Intracellular mechanisms in the activation of human platelets by low-density lipoproteins

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Low-density lipoproteins (LDL) have been shown to cause aggregation of human blood platelets at concentrations above 2 g of protein/l. The secretion of the contents of platelet dense granules was detected, but not that of the lysosomes. LDL gave rise to a mobilization of [³H]arachidonic acid from phospholipids and the appearance of products of the cyclo-oxygenase pathway after only 10 s. LDL-promoted aggregation was inhibited by both aspirin and indomethacin. There was an increase in ³H-labelled diacylglycerols and the phosphorylation of 47 kDa proteins. LDL therefore shares at least some of the mechanisms of stimulus/response coupling with those of other agonists.

INTRODUCTION

Low-density lipoproteins (LDL) sensitize isolated platelets to other agonists (Koller *et al.*, 1979; Aviram & Brook, 1982) and may act as agonists in their own right (Hassall *et al.*, 1983). The mechanism of action of the LDL is uncertain, but there are LDL receptors on the platelet surface (Koller *et al.*, 1982) which are different from the classical 'Brown & Goldstein' receptors found on other cell types (Shmulewitz *et al.*, 1984; Hassall & Bruckdorfer, 1985). There is some doubt whether the high-affinity binding of LDL on platelets is responsible for its effects on platelet activation, but low-affinity or non-specific binding has also been shown to occur (Hassall & Bruckdorfer, 1985).

Progress has been made in the understanding of receptor-mediated stimulus-response coupling. Essentially it has been proposed that, after the stimulus, phosphatidylinositol 4,5-bisphosphate is hydrolysed by a phosphodiesterase to release inositol 1,4,5-trisphosphate and diacylglycerols, and that inositol trisphosphate releases Ca²⁺ from intracellular stores (Berridge, 1984). The increased [Ca²⁺] may promote the hydrolysis of membrane phospholipids and the metabolism of the released arachidonic acid. Furthermore, a calmodulin-sensitive protein kinase is considered to stimulate the phosphorylation of light-chain myosin (Daniel *et al.*, 1981). The release of diacylglycerols also may be involved in the activation of a protein kinase C and the phosphorylation of a protein(s) of *M_r* 47000, of unknown function (Nishizuka, 1984). The aim of the present paper was to study in greater detail some aspects of the mechanisms by which LDL stimulates platelet aggregation in the absence of other known platelet agonists.

EXPERIMENTAL

Isolation of platelets and lipoproteins

Normal blood was collected from volunteers into acid-citrate-dextrose anticoagulant and centrifuged for

5 min at 750 *g* to yield platelet-rich plasma. This was centrifuged again to remove traces of other blood cells and then re-centrifuged at 750 *g* for 10 min in the presence of 300 nM-prostacyclin (prostaglandin I₂). The platelets were resuspended in buffer (160 mM-NaCl/2.7 mM-KCl/7 mM-NaHCO₃/0.5 mM-MgCl₂/5 mM-Hepes/5 mM-glucose, pH 7.4), centrifuged again in the presence of prostacyclin and resuspended again in the buffer to give a final platelet count of (4–8) × 10¹¹ cells/l. In each of the tests described below there were some variations in this procedure, which are indicated.

LDL were prepared from fresh plasma by differential ultracentrifugation (Hatch & Lees, 1968). They were dialysed against buffer (see above) and concentrated with poly(ethylene glycol) to a final concentration of 8–20 g of protein/l.

Platelet aggregation and granule release

Platelet-aggregation tests were performed in a conventional Payton dual-channel aggregometer as described by Hassall *et al.* (1983), by addition of LDL (final concn. 0–3 g of protein/l). Alternatively a Chronolog 'lumi-aggregometer' (Coulter Electronics, Luton, Beds., U.K.) was used for simultaneous measurement of aggregation and release of ATP from dense granules by the luciferin-luciferase bioluminescence reaction in the presence of 'Chromolume' reagent supplied by the manufacturers. In each case 4 μM-ATP was added to calibrate the magnitude of the response.

