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The role of apoE in inhibitory effects of apoE-rich HDL on platelet function

Masaaki Higashihara[1], Makoto Kinoshita[1], Tamio Teramoto[1], Shoji Kume[2] and Kiyoshi Kurokawa[1]

[1]*First Department of Internal Medicine, Faculty of Medicine, University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo* and [2]*Central Clinical Laboratory, Yamanashi Medical College, Yamanashi, Japan*

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Apolipoprotein E- (ApoE-) rich high-density lipoprotein (HDL), which was prepared from the bound fraction of normolipemic volunteers on heparin-Sepharose and from a hyperalphalipoproteinemic patient, potently inhibited aggregation of human platelets in a dose-dependent fashion. Dimyristoyl phosphatidylcholine liposome with apoE (apoE·DMPC) also inhibited platelet aggregation, and incubation of washed platelets with apoE·DMPC resulted in the release of cholesterol into the supernatant in a time- and dose-dependent manner. These results suggest that apoE plays a major role in the inhibitory effect of apoE-rich HDL in platelet function, presumably due to the release of cholesterol from the plasma membrane.

High-density lipoprotein; Cholesterol; Platelet aggregation; Apolipoprotein E

1. INTRODUCTION

Platelets play an important role in the formation of thrombus and atherosclerotic lesions [1]. Platelets from the patients with hypercholesterolemia show an increase in aggregability, especially for epinephrine [2,3] and their plasma membranes contain a great deal of cholesterol, suggesting that the content of cholesterol in the plasma membrane has a critical role in determining the sensitivity to stimuli. A number of reports have indicated that low-density lipoprotein (LDL) enhances agonist-induced platelet aggregation, whereas high-density lipoprotein (HDL) has an opposite effect [4–7]. Recently, Desai et al. [8] showed that one of the critical factors of HDL in reducing the aggregability was the content of apoE, because HDL_2 showed more potent inhibition than HDL_3. However, the mechanism of potent inhibition by apoE-rich HDL remains unknown.

In this report, we focus on the role of apoE in the inhibitory effects of apoE-rich HDL on platelet function and investigate the interaction of dimyristoyl phosphatidylcholine liposome with apoE (apoE·DMPC) with platelets, especially in terms of cholesterol exchange in the plasma membrane.

2. MATERIALS AND METHODS

2.1. *Materials and proteins*

Dimyristoyl phosphatidylcholine (DMPC) and luciferine-luciferase were purchased from Sigma (St. Louis, MO). α-thrombin was purchased from Mochida Pharmaceutical (Tokyo, Japan). $Na^{125}I$ was obtained from New England Nuclear (Boston, MA). Recombinant apoE was a gift from Mitsubishi Kasei (Kanagawa, Japan). Apolipoprotein A-I (ApoA-I) was purified from normal plasma according to the method of Schonfeld et al. [9]. 1,3,4,6-tetrachloro-3α-6α-diphenylglycouril (IODO-GEN) was purchased from Pierce (Rockford, IL). Heparin-Sepharose was made according to the method of Iverius et al. [10]. ConA-Sepharose was purchased from Pharmacia, Uppsala, Sweden.

2.2. *Isolation of plasma lipoproteins*

Human HDL ($d = 1.063$–1.21 g/ml) was isolated from plasma obtained from normolipemic fasted volunteers by ultracentrifugation in a 50 Ti rotor (Hitachi) [7]. ApoE-rich and apoE-poor HDL fractions of normal plasma were prepared by heparin-Sepharose column chromatography as described by Weisgraber et al. [11]. The ApoE/ApoA-I ratio in apoE-rich HDL fraction was calculated to be about 0.65.

The ApoE-rich HDL fraction was also isolated from plasma of a hyperalphalipoproteinemic patient with cholesteryl ester transfer protein deficiency. A fraction of $d < 1.063$ g/ml of the patient's plasma was applied to the ConA-Sepharose column to remove apoB as described previously [12], and flow-through fractions (apoE-rich HDL) were collected. The ApoE/ApoA-I ratio of this fraction was about 0.9.

The ApoE·DMPC complex or apoA-I·DMPC complex was prepared by incubating DMPC with recombinant apoE or purified apoA-I overnight at room temperature with a lipoprotein/DMPC ratio of 1:3.75 (weight/weight) according to the method of Innerarity et al. [13]. Free apolipoprotein was removed by ultracentrifugation.

The electron micrography of negatively stained apoE·DMPC was carried out according to the method of Basu et al. [14].

2.3. *Iodination and binding experiments*

LDL was iodinated by using IODO-GEN [15]. The specific activities were 200–400 cpm/ng protein. [^{125}I]LDL binding was carried out at 37°C as described previously [7].

2.4. *Platelet function*

Washed platelets were prepared as described previously [16].

Correspondence address: M. Higashira, First Department of Internal Medicine, Faculty of Medicine, University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113, Japan

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Platelet