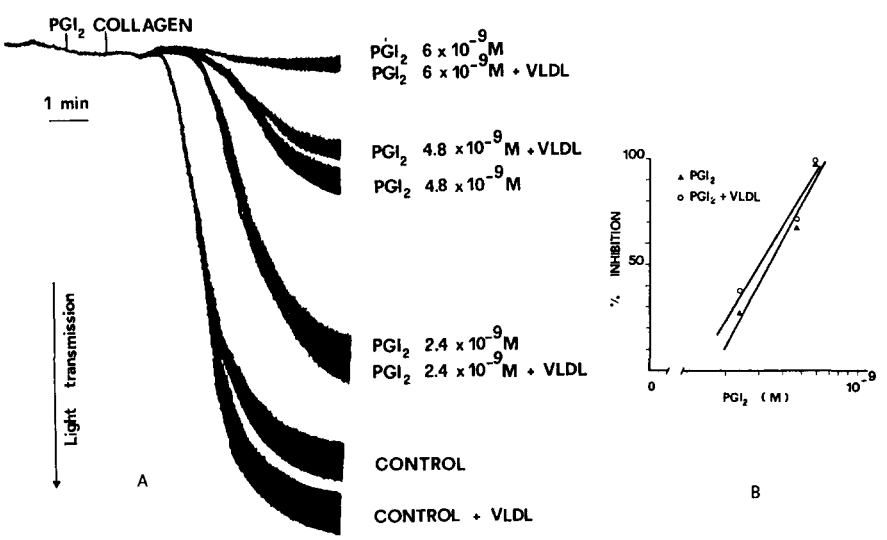

Fig. 3. Effects of VLDL (0.1 mg protein/ml) on platelet aggregation induced by collagen and on its inhibition by PGI_2. For details see legend to Fig. 1.

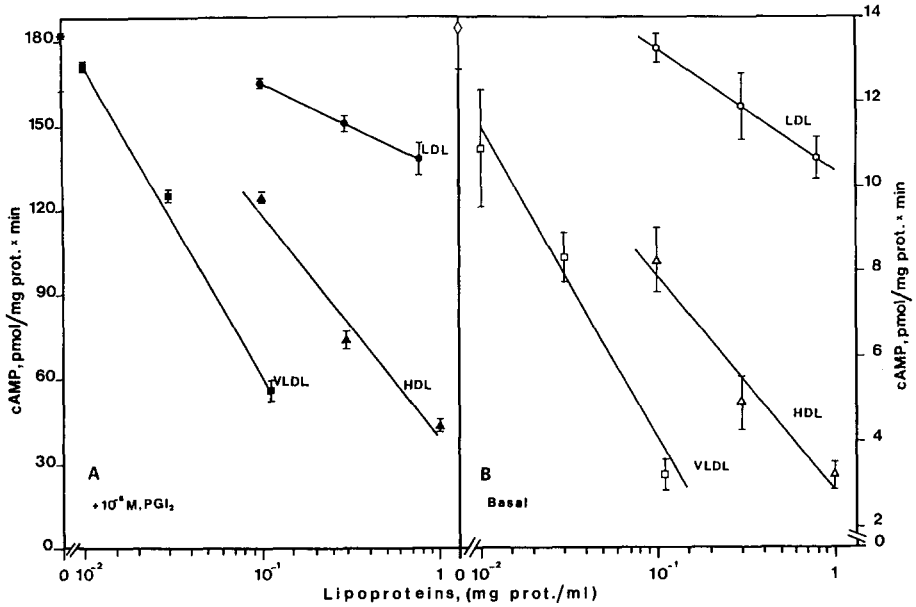

Fig. 4. Dose–response curves for the inhibition of PGI_2-sensitive (panel A) and basal (panel B) adenylate cyclase activity by lipoprotein fractions.

have been shown to affect the aggregation of platelets directly [26, 27]. *In vitro* studies using WP, in fact, have shown that LDL stimulate platelet aggregation, both in the presence [14] and in the absence of aggregating agents [28]. On the contrary, an inhibitory activity of HDL has been described [14, 29]. The opposite effects of the different lipoprotein fractions on platelet response to aggregating agents, i.e. stimulation by LDL and inhibition by HDL, appear to be well in line with their relative contribution to the atherosclerotic disease, since LDL are considered to be atherogenic whereas HDL exert a protective effect against cholesterol deposition at the level of vascular tissue [30, 31].