The secretion of lysosomes was detected by measurement of *N*-acetyl-β-D-glucosaminidase (EC 3.2.1.30). Platelet suspensions were stimulated either by thrombin (1 unit/ml) or by LDL (2.75 g/l) for 0–3 min, and the activation was terminated by addition of cold formaldehyde/EDTA (final concn. 0.127 M and 0.01 M respectively). After centrifugation at 4 °C at 2000 *g* for 2 min, quadruplicate samples were removed into a 96-well Elisaplate (Nunc), buffered with citrate/phosphate buffer, pH 4.5 (final concns. 0.067 M for each), and assayed for *N*-acetylglucosaminidase by incubation with *p*-nitrophenol *N*-acetyl-β-D-glucosaminide (final concn. 3.3 mM) for

Abbreviation used: LDL, low-density lipoproteins.

50 min at 37 °C. The reaction was stopped by the addition of NaOH (final concn. 0.073 M), and the A_{405} was measured on a Bio-Rad 2550 E1A Microtiter plate reader and compared with a range of standards. Results are expressed as μmol of *p*-nitrophenol released/min per 10^{11} cells.

Release of arachidonic acid and its metabolites

Isolated platelets ($4 \times 10^{11}/\text{l}$) were incubated with [5,6,8,9,12,14,15- ^3H]arachidonic acid (1 mCi/l; sp. radioactivity 130 Ci/mmol; Amersham International, Amersham, Bucks., U.K.) for 90 min at 37 °C. Most of the free acid is esterified in the platelet phospholipids during this time. Samples (0.5 ml) of platelets were activated by addition of LDL (final concn. 2.75 g/l) or thrombin (2 units/ml) for up to 120 s in a platelet aggregometer cuvette at 37 °C with stirring. The incubations were stopped by addition of 30 μl of 2 M-citric acid and by placing the cuvettes in an ice bath. The extraction of metabolites was a two-stage process. First 5 ml of diethyl ether was added and vortex-mixed for 1 min, followed by centrifugation at 3000 g for 5 min, and the upper ether layer was set aside. The ether extraction was repeated and the two extracts were combined. The lower layer was then re-extracted with chloroform/methanol by the method of Bligh & Dyer (1959). The total dried extract was dissolved in 200 μl of ether/methanol (9:1, v/v) and run on Kieselgel 60 0.2 mm-thickness aluminium-backed t.l.c. plates (Merck, Darmstadt, Germany) in the solvent chloroform/methanol/acetic acid/water (450:40:5:4, by vol.). Radioactive products were detected with a t.l.c. radioactivity scanner (model F0112/F3222/RRG-121; Panax Equipment, Mitcham, Surrey, U.K.). The products were compared with the R_F values for known standards for identification. The radioactive areas were eluted from the plates and counted for radioactivity on a Packard Tri-Carb spectrometer model 3385.

Measurement of diacylglycerol synthesis

In the samples above that were run on t.l.c., the zone between arachidonic acid and the solvent front was eluted and subjected to further separation by t.l.c. on Kieselgel 60 plates in a solvent system containing light petroleum (b.p. 40–60 °C)/diethyl ether/acetic acid (35:10:2, by vol.). Diacylglycerols were identified by comparison of R_F values with those of reference standards and the amount of radioactivity in the eluted spots was counted as described above.

Phosphorylation of platelet proteins

Platelets from 20 ml of platelet-rich plasma were isolated and resuspended in 3 ml of phosphate-free buffer (145 mM-NaCl, 5 mM-KCl, 1 mM-MgSO₄, 10 mM-Hepes, pH 7.4), and incubated with 0.5 ml of Na₃³²PO₄ for 1 h at 37 °C. Platelets were re-isolated with the presence of prostacyclin, and resuspended platelets (final concn. $4 \times 10^{11}/\text{l}$) were aggregated with LDL (0–3 g of protein/l) in 200 μl portions in an aggregometer. The reactions were stopped after 30 s by addition of 10 μl of a solution containing 5% (w/v) SDS, 0.1% 2-mercaptoethanol and 20% (w/v) sucrose. Then 60 μg of platelet protein was loaded (with 5 μl of 3.3 mM-Tris/HCl buffer, pH 6.8, containing 1.3% SDS, 13% sucrose and 0.00025% Bromophenol Blue) on to polyacrylamide slab gels (13% polyacrylamide, 0.15% acrylamide and 0.1% SDS, with

a 5%-polyacrylamide stacking gel). The proteins were separated by electrophoresis in a Protean II apparatus (Bio-Rad) at 25 mA in a buffer containing 25 mM-Tris, 150 mM-glycine and 1% SDS, pH 8.3. M_r values were estimated by comparison with lanes in which appropriate protein markers were run. The protein bands were stained in Coomassie Blue and recorded densitometrically with a Chromoscan III scanner (Joyce-Loebl). Autoradiography on X-ray film (Kodak Safety Film RPO3) revealed sites of phosphorylation and were recorded by densitometry as for the protein bands.

