The aggregation of isolated human platelets in the presence of lipoproteins and prostacyclin

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Addition of prostacyclin (PGI₂) temporarily inhibits platelet aggregation and permits the isolation of platelets free from plasma proteins, which have the same sensitivity as those in plasma [Moncada, Radomski & Vargas (1982) *Br. J. Pharmacol.* **75**, 165P]. By using a modification of this technique we have established that platelets isolated from normal subjects aggregate more readily in response to ADP and adrenaline when physiological concentrations of low-density lipoproteins (LDL) are present. At high LDL concentrations spontaneous aggregation occurs. High-density lipoproteins (HDL) and very-low-density lipoproteins (VLDL) had no effect on agonist-induced platelet aggregation at normal concentrations, but HDL sensitized at higher concentrations. These effects by lipoproteins are not accompanied by changes in platelet lipid content. Cyclohexanedione treatment of LDL to modify apolipoproteins appeared to abolish the sensitization effect, indicating that binding to receptors was essential for the effects of LDL. LDL, but not HDL, overcame the inhibitory effect of PGI₂ on platelet aggregation, except at very high concentrations of PGI₂. PGI₂ raised the cyclic AMP content of isolated platelets, but LDL only partially prevented this rise. These results suggest that LDL may have a greater role in platelet aggregation than previously recognized and may also regulate effects of PGI₂. These findings may be of relevance to an understanding of cardiovascular diseases.

Familial hypercholesterolaemia is characterized by elevated levels of plasma LDL. Platelets from such patients aggregate more readily than those from normal subjects when ADP or adrenaline are added to PRP (Carvalho *et al.*, 1974). By contrast, low plasma LDL concentrations are associated with reduced platelet sensitivity in a cohort of healthy volunteers (Hassall *et al.*, 1983a). The underlying mechanism for these observations is not clear. Early reports on the effects of adding LDL to PRP were conflicting (Farbiszewski *et al.*, 1969; Miller & Nordoy, 1977). In other studies LDL have been added to isolated platelet preparations known to have impaired responses; small increases in sensitivity were noted, but only single, high doses of agonist were used, which might have obscured the real extent of the effect (Koller *et al.*, 1979; Aviram & Brook, 1982).

Abbreviations used: PGI₂, prostaglandin I₂ (prostacyclin); LDL, low-density lipoproteins; HDL, high-density lipoproteins, VLDL, very-low density lipoproteins; PRP, platelet-rich plasma.

In the present study the platelet-isolation procedure of Moncada *et al.* (1982) has been modified using chromatography on Sepharose 2B (Tangen *et al.*, 1971) to yield fully responsive platelets. The modification was introduced to permit the more rapid recovery of platelet response to adrenaline in particular and to avoid damage by repeated centrifugation. The procedure depends on the presence of PGI₂ throughout the isolation to inhibit temporarily platelet aggregation by raising intracellular cyclic AMP concentrations (Gorman *et al.*, 1977; Tateson *et al.*, 1977). The effects of LDL and other individual lipoproteins on the ability of these isolated platelets to aggregate were studied over a wide range of concentrations. The normal physiological ranges of lipoprotein concentrations expressed in terms of protein are: LDL, 0.6–3.0; HDL, 0.6–1.7; VLDL (non-starved), 0.01–0.6 g of protein/litre, based on known lipoprotein compositions and concentrations (Eisenberg & Levy, 1975). The effect of lipoproteins on the inhibition of platelet aggregation by PGI₂ was also examined. These findings have already been presented in abstract form (Hassall *et al.*, 1983b).

Materials and methods

Normal blood was collected from volunteers into acid citrate/dextrose anticoagulant and centrifuged for 10 min at 200g to yield PRP. The volunteers had not taken aspirin for at least 10 days before withdrawal of blood.