PGI_2 plays a major role in the homeostatic balance regulating the platelet response in the event of endothelial lesion [5, 32], therefore PGI_2–lipoprotein interactions at platelet level might bear some relevance in the onset of atherosclerosis.

The results reported here show that PGI_2 is apparently less effective in inhibiting WP aggregation when LDL are present. This phenomenon has to be ascribed to the stimulatory effect of LDL on platelet aggregation since no activity of this lipoprotein fraction on the IC_{50}s for PGI_2 was detectable.

The activity of HDL on platelet–PGI_2 interaction was opposite compared to that of LDL. In fact, HDL appeared to increase the PGI_2 inhibitory effect on WP aggregation. This finding is probably related to the direct inhibition of platelet aggregation exerted by this lipoprotein. Similarly to LDL, no change in the IC_{50} for PGI_2 was detected in the presence of HDL.

Stimulation of platelet aggregation by VLDL was

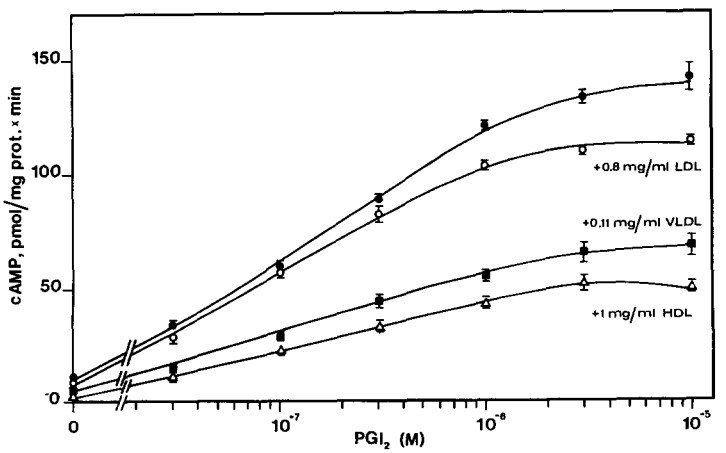

Fig. 5. Dose–response curves for the stimulation of adenylate cyclase activity by PGI_2 in the absence and presence of a single lipoprotein concentration.

Table 2. Inhibition of adenylate cyclase (A.C.) activity by different lipoprotein fractions

	Basal A.C.	PGI_2-sensitive A.C.
VLDL	0.039 (0.011–0.142)	0.057 (0.041–0.079)
HDL	0.162 (0.046–0.551)	0.237 (0.144–0.392)
LDL*	15.8 (1.3–86.5)	27.3 (3.4–70.2)

The results are IC_{50}s expressed in mg protein/ml (mean of three values from one experiment representative of at least two others). In parentheses are the 95% confidence limits.

* Extrapolated.

described by Aviram *et al.* [14], but this effect was not confirmed by other groups [28]. In our experimental conditions no reproducible activity of VLDL was evident either on platelet aggregation or on the IC_{50} for PGI_2.

Increased platelet response to aggregating agents have been reported in type IIa hypercholesterolemia [27, 33]. The above mentioned LDL–platelet interactions might explain, at least in part, this clinical finding, since in type IIa patients LDL levels are higher than in normal subjects. The effects of the lipoprotein fractions we demonstrate here, however, cannot explain the lower sensitivity to PGI_2 of platelet from type IIa as compared to control subjects [10]. In fact LDL do not modify the antiaggregatory potency of PGI_2.

As far as HDL are concerned, their inhibitory effect on platelet aggregation cannot be simply extrapolated to an *in vivo* situation because no significant modification of platelet function was detected in subjects with HDL concentration lower than normal [34].