RESULTS

Aggregation and secretion

Spontaneous aggregation of isolated platelets occurred when they were incubated with LDL at concentrations above 2 g/l (Fig. 1a). The time to onset of aggregation was decreased with increasing LDL concentrations. No turbidometrically determined changes in platelet shape were observed, although this can be detected under the electron microscope (Hassall *et al.*, 1983). Aggregation was followed by secretion of dense granules, since ATP was released after addition of LDL (3 g/l). In these experiments (Fig. 1b) LDL was added at the same time as the Chromolume reagent, which tended to inhibit the aggregation response to LDL. A similar effect of Chromolume has been shown on platelet response to other agonists (Thompson & Scrutton, 1985). LDL caused a release of ATP which ranged between 60 and 90% of that released by 0.1 unit of thrombin/ml.

In parallel experiments thrombin caused the release of lysosomal *N*-acetyl- β -D-glucosaminidase. No significant activity was detected, even after 3 min incubation with LDL (2.75 g/l), which causes aggregation, indicating that lysosomal release does not occur (Fig. 1c).

The addition of isolated LDL to platelet-rich plasma (final total LDL concn. 5 g of protein/l) gave rise to spontaneous aggregation of blood platelets: lower concentrations had no effect.

Metabolism of arachidonic acid

Platelets were preincubated with radioactively labelled arachidonic acid, which was esterified in membrane phospholipids. The platelets were stimulated with thrombin (2 units/ml) or LDL for 10 or 120 s at lipoprotein concentrations that would (a) induce or (b) not induce aggregation within 2 min. After 10 s both thrombin and LDL (2.75 g of protein/l) initiated the hydrolysis of membrane phospholipids, and an increase in the radioactivity found in metabolites of arachidonic acid (Table 1). After 2 min this hydrolysis increased, and was greater with LDL than with thrombin, so that one-third of the arachidonic acid-containing phospholipids had become hydrolysed. There was a marked increase in the formation of cyclo-oxygenase products, particularly thromboxane B₂, and also in 12-hydroxy-heptadecatrienoic acid. The principal product of the lipoxygenase pathway, 12-hydroxyeicosatetraenoic acid, was also increased. At sub-aggregatory concentrations of LDL (2 mg of protein/l) no hydrolysis of phospholipids or metabolism of arachidonic acid occurred.

In other experiments it was shown that LDL-induced aggregation was inhibited by aspirin (100 μM) or indomethacin (100 μM), but not by dazoxiben [4-(2-

Fig. 1. Effects of low-density lipoproteins on aggregation and secretion in isolated platelets

Platelets were isolated from plasma (see the Experimental section) and incubated (final concn. $2 \times 10^{11}/l$) with a range of concentrations of LDL (0–3 g of protein/l final concn.). The effects of LDL were studied on (a) platelet aggregation, (b) secretion of ATP from dense granules measured by the luciferin–luciferase reaction and (c) the release of lysosomal contents by measurement of *N*-acetyl- β -D-glucosaminidase (NAG) activity. In (c) the results for LDL (■; 3 g of protein/l) were compared with the effect of addition of thrombin (□; 1 unit/ml). The results are typical of those obtained by the following numbers of experiments (a) $n = 12$; (b) $n = 5$; (c) $n = 3$.

imidazol-1-ylethoxy)benzoic acid] hydrochloride, a thromboxane synthetase inhibitor, at a concentration ($10 \mu M$) that in separate experiments completely inhibited thromboxane biosynthesis in isolated platelets.

Diacylglycerol formation

In some experiments neutral products of phospholipid hydrolysis running near the solvent front were eluted and rechromatographed in a separate t.l.c. system (see the

Experimental section) to obtain a better separation. By this means it was possible to isolate labelled diacylglycerols to ascertain whether there were increases in the concentrations of these lipids after stimulation with LDL. This could not be achieved by direct assay of mass, because of the presence of diacylglycerols in LDL.