Isolation of platelets

PRP containing 300 µg of PGI₂/litre was centrifuged at 800g for 10 min to obtain a platelet pellet. The platelets were resuspended in 1 ml of Tyrode's buffer, pH 7.4 (10 µg of PGI₂/litre), applied to a small plastic column containing Sepharose 2B (Pharmacia, Uppsala, Sweden) and eluted in Tyrode's buffer (no added PGI₂). They were diluted to a count of 4×10^8 platelets/ml and, after addition of human albumin (0.3 g/litre) and fibrinogen (0.4 g/litre), left to stand for 1–2 h to recover sensitivity to all aggregating agents.

Preparation of lipoproteins

VLDL ($\rho > 1.006$ g/ml), LDL ($\rho = 1.019$ – 1.063 g/ml) and HDL ($\rho = 1.063$ – 1.21 g/ml) were isolated from normal plasma by differential ultracentrifugation (Hatch & Lees, 1968) from different donors than those used for platelet aggregation. They were washed by re-centrifugation at the appropriate densities, dialysed against Tyrode's buffer, pH 7.4, and passed through a 0.22 µm filter. LDL modified by cyclohexanedione were prepared by the method of Mahley *et al.* (1977).

Aggregation tests

Isolated platelets were incubated for 5 min at 20°C with either an equal volume of Tyrode's buffer, pH 7.4, or buffer containing lipoproteins at a series of concentrations. Platelet aggregation was initiated by addition of adrenaline or ADP at various concentrations at 37°C in a Payton dual-channel aggregometer (Centronic Sales, Croydon, Surrey, U.K.) fitted to use 0.1 ml cuvettes (Born & Cross, 1963). Appropriate blanks were prepared without platelets to allow for lipoprotein turbidity. The minimum concentration of agonist required to induce secondary aggregation within 2 min was ascertained and used as an index of platelet sensitivity (Hardisty *et al.*, 1970).

Analysis of platelets

After incubation with lipoproteins, platelets were washed free of lipoproteins by centrifugation at 1000g in buffer (4.9 mM-KH₂PO₄/35 mM-NaH₂PO₄/40 mM-NaOH/103 mM-NaCl/5.5 mM-glucose/5.3 mM-EDTA, pH 7.6) (Gaintner *et al.*, 1962), extracted into chloroform/methanol (Bligh & Dyer, 1959) and the cholesterol (Richmond, 1973) and phospholipid (Bartlett, 1959) contents

measured. Cyclic AMP assays on platelets were performed by radioimmunoassay (Harper & Brooker, 1975).

Electron microscopy

Platelet samples were fixed in 3% (v/v) glutaraldehyde in 0.1 M-sodium cacodylate buffer, pH 7.4, containing 0.1 M-sucrose (300 mosM). The samples were dehydrated through ethanol mixtures and critical-point-dried before being coated in carbon and gold in an Ion Tech Saddle field using an Ion Source Sputter Coater (Ion Tech, Teddington, Middx., U.K.). The platelets were then viewed in a Phillips 501 scanning electron microscope (Pye-Unicam, Cambridge, U.K.).

Results and discussion

Comparison of platelet-rich plasma and isolated platelets

There was considerable individual variation between donors in the responsiveness of PRP to the agonists, especially ADP and adrenaline (Hardisty *et al.*, 1970). This was reflected in the aggregation of isolated platelets, which had the same sensitivity as the PRP; these properties were retained for at least 6 h. For this study the rather less sensitive individuals were chosen.

The appearance of the isolated platelets under scanning electron microscopy shows that most of the platelets are not in an activated state as is the case for PRP (Fig. 1).

Isolated platelets have a slightly slower shape change before aggregation with ADP than that normally observed in PRP (Fig. 2). No spontaneous aggregation occurred even after 10 min stirring in the aggregometer.