In an attempt to elucidate the mechanism of action of the lipoproteins on platelet aggregation, and their interaction with PGI_2, we investigated the effect of the different lipoprotein fractions on basal and PGI_2-sensitive adenylate cyclase. An effect of lipoprotein fractions on cAMP levels or adenylate cyclase activity in various tissues has been already demonstrated by us [18] and by other laboratories [15–17]. However, in most cases it was found that the various lipoproteins stimulated adenylate cyclase or cAMP accumulation, at variance with the results of the present report. Indeed, we demonstrate here that the lipoprotein fractions tested inhibit adenylate cyclase activity, both basal and PGI_2-stimulated, in membranes from human platelets. Such inhibition is brought about by either a specific lipoprotein component or by the whole lipoprotein aggregate, since neither bovine serum albumin nor the lipid emulsion Intralipid® displayed any effect.

The mechanism of lipoprotein-induced inhibition has not yet been elucidated, but a direct effect on the receptor for PGI_2, coupled to adenylate cyclase, can be ruled out. In fact, neither the EC_{50} for PGI_2, related to the prostaglandin receptor affinity and to its coupling with the enzyme, nor the maximal fold stimulation are affected. It is more likely, therefore,

Table 3. Effect of bovine serum albumin and of Intralipid® on adenylate cyclase activity

	Additions							
		Intralipid® (mg/ml)			BSA (mg/ml)			
PGI_2, M	None	0.1	0.3	1	0.1	0.3	1
0	16.6 ± 1.81	17.03 ± 1.08	15.12 ± 0.93	12.34 ± 0.39	22.78 ± 1.07*	17.84 ± 0.85	20.54 ± 0.23
10^{-5}	148.21 ± 1.78	135.64 ± 8.16	138.14 ± 2.60	142.83 ± 2.35	166.01 ± 0.83*	140.44 ± 0.39	139.83 ± 5.35

Results are means ± S.E.M. (N = 3), expressed in pmoles cAMP/mg protein × min. * Significantly different from sample without BSA, $P < 0.05$.

that the effect of the lipoproteins is elicited mainly at the level of other adenylate cyclase components, such as the enzyme catalytic unit or the *N*-protein. This might explain why no effect of LDL or HDL on basal cAMP levels was detected in intact human platelets [28]. In intact platelets, these two enzyme components, which are located on the cytoplasmic side of the membrane, would not be accessible to lipoproteins, unless internalization occurs.

The lack of effect of the various lipoprotein fractions on the EC_{50} for PGI_2 allows us to rule out the possibility that the lipoproteins simply remove some PGI_2 from its receptor as suggested by others [28]. If this were the case, then the dose–response curves for PGI_2 would be shifted to the right and the EC_{50} would consequently increase.

The observed effects of the lipoproteins on basal and PGI_2-stimulated adenylate cyclase activity do not seem to be correlated with their actions on platelet aggregation. Indeed, a reduction in the stimulated adenylate cyclase activity should bring about an enhancement of the aggregatory response, even if the role for a decreased cAMP level in platelets has been questioned [35]. On the contrary, LDL, which among the lipoprotein fractions are the least potent inhibitors of adenylate cyclase, are the most effective in increasing WP aggregation, both in the presence and absence of PGI_2. VLDL, which inhibit adenylate cyclase to a larger extent than the other lipoproteins, only slightly and inconsistently affect platelet aggregation. Finally, HDL are even inhibitors of platelet aggregation, an effect which is usually associated with increased cAMP levels while they are shown here to decrease adenylate cyclase activiy.

In conclusion, this study shows that: (1) the lipoprotein fractions affect platelet aggregation; (2) they inhibit the activity of platelet adenylate cyclase; (3) the potency of PGI_2 both as inhibitor of platelet aggregation and as stimulator of adenylate cyclase is not directly modified by the lipoproteins. Therefore, it can be hypothesized that the effect of lipoproteins in intact cells involves more steps than the interaction with the adenylate cyclase system and that mediators other than cAMP might play a role in the control of platelet aggregation under these conditions.

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