Stimulation of platelets with LDL or thrombin increased the radioactivity in the diacylglycerols and free arachidonic acid after 10 s: this was even more marked

Table 1. The effect of LDL on the metabolism of arachidonic acid

Isolated platelets (4×10^{11} /l) were incubated with [3 H]arachidonic acid at 20 °C for 90 min until most of the acid was esterified into membrane phospholipids. Samples (0.5 ml) were incubated by stirring in a Payton aggregometer cuvette for 10 or 120 s with either thrombin (2 units/ml) or LDL at either 2 g of protein/l (no aggregation within 2 min) or 2.75 g of protein/l (aggregatory). The reactions were stopped by addition of 2 M-citric acid and extracted with diethyl ether and then by the method of Bligh & Dyer (1959). The combined extracts were run on t.l.c. in chloroform/methanol/acetic acid/water (450:40:5:4, by vol.) and the radioactivity in spots (recognized by running appropriate standards) was determined. The results are expressed as a percentage of the total radioactivity (c.p.m.) in the platelets, which stayed constant irrespective of treatment, and given as means $\pm$ S.D. for three experiments each performed in triplicate. Abbreviations: HHT, heptadecatrienoic acid; 12-HETE, 12-hydroxyeicosatetraenoic acid; AA, arachidonic acid; PG, prostaglandin.

Agonist	Time after agonist addition (s)	Radioactivity in arachidonic acid metabolites and esters (% of total platelet radioactivity)					
		Phospholipid	Thromboxane B ₂	PGD ₂ +PGE ₂	HHT	12-HETE	AA
None	–	85.6 $\pm$ 2	2.7 $\pm$ 0.8	2.1 $\pm$ 0.4	3.8 $\pm$ 0.4	3.5 $\pm$ 0.3	1.2 $\pm$ 0.1
Thrombin (2 units/ml)	10	79.6 $\pm$ 1.8	3.8 $\pm$ 1.2	2.9 $\pm$ 0.5	4.7 $\pm$ 0.2	5.8 $\pm$ 1.3	1.5 $\pm$ 0.8
Thrombin (2 units/ml)	120	68.4 $\pm$ 5.3	5.9 $\pm$ 1.7	3.6 $\pm$ 0.1	7.0 $\pm$ 1.3	9.2 $\pm$ 3.6	3.6 $\pm$ 3.8
LDL (2.75 g of protein/l)	10	75.1 $\pm$ 5.3	4.5 $\pm$ 0.2	4.7 $\pm$ 1.5	6.3 $\pm$ 1.7	5.5 $\pm$ 0.8	2.3 $\pm$ 1.2
LDL (2.75 g of protein/l)	120	58.6 $\pm$ 9.9	9.9 $\pm$ 2.4	5.2 $\pm$ 1.6	9.5 $\pm$ 2.4	9.6 $\pm$ 1.9	3.3 $\pm$ 1.2
LDL (2 g of protein/l)	10	83.8 $\pm$ 3.0	3.0 $\pm$ 0.5	2.8 $\pm$ 0.7	4.0 $\pm$ 0.8	3.9 $\pm$ 0.6	1.4 $\pm$ 0.2
LDL (2 g of protein/l)	120	84.7 $\pm$ 3.0	2.8 $\pm$ 0.6	2.7 $\pm$ 0.7	3.9 $\pm$ 0.9	3.6 $\pm$ 0.5	1.2 $\pm$ 0.2

Table 2. Diacylglycerol synthesis in platelets stimulated by LDL

Isolated platelets were labelled with [3 H]arachidonic acid, then stimulated, extracted and chromatographed on t.l.c. as described in Table 1. Lipids running above arachidonic acid were re-extracted and run on another t.l.c. plate in light petroleum/diethyl ether/acetic acid (70:20:4, by vol.). Radioactivity in the diacylglycerol spots was identified by reference to standards and counted. In the incubations the platelets were stimulated either with thrombin or with LDL at two different concentrations, only the higher one being sufficient to induce platelet aggregation. Some incubations were performed in the presence of 1 mM-EGTA. The results are expressed as the radioactivity in diacylglycerols as a percentage of that in total lipids and given as means $\pm$ S.D. for three separate experiments.

