BIOPHYSICS AND BIOCHEMISTRY

Contribution of Arachidonic Acid Cycle Enzymes to Platelet Activation by Low-Density Lipoproteins

I. I. Vlasova and O. A. Azizova

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The mechanism of platelet activation by low-density lipoproteins (LDL) was studied using inhibitors of arachidonic acid cycle enzymes. Lipoxygenase and cyclooxygenase inhibitors (indomethacin, acetylsalicylic acid, NDGA, and BW755C) inhibited LDL-induced platelet aggregation to a small extent, as was indicated by mere 20% to 30% decreases in the maximal rate of change in light transmission. 4-Bromophenacyl bromide inhibited LDL-induced platelet aggregation in a dose-dependent manner, almost complete inhibition occurring at concentrations in excess of 20 μM. The results support the conclusion that enzymes of the arachidonic acid cycle do not contribute substantially to platelet activation by LDL.

Key Words: *platelet aggregation; low-density lipoproteins; lipoxygenase; cyclooxygenase; phospholipase*

Low-density lipoproteins (LDL) are plasma components that have important effects on platelet activity. In addition to exhibiting aggregation-promoting activity, they have been found capable, in high concentrations (1-2 g/liter), of acting as inducers of platelet aggregation [6,9]. In an attempt to explain this effect, LDL have been tested for effects on platelet secretion [3,10], intracellular calcium [13], products of the phosphoinositide cycle [11], cell membrane lipids [4], etc. Nevertheless, the mechanism of platelet activation by LDL has been studied inadequately, and the data are contradictory [3,4,11,13].

In recent years, high priority has been given to research into the role of oxidized LDL in the development of atherosclerosis. It has been shown [4,5] that platelet aggregation can be induced by oxidized LDL, but not by their native counterparts. In our previous work we found that platelet aggregation parameters depend on the LDL concentration and on the degree to which lipids have been modified by peroxidation in LDL [1]. The present study was designed to determine, using various inhibitors of enzymes involved in the known pathways of platelet activation, whether the phosphoinositide or arachidonic acid cycle is the major mechanism underlying platelet activation by LDL, both native and modified through lipid peroxidation.

Institute of Physicochemical Medicine, Ministry of Health and the Medical Industry of Russia, Moscow (Presented by Yu. M. Lopukhin, Member of the Russian Academy of Medical Sciences)

MATERIALS AND METHODS

LDL were isolated from the serum of healthy human donors by preparative ultracentrifugation [8] and dialyzed for 18-20 h at 4°C against a 1000-fold volume of 10 mM Tris-HCl buffer, pH 7.4, containing 140 mM NaCl and 7.6 mM KCl. The isolated LDL were stored at 4°C and used within 48 h.

The LDL concentration was estimated from the protein level in the LDL as measured by the biuret

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test [7]. LDL were oxidized by incubation under aerobic conditions at 37°C with 5 μM $CuSO_4$ for 2-4 h, and the degree of their oxidation was assessed by the accumulation of 2-thiobarbituric acid-reactive products (expressed in μmoles of malonic dialdehyde - MDA per gram protein) in the LDL [12]. LDL containing MDA at levels of ≤ 1 μmol/g protein were referred to as native.

Platelets were isolated from the venous blood of healthy donors taken in a 6:1 ratio with an anticoagulant composed of 85 mM sodium citrate, 71 mM citric acid, and 111 mM glucose. Platelet-enriched plasma was produced by centrifugation of blood samples at 100 g for 15 min. Washed platelets, obtained by a 10-min centrifugation of plasma samples at 350 g, were suspended in a buffer solution of the following composition (mM): 138 NaCl, 3 KCl, 1 $MgCl_2$, 10 glucose, 10 HEPES, and 0.37 NaH_2PO_4 (pH 7.4). Platelets were present in solution in a concentration of 3×10^8 cells/ml.

Measurements were made at 37°C and a stirring rate of 800 rpm using an automated analyzer of platelet aggregation (Biola Co., Moscow) equipped with a computerized data-processing system. Platelet suspensions (0.15 ml each with 0.15 ml of added dialysis buffer or LDL solution) were placed in cuvettes of an aggregometer, after which 1 mM $CaCl_2$ and 0.4 mg/ml fibrinogen were added to each cuvette. In the absence of LDL, platelet aggregation was stimulated by adding 1 μM ADP to the cuvette. Microquantities of inhibitors were placed in empty cuvettes and, in the case of alcoholic solutions, evaporated at 37°C, followed by the addition of a platelet suspension and LDL solution to the cuvettes. LDL and cells were preincubated for 5-10 min.