The effects of lipoproteins

After 5–10 min pre-incubation with LDL (2.0 g of protein/litre) there was a highly significant increase

Fig. 1. Scanning electron micrographs of isolated platelets incubated in the presence or in the absence of lipoproteins

A suspension of isolated platelets (4×10^8 /ml in Tyrodes buffer, pH 7.4) was incubated with an equal volume of LDL, HDL (final concentration 2 g of protein/litre) or with buffer alone at 20°C. After 5 min incubation the platelets were fixed in 3% glutaraldehyde and prepared for scanning electron microscopy (Phillips 501). The micrographs depict: (a) PRP; (b) isolated platelets in the absence of lipoproteins; (c) after incubation with 1 g of LDL protein/litre; (d) after incubation with 2 g of LDL protein/litre; (e) after incubation with 2 g of HDL protein/litre. The magnifications are: (a), $\times 12\,300$; (b) and (d), $\times 11\,150$; (c) and (e), $\times 8000$.

in sensitivity to both ADP and adrenaline, but the initial response due to shape change had disappeared (Fig. 2). In the absence of agonist, spontaneous aggregation occurred after stirring in the aggregometer for 4 min and after shorter intervals at higher concentrations of LDL. The scanning electron micrographs suggest that at 2 g of protein/litre or above, LDL activate platelet shape change before addition of agonist (Fig. 1).

The platelet sensitivity, i.e. the minimum concentration of adrenaline or ADP required to produce secondary aggregation, was determined at various concentrations of LDL, HDL and VLDL after 5–10 min pre-incubation (Fig. 3). Concentrations of LDL above 0.5 g of protein/litre sensitize the platelets to adrenaline and ADP. With adrenaline there was little change in the sensitivity of the platelets over the range 0.8–1.3 g LDL protein/litre, which were nevertheless 10-fold more sensitive than at 0.5 g of LDL/litre. The shape change before aggregation was diminished but still evident at these low concentrations of LDL, which reflects the lesser degree of stimulation of the platelets shown in the scanning electron micrographs at 1 g of LDL protein/litre before the addition of agonists (Fig. 1). However, at concentrations above 1.5 g of LDL protein/litre even greater hypersensitivity to adrenaline and ADP was observed. LDL also sensitized platelets to thrombin, collagen and arachidonic acid and reduced the threshold concentration for these agonists 100-fold at concentrations of LDL above 1.5 g of protein/litre.

In the case of HDL, no changes in platelet sensitivity to adrenaline were observed below 1.5 g of protein/litre, but above this concentration there was a gradual increase in sensitivity, which was always less than that induced by equivalent concentrations of LDL protein (Fig. 3). At 2 g of protein/litre, HDL also activated platelets before addition of agonists (Fig. 1). The VLDL concentration that could be employed was limited because the increasing turbidity interfered with the aggregation tests. Nevertheless, the concentrations used covered the normal physiological range and these had no effect on platelet sensitivity to adrenaline or ADP (Fig. 3).

The sensitizing effects of LDL (1 or 2 g/litre) were not antagonized by the presence of HDL (1 g/litre) or VLDL (0.5 g/litre), but were enhanced if the protein concentration of HDL was high (2 g/litre). Combined incubations with subthreshold concentrations of LDL (0.5 g/litre) and HDL (1 g/litre) did not produce a sensitization of the platelets at these protein concentrations.

Platelet lipids and LDL binding

The effects of LDL on aggregation may be exerted in at least two ways. In Type II hyperlipoproteinemia LDL are elevated in concentration and are abnormally rich in non-esterified cholesterol; this increases the cholesterol content and cholesterol/phospholipid ratio of the platelets which become more sensitive to adrenaline and ADP (Shattil *et al.*, 1975). In the experiments described above, no change was observed in the cholesterol content or in

Fig. 2. The aggregation of isolated human platelets initiated by adrenaline and ADP in the presence of LDL. Isolated platelets (2×10^8 /ml) were incubated for 5 min at 20°C in Tyrode's buffer, pH 7.4 (a), or buffer containing LDL (2 g of protein/litre) (b). Platelet aggregation was initiated by addition of various concentrations of adrenaline or ADP at 37°C in an aggregometer. The results have been reproduced in at least 10 experiments.