Addition	Time after stimulus (s) ...	3 H in diacylglycerols (% of total)	
		10	20
None		0.8 $\pm$ 0.1	0.82 $\pm$ 0.1
LDL (2 g of protein/l)		0.9 $\pm$ 0.2	1.0 $\pm$ 0.3
LDL (2.75 g of protein/l)		1.5 $\pm$ 0.3	3.6 $\pm$ 0.7
LDL (2.75 g of protein/l) + 1 mM-EGTA		0.6 $\pm$ 0.1	0.5 $\pm$ 0.1
Thrombin (2 units/ml)		1.5 $\pm$ 0.3	2.2 $\pm$ 0.3
Thrombin (2 units/ml) + 1 mM-EGTA		1.3 $\pm$ 0.1	0.8 $\pm$ 0.1

after 2 min (Table 2). However, if the same experiments were conducted in the presence of 1 mM-EGTA, there was still a transitory increase in diacylglycerols after 10 s after thrombin stimulation, which was eliminated in the case of LDL.

Protein phosphorylation

Stimulation of platelets with LDL for 30 s brought about a significant increase in the phosphorylation of a 47 kDa platelet protein (Fig. 2), which is known to result from the activity of protein kinase C (Kawahara *et al.*,

1980). No increase was noted in the phosphorylation of myosin light chain by the calmodulin-linked kinase (Daniel *et al.*, 1981). The effect of LDL was considerably decreased by the presence of 1 mM-EGTA.

DISCUSSION

LDL have been shown to act as direct agonists on isolated platelets, as well as potentiating the action of other well-known promoters of platelet aggregation at lower concentrations of LDL (Hassall *et al.*, 1983). This

Fig. 2. Phosphorylation of platelet proteins by low-density lipoproteins

Isolated platelets were incubated in a buffer (145 mM-NaCl/5 mM-KCl/1 mM-MgSO₄/10 mM-Hepes), pH 7.4, containing Na₃³²PO₄, for 1 h at 37 °C. Samples were stimulated with LDL (3 g of protein/l) for 30 s in the absence (b) or presence (c) of 1 mM-EGTA, and the phosphorylation of proteins was compared with that in unstimulated platelets (a). The proteins were separated on 13%-polyacrylamide/SDS gels (see the Experimental section) by electrophoresis at 25 mA. The sites of phosphorylation were detected on X-ray film and compared with the location of protein bands and known *M_r* markers. The densitometric traces of the X-ray film are shown with the location of the markers: the traces represent one typical experiment out of four.

was shown more clearly in the present study, when concentrations of LDL above 2 g of protein/l readily caused platelet aggregation. This concentration of LDL is above the normal physiological concentration range of the lipoprotein in human plasma, but would be typical of the pathological concentrations observed in patients with familial hypercholesterolaemia. Even in these patients, platelet concentrations appear to be within the normal range, although the platelets are sensitized to other agonists (Carvalho *et al.*, 1974). The fact that even higher concentrations (5 g of protein/l) of LDL are required to induce spontaneous aggregation in platelet-rich plasma indicates the presence of materials in plasma which attenuate the effects of LDL. An example of this is a sub-fraction of plasma high-density lipoproteins (HDL₂) which will decrease platelet aggregability by LDL, whereas the major HDL₃ fraction has little effect

(Desai *et al.*, 1986). There may be other proteins in the plasma which are inhibitory.

LDL cause degranulation of platelet dense bodies, leading to secondary aggregation of platelets. They have been shown to enhance slightly the release of β -thromboglobulin from isolated platelets, but at concentrations below that required for direct aggregation (Aviram & Brook, 1984). The effects of LDL are clearly not potent enough to bring about the release of lysosomal contents, which can be achieved with thrombin. Such differences may be explained in terms of the rate of ATP hydrolysis in platelets stimulated by thrombin (Verhoeven *et al.*, 1984).