The enzyme inhibitors indomethacin and acetylsalicylic acid were used for cyclooxygenase, nordihydroguaiaretic acid (NDGA) for lipoxygenase, and BW-755C for both these enzymes. The liberation of arachidonic acid from cellular phospholipids by phospholipase A_2 can be blocked by the nonspecific phospholipase inhibitor 4-bromophenacyl bromide.

The maximal rate of change in light transmission was determined as the mean for three samples. The standard error in measuring platelet aggregation rates did not exceed 10% in absolute terms. Each curve in Figs. 1-3 represents the results of only one assay (curves similar to each of those shown were obtained in at least 5 independent assays). LDL with oxidation degrees ranging from 0.5 to 12 μmol MDA/g protein were assayed. ADP-induced aggregation was used as an example of the influence exerted by inhibitors on the aggregation parameter in the case of an inducer for which the main pathway of cell activation is arachidonic acid metabolism [2].

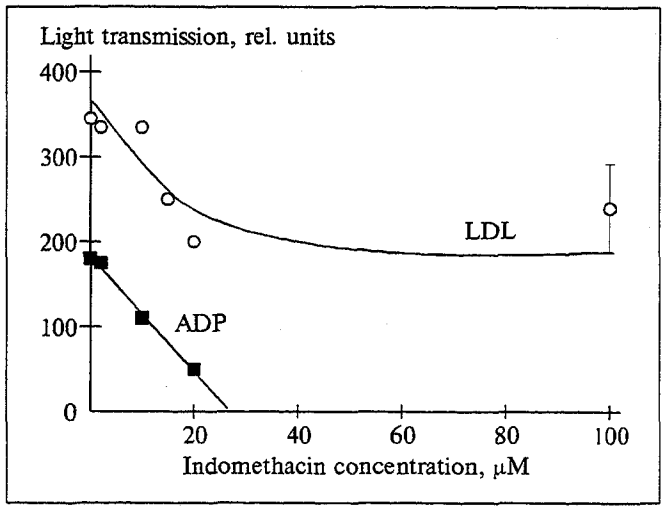

Fig. 1. Rate of change in light transmission by platelet suspension as a function of indomethacin concentration. The inducers used were 0.8 g/liter LDL, 5.6 μmol/g protein MDA, and 1 μM ADP.

RESULTS

In this experiment we recorded the total platelet response (aggregation) as the variation with time in the light transmission by a platelet suspension under the action of an inducer (LDL or ADP). The aggregation parameter used was the maximal rate of change in light transmission, defined as the maximal slope of the light transmission curve [1]. Light transmission rates were plotted against concentrations of the inhibitors of arachidonic cycle acid enzymes (Figs. 1-3).

Figure 1 presents plots of the aggregation parameter against the concentration of the cyclooxygenase inhibitor indomethacin. The inhibition of

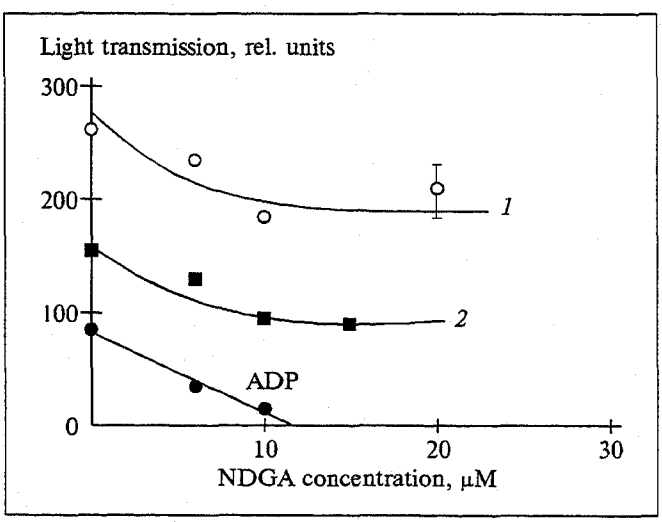

Fig. 2. Rate of change in light transmission by platelet suspension as a function of NDGA concentration when platelets were activated with 1 μM ADP or with LDL (0.7 g/liter). *1*) 0.8 μmol/liter MDA; *2*) 3.7 μmol/liter MDA.

