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Low-density lipoproteins increase intracellular calcium in aequorin-loaded platelets

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Low-density lipoproteins activate isolated human platelets. The mechanism of this activation is unknown, but may involve increased phosphoinositide turnover. We have examined the effect of low-density lipoproteins on intracellular calcium concentrations in platelets loaded with the photoprotein aequorin. The lipoproteins induced concentration-dependent increases in intracellular calcium, associated with shape change and aggregation. These responses could be partially inhibited by the removal of extracellular calcium and by pre-incubation with acetylsalicylic acid. They were also antagonised by agents which increase cellular concentrations of cyclic adenosine and guanosine monophosphates. It is not clear whether the platelet-lipoprotein interaction involves a 'classical' lipoprotein receptor.

LDL; intracellular Ca^{2+}; Aequorin; (Human platelet)

1. INTRODUCTION

There is considerable evidence that platelets from hypercholesterolaemic patients are abnormally sensitive to several agonists in vitro [1,2]. These patients have elevated levels of circulating thromboxane B_2 and β-thromboglobulin, suggesting that in vivo platelet activation may also be abnormal [3]. The abnormal platelet activation appears to be related to high blood levels of LDL and VLDL, particularly the former [4], and LDL has been shown in vitro to be a platelet activator in its own right [5]. The mechanism of the LDL-platelet interaction is poorly understood. However, it is known that there are specific binding sites for LDL on the platelet [6] and that LDL causes early activation of the cyclo-oxygenase pathway, increased diacylglycerol concentrations, and the phosphorylation of 47 kDa proteins [7]. This may be associated with enhanced membrane phosphoinositide turnover, which would usually involve an increase in $[Ca]_i$. We have examined the $[Ca]_i$ response to LDL in isolated human platelets using the photoprotein aequorin. The more commonly used fluorescent calcium probes, such as quin-2 and fura-2, could not be used satisfactorily in these experiments because of the intense autofluorescence of LDL itself at physiological concentrations.

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Abbreviations: LDL, low-density lipoprotein; HDL, high-density lipoprotein; VLDL, very low density lipoprotein; $[Ca]_i$, intracellular calcium concentration

2. MATERIALS AND METHODS

2.1. *Materials*

Aequorin was purchased from Dr J. Blinks, Mayo Clinic, Rochester, MA. Nifedipine was a gift from Bayer, England. HL725 was from Calbiochem. Other chemicals were from Sigma or British Drug Houses Ltd.

2.2. *Preparation of LDL*

LDL was prepared by differential ultracentrifugation from the fresh plasma of normal volunteers by a modification of the method of Chung et al. [8]. After extensive dialysis the LDL was concentrated to a protein concentration of 15–30 g LDL/l.

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Until the final period of dialysis EDTA was present to minimise LDL oxidation. The loading procedure was modified from those of Vickers and Mustard [9] and of Johnson et al. [10]. All procedures were performed at room temperature. Fresh blood (usually 20 ml) was anticoagulated with 3.15% trisodium citrate (9:1 by vol.). The blood was centrifuged at 180 × *g* for 15 min at room temperature and the platelet-rich plasma (PRP) was removed. Forskolin (10 μM) was added and the PRP was centrifuged at 600 × *g* for 10 min at room temperature: all the following procedures were also performed at room temperature. The plasma was removed as completely as possible and the pellet resuspended in a hypotonic loading buffer [9], to which EGTA (10 mM final concentration) was added. After 10 min, disodium ATP (5 μM) and aequorin (17.5 μl of a 3 g/l solution) was added. 5 min later, 42 μl hypertonic buffer and 2.5 μl of 200 mM magnesium chloride were added. After a further 15 min, the platelet suspension was filtered through a Sepharose CL-4B column pre-equilibrated with a modified Hepes-Tyrode's buffer. The gel filtered platelets were then made up to 20 ml with buffer (at a platelet concentration of about 2.5×10^{11} cells/l) and calcium chloride (to 1 mM) was added. The platelets were used after a further 30 min equilibration, with or without 0.1 mM acetylsalicylic acid.

2.3. *Measurement of platelet responses*

Platelet aggregation and luminescence responses were recorded using a Chrono-Log PICA lumiaggregometer (Coulter Electronics). $[Ca]_i$ values were calculated as described by Johnson et al. [10], but assuming an intracellular magnesium concentration of 0.1 mM [11]. Where appropriate, potential antagonists were added 2 min before stimulation with LDL.

3. RESULTS

LDL caused a concentration-dependent increase in $[Ca]_i$ in isolated human platelets (fig.1). For comparison, an aggregatory concentration of thrombin (0.5 U/ml) produced a mean $[Ca]_i$ of 9.1 μM (± 0.8, n = 9). Low LDL concentrations (0.25–0.5 g/l) induced only shape change, whereas higher concentrations (≥0.75 g/l) induced both shape change and secondary aggregation. In some instances the two phases of the platelet response coincided with two peaks in the luminescence signal. In most experiments, however, only a single peak was observed in the luminescence trace, corresponding to the shape change at low concentrations of LDL and to the aggregation response at higher LDL concentrations. Addition of 2.5 mM EGTA to the platelet suspension immediately before stimulation with LDL (1 g/l) reduced the $[Ca]_i$ by about 30% and inhibited secondary aggregation, without affecting the shape change (fig.2).