These events were preceded by an increased formation of arachidonic acid metabolites. The hydrolysis of phospholipids containing labelled arachidonic acid was detectable after only 10 s incubation with LDL, at a rate comparable with that obtained with a large dose of thrombin. The principal products of arachidonic acid metabolism by the cyclo-oxygenase and lipoxygenase pathways, thromboxane B₂, 12-hydroxyheptatrienoic acid and 12-hydroxyeicosatetraenoic acid, were all increased after this short time interval. After 120 s almost 35% of the labelled arachidonic acid had been released and metabolized. The aggregation of platelets by LDL was inhibited by both aspirin and indomethacin, indicating an absolute requirement for cyclo-oxygenase activity. The generation of thromboxane was dispensable, since dazoxiben did not inhibit aggregation. Prostaglandin endoperoxides, which accumulate if thromboxane synthetase is blocked, are also potent activators of platelet aggregation. At concentrations of LDL just insufficient to induce aggregation, but enough to sensitize platelets to other agonists, no mobilization of arachidonic acid occurred. This is further evidence that granular exocytosis is mediated by early formation of metabolites of the cyclo-oxygenase pathway. This agrees with previous findings that low concentrations of LDL cause only marginal increases in malondialdehyde formation or in the synthesis of thromboxane B₂ determined by specific radioimmunoassay (Aviram *et al.*, 1985). Similar results were found in patients with familial hypercholesterolaemia, where malondialdehyde production by platelets was only slightly increased (Zahavi *et al.*, 1981). This suggests that the potentiation of LDL of aggregation induced by other agonists does not require the initial mobilization of arachidonic acid. LDL also over-rides the inhibitory effects of prostacyclin on the aggregation of isolated platelets (Bruckdorfer *et al.*, 1984) even at these lower concentrations of LDL. In hypercholesterolaemic subjects with increased LDL, there is a lower sensitivity of platelets to prostacyclin (Colli *et al.*, 1983). LDL therefore has an important effect on the response of platelets to arachidonic acid metabolites.

The activation of platelets with both thrombin and LDL resulted in an increase in radioactivity in the diacylglycerols within 10 s and persisting for 2 min. In the presence of EGTA this effect was transitory, as described previously (Rittenhouse-Simmons, 1979) for thrombin-treated platelets, but if LDL were used as agonists EGTA abolished the apparent synthesis of diacylglycerols and platelet aggregation. EGTA is unlikely to act by inhibition of binding of LDL to the platelets, since 3 mM-EDTA had no effect on this (Curtiss & Plow, 1984). Diacylglycerols arise in platelets

because of the action of phosphodiesterase (phospholipase C), which is specific for phosphoinositides (Rittenhouse, 1983), and which will hydrolyse phosphatidylinositol, its 4-phosphate and 4,5-bisphosphate. The requirements for bivalent ions are different for this enzyme according to the substrate used, both in platelets (Low *et al.*, 1986) and in rat brain (Irvine *et al.*, 1984). In other studies (Bruckdorfer & Giraud, 1985), paradoxically, no hydrolysis of phosphatidylinositol bisphosphate was observed, and therefore the origin of these diacylglycerols is uncertain. Siess *et al.* (1983) have suggested that arachidonic acid and its metabolites may induce diacylglycerol formation and phosphorylation of a 40 kDa protein, but this still begs the question which phospholipid is rapidly hydrolysed to release arachidonic acid.

The generation of diacylglycerols is reflected in the phosphorylation of 47 kDa protein after the incubation of platelets with LDL, a phenomenon noted with other platelet agonists (Nishizuka, 1984). The unusual feature here is the absence of any significant phosphorylation of myosin light chain, which is associated with increases in intracellular Ca^{2+} concentrations. It has proved difficult to determine whether LDL does bring about any mobilization of intracellular Ca^{2+} by using standard techniques with fluorophores, because of the strong fluorescence of LDL itself (R. C. Dunn & K. R. Bruckdorfer, unpublished work). The effect of EGTA in eliminating phosphorylation may be simply because diacylglycerols are not generated and also because the Ca^{2+} concentrations have been decreased to below the minimum required for protein kinase C activity (Wolf *et al.*, 1985).

LDL are a heterogeneous group of lipoproteins (Krauss & Burke, 1982), which can act as potent activators of blood platelets. They appear to share at least some of the mechanisms of stimulus-secretion coupling of other platelet agonists, and act primarily by the synthesis of arachidonic acid metabolites.

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