Fig. 3. Sensitivity of isolated human platelets to adrenaline and ADP in the presence of VLDL, LDL and HDL. Isolated platelets were incubated with lipoprotein for 5 min at 20°C at final concentrations of 0–3 g of lipoprotein protein/litre of Tyrode's buffer, pH 7.4. For each incubation, aggregation tests were performed at 37°C with a range of adrenaline (a) and ADP (b) concentrations. Appropriate blanks were prepared without platelets to allow for lipoprotein turbidity. Each point is the mean value ($\pm$ s.d.) of between three and ten experiments and represents the minimum concentration of adrenaline or ADP required to induce aggregation within 2 min (sensitivity). $\blacktriangle$, VLDL; $\bullet$, LDL; $\square$, HDL; $\diamond$, cyclohexanedione-modified LDL.

the cholesterol/phospholipid ratio (0.60) of isolated platelets incubated for 5 or 80 min with normal lipoproteins. Therefore, alterations in lipid content do not account for the effects of LDL after short incubations, although this may contribute to the mechanism in type II hyperlipoproteinaemia (Shattil & Cooper, 1976). It has been noted previously that increased platelet cholesterol content does not enhance platelet aggregation in all cases (Owen *et al.*, 1981).

Another possibility is that LDL binds to the platelet membrane and enhances aggregation. Cyclohexanedione treatment of LDL modifies the amino groups of arginine and prevents the binding of the lipoproteins to their receptors on cultured skin fibroblasts (Mahley *et al.*, 1977) and on platelets (Aviram *et al.*, 1981). LDL modified by cyclohexanedione had no effect on platelet aggregation (Fig. 3), indicating that binding of LDL is an essential requirement for their action on platelet aggregation.

Prostacyclin and lipoproteins

It was noted that when LDL was added to isolated platelets immediately after elution from the Sepharose column, they responded to all agonists. Therefore active platelets could be prepared from PRP in 20 min without a refractory period by

addition of LDL. Incubation with LDL (2 g of protein/litre) before addition of PGI_2 prevented the inhibition of adrenaline-induced aggregation by $10 \mu\text{g}$ of PGI_2 /litre, but not by $100 \mu\text{g}$ of PGI_2 /litre (Fig. 4). Similarly addition of LDL (2 g of protein/litre) after inhibition by PGI_2 reversed the inhibition only at the lower PGI_2 concentration: HDL had no such effect. Similar results were obtained with ADP as the aggregating agent or if 1 g of LDL or HDL/litre was used.

Total cyclic AMP assays were performed on active or PGI_2 -inhibited, isolated platelet suspensions after incubation with LDL or HDL (2 g of protein/litre each) or buffer. As expected, PGI_2 ($10 \mu\text{g}$ /litre) increased basal cyclic AMP levels from $7.1 \pm 2.1 \text{ pmol}/10^8$ platelets to $27.2 \pm 7.9 \text{ pmol}/10^8$ platelets ($n = 3$; means $\pm$ s.d.) within 5 min or less. Neither LDL nor HDL altered the basal cyclic AMP levels. In the presence of PGI_2 , LDL limited the increase ($12.1 \pm 3.1 \text{ pmol}/10^8$ platelets); HDL were also only partially effective in doing this ($14.3 \pm 1.1 \text{ pmol}/10^8$ platelets).

We have demonstrated the potential of LDL and HDL for sensitization of platelets. At low concentrations LDL has little effect, which is consistent with the observation in PRP that low LDL concentration is related to low platelet sensitivity in a cohort of healthy volunteers (Hassall *et al.*, 1983a).