Preincubation with 0.1 mM acetylsalicylic acid also reduced the peak $[Ca]_i$ response to LDL (1 g/l) by about 20% and abolished or reduced secondary aggregation (fig.3). LDL-induced activation, both the shape change and the increase in

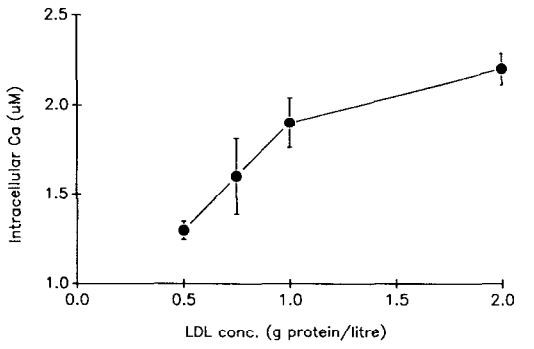

Fig.1. Concentration-response curve for LDL-induced increase in $[Ca]_i$ in aequorin-loaded platelets. Points are means ± SE (n = 11–18).

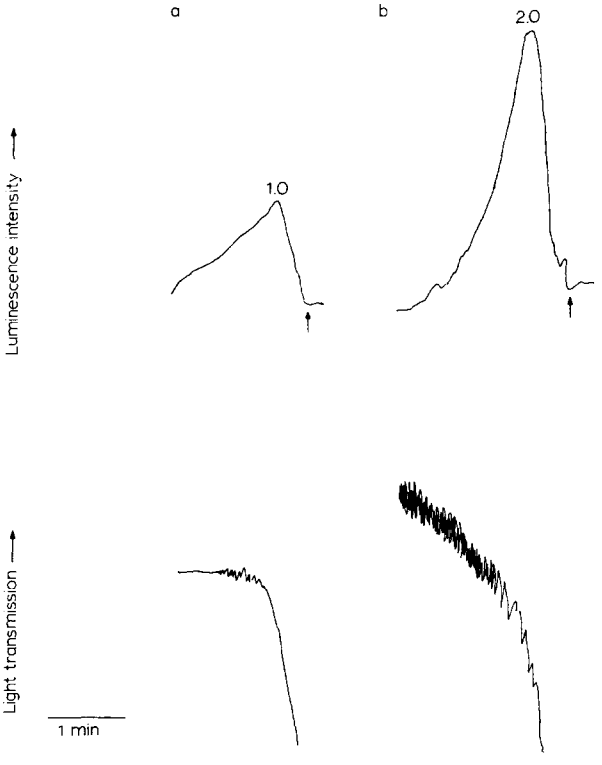

Fig.2. Platelet $[Ca]_i$ and aggregation responses to 1 g/l LDL (a) in the presence of 2.5 mM EGTA added immediately before LDL and (b) without EGTA, in the presence of 1 mM Ca^{2+}. Arrow indicates addition of LDL. Figures above luminescence peaks are $[Ca]_i$ (μM). Each trace is representative of 4 experiments.

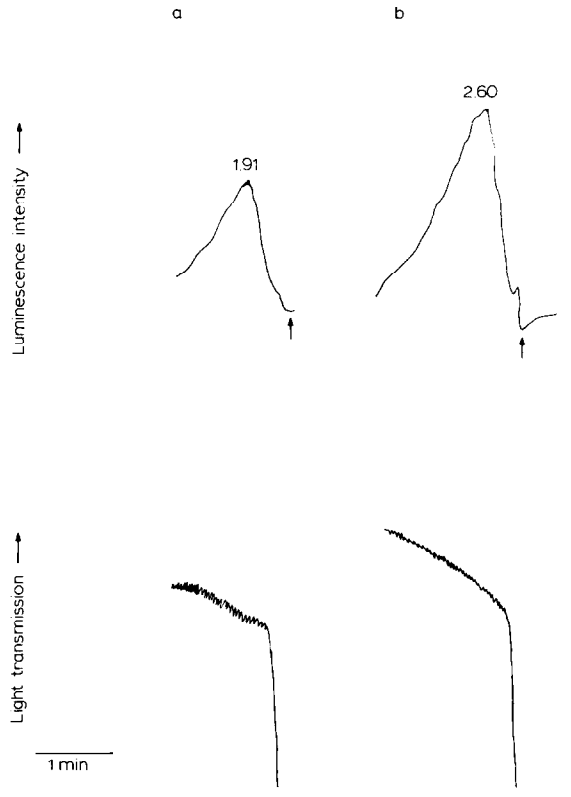

Fig.3. Platelet $[Ca]_i$ and aggregation responses to 1 g/l LDL (a) with preincubation for 30 min with 100 μM acetylsalicylic acid and (b) without acetylsalicylic acid. Arrow indicates addition of LDL. Figures above luminescence peaks are $[Ca]_i$ (μM). Each trace is representative of 6 experiments.