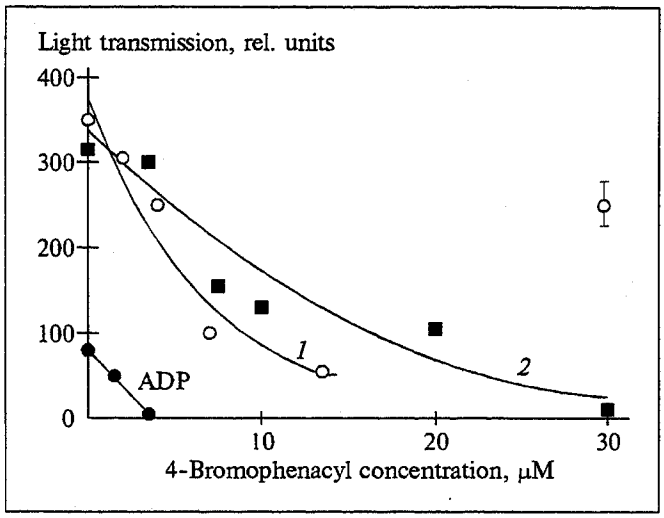

Fig. 3. Rate of change in light transmission by platelet suspension as a function of 4-bromophenacyl bromide concentration when platelets were activated by inducers: *1*) 0.6 g/liter LDL, 1 μmol/g MDA; *2*) 2.5 g/liter LDL, 2.3 μmol/g MDA, 1 μM ADP.

LDL-induced aggregation was maximal (25-30%) at indomethacin concentrations of 20-40 μM. For comparison, this figure also shows the results for ADP-induced platelet aggregation, which agree well with those from an earlier study [2]. Our results are also consistent with the finding by Ardlie *et al.* [4] that indomethacin (2 μM) inhibits LDL-induced platelet aggregation by 20%. In concentrations above 5 μM, it can also inhibit lipoxygenase, and for this reason the contribution of cyclooxygenase was also checked using another inhibitor, acetylsalicylic acid. The inhibition of LDL-induced aggregation reached the highest levels at acetylsalicylic acid concentrations of 120-150 μM. At these concentrations, the rate of change in light transmission decreased by 15-20%.

Figure 2 shows the rate of change in light transmission plotted against the concentration of the lipoxygenase inhibitor NDGA for platelets activated by native and oxidized LDL (these LDL were from a single donor) and by ADP. Changes in the aggregation parameter in the case of LDL-induced aggregation did not exceed 30%, and the inhibition of aggregation was maximal at inhibitor concentrations of 10-15 μM. A comparison of plots obtained for LDL-induced platelet aggregation *vs.* inhibitor concentration for LDL with different levels of oxidation products showed that the degree of inhibition does not depend on the extent to which lipids of the inducer have been modified by peroxidation. Strong inhibition at micromolar NDGA concentrations is typical for ADP [2]. Similar plots of aggregation parameter *vs.* inhibitor concentration were obtained for BW755C.

Figure 3 depicts the aggregation parameter as a function of the concentration of 4-bromophenacyl bromide, a nonspecific phospholipase inhibitor, for ADP- and LDL-induced platelet aggregation (the LDL were from two donors). The result for ADP is in line with that reported by Borin *et al.*: a virtually complete inhibition occurred at micromolar concentrations of the inhibitor. 4-Bromophenacyl bromide inhibited LDL-stimulated aggregation in a dose-dependent manner, the maximal inhibition amounting to 90% of the initial value at inhibitor concentrations of 10-20 μM. The differences between the curves of LDL-induced aggregation shown in Fig. 3 as representative examples are not determined by differences in the concentrations of the LDL or in the degree of their modification by peroxidation. Overall, our results suggest that these differences are due to individual cellular responses recorded for different donors.

The weak inhibition of LDL-induced platelet aggregation observed following inhibition of arachidonic acid cycle enzyme warrants the following three conclusions:

- arachidonic acid metabolism is not the principal pathway of cellular signal transmission in the case of platelet activation by LDL;
- the strong inhibition of LDL-induced aggregation by 4-bromophenacyl bromide suggests that phospholipase C, a key enzyme of the phosphoinositide cycle, is implicated in platelet activation by LDL;
- native and oxidized LDL activate platelets by the same mechanisms.

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