Fig. 4. The effect of LDL on inhibition of platelet aggregation by PGI_2

Isolated platelets (IP) were incubated with PGI_2 (10 or $100\text{ }\mu\text{g/litre}$ final concentration) for 1 min at 20°C before transfer to an aggregometer and addition of adrenaline ($5\text{ }\mu\text{M}$) at 37°C after a further min. In some incubations LDL or HDL (final concentration 2 g of protein/litre) were added 5 min before or 1 min after addition of PGI_2 and tested for aggregation with adrenaline. (a) IP + $10\text{ }\mu\text{g}$ of $\text{PGI}_2/\text{litre}$; (b) IP + $100\text{ }\mu\text{g}$ of $\text{PGI}_2/\text{litre}$; (c) IP + $100\text{ }\mu\text{g}$ of $\text{PGI}_2/\text{litre}$ + LDL (2 g/litre); (d) IP + LDL (2 g/litre added first) + $100\text{ }\mu\text{g}$ of $\text{PGI}_2/\text{litre}$; (e) IP + HDL (2 g/litre added first) + $10\text{ }\mu\text{g}$ of $\text{PGI}_2/\text{litre}$; (f) IP + $10\text{ }\mu\text{g}$ of $\text{PGI}_2/\text{litre}$ + HDL (2 g/litre); (g) IP + $10\text{ }\mu\text{g}$ of $\text{PGI}_2/\text{litre}$ + LDL (2 g/litre); (h) IP + LDL (2 g/litre added first) + $10\text{ }\mu\text{g}$ of PGI_2 . The results have been obtained in at least five experiments with different preparations of LDL and HDL.

In contrast with previous work using lipoproteins and platelets from hypercholesterolaemic guinea-pigs (Fujitani *et al.*, 1979), VLDL did not sensitize human platelets. HDL only affected aggregation at high concentrations above the physiological range, which agrees with a previous study where no relationship was found between HDL concentration and platelet aggregation in human volunteers (Nordoy *et al.*, 1979). The mechanism for the sensitization by LDL is unknown, although binding of LDL to a receptor appears to be involved. Human platelets are known to have receptors that bind (Aviram *et al.*, 1981), but do not internalize LDL (Masurov *et al.*, 1982). The saturating concentration of LDL for these receptors ($0.1\text{ g total of LDL/litre}$) is considerably below that required to affect platelet

aggregation (Masurov *et al.*, 1982). Koller *et al.* (1982) showed that HDL_3 the most abundant of the three subfractions of HDL ($\rho = 1.125\text{--}1.21\text{ g/ml}$) (Nichols, 1967) inhibited the specific binding of LDL to platelets. We can find no effect of HDL or of HDL_3 (D. G. Hassall & K. R. Bruckdorfer, unpublished work) in reversing the effects of LDL on platelet aggregation; in fact HDL enhanced the action of LDL and were themselves promoters of aggregation at high concentrations. It is possible that non-specific binding sites or a small number of additional specific receptors are involved in the effects of LDL.

It is uncertain whether the mechanism by which LDL reverse the inhibitory action of PGI_2 on platelet aggregation involves the regulation of cyclic AMP levels; although LDL do not themselves reduce basal cyclic AMP levels, LDL may remove some PGI_2 from receptors on the platelet membrane, thereby partially reducing PGI_2 effects on adenylate cyclase activity.

It has been proposed that prostacyclin plays a key role in regulating a haemostatic mechanism that limits the response of platelets in the event of endothelial injury (Moncada, 1982). It has been reported that HDL, but not LDL, stimulated the synthesis of PGI_2 by endothelial cells (Fleischer *et al.*, 1982) and as shown above do not affect the inhibitory action of PGI_2 on platelets. LDL oppose the inhibition of platelet aggregation by PGI_2 and are believed to play an important role in the development of atherosclerosis (Kannel *et al.*, 1971). These results may be of significance in the study of cardiovascular diseases. Clearly platelet-lipoprotein interactions will be modified by other plasma factors and this should be the subject of further study.

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