$[Ca]_i$, was completely inhibited by the cyclic AMP phoshodiesterase inhibitor HL725 (1 μM) [12] and by the permeant cyclic GMP analogue 8-bromo-cyclic GMP (100 μM) (n = 3 for each). Two calcium channel blockers, nifedipine and diltiazem (each at 10 μM), which have been reported to inhibit in vitro platelet activation by other agonists such as adenosine diphosphate and arachidonic acid [13], failed to influence the calcium and aggregation responses to LDL (n = 4 for each). All the above compounds were incubated with the platelet suspension for 2 min before the addition of LDL.

4. DISCUSSION

Our results confirm that LDL produces a concentration-dependent activation of isolated human platelets, as judged by shape change and aggregation. We have also shown that this activation is accompanied by an increase in $[Ca]_i$ which approximately parallels the extent of activation. Some of the rise in $[Ca]_i$ is due to influx from the external medium, since it can be partially inhibited by EGTA: longer pre-incubation with EGTA may have produced more marked inhibition. However, some of the calcium must originate from intracellular stores. It would be anticipated that such calcium mobilisation may be associated with enhanced phosphoinositide turnover, and preliminary experiments using platelets prelabelled with ^{3}H-inositol suggest that this is the case (Tranter, unpublished). Earlier studies, using ^{32}P-labelled phospholipids, had failed to demonstrate significant breakdown of phosphatidylinositol 4,5-bisphosphate [14]. This probably reflects the greater sensitivity of the inositol-labelling technique as an index of phosphoinositide turnover, and perhaps the small magnitude of the LDL-induced hydrolysis. It should be noted that the $[Ca]_i$ increase in response to LDL (up to 2 g/l) was much smaller than that to thrombin (0.5 U/ml), though LDL (1–2 g/l) consistently induces secondary aggregation.

Since acetylsalicylic acid is an effective but incomplete inhibitor of LDL-induced activation, we agree with earlier findings that some of the activation is due to a cyclo-oxygenase product, probably thromboxane A_2 [7]. This will itself promote calcium mobilisation and influx by a phosphoinositide-linked mechanism. The inhibitory effects of increased cyclic nucleotide levels, though this is only inferred in the case of phosphodiesterase inhibition, are similar to interactions with other platelet agonists. In these cases it is thought that elevated levels of cyclic nucleotides may inhibit phosphoinositide breakdown and also promote the removal of free calcium from the cytoplasm, probably into membrane binding sites [15]. LDL itself inhibits the prostacyclin-induced synthesis of cyclic nucleotides [16]. The lack of effect of the two calcium channel blockers is interesting as these drugs are widely used in the therapy of cardiovascular disease. Higher concentrations of these compounds may be inhibitory, but would have been very much higher than plasma levels attained in clinical practice. The mechanism of action of

calcium channel blockers is unclear even in instances where they appear to be effective antiaggregants: there do not appear to be any voltage-dependent calcium channels in the platelet, though extracellular calcium is obviously very important for normal platelet function.

Our findings differ in several respects from the results of Block and co-workers [18,19]. They also found that LDL increased $[Ca]_i$ and phosphoinositide breakdown in the human platelet, though they used quin-2 as an indicator of free calcium concentration. This proved to be possible because of the very low concentrations of LDL used in their study (10 mg protein/l). However, in our series of experiments we have not seen detectable calcium or shape-change responses at LDL concentrations below 50 mg/l: occasionally responses were seen at a concentration of 100 mg/l, while consistent responses were only obtained at concentrations greater than 250 mg/l. It is not clear whether this discrepancy is related to different techniques for the preparation of LDL or to some other technical factor. Furthermore, the LDL-induced increases in $[Ca]_i$ described by Block and colleagues are comparable to those produced by thrombin, though it should be noted that the concentration of thrombin used in their studies (0.1 U/ml) was lower than that in our experiments. We found a much greater discrepancy between $[Ca]_i$ responses to these agonists, even at LDL concentrations as high as 2 g/l, and it may be surprising that relatively modest increases in $[Ca]_i$ can be associated with secondary aggregation responses. As might be expected the absolute values of $[Ca]_i$ reported by aequorin are much higher than those with fluorescent indicators, even with a revised estimate of intracellular magnesium concentration. The reasons for this remain unclear.

In conclusion, we have shown that LDL may activate human platelets partly by modestly increasing $[Ca]_i$, possibly by enhancing phosphoinositide breakdown and by generating cyclooxygenase-derived secondary agonists. We have yet to establish whether this response is mediated by the type of LDL receptor described by Brown and Goldstein [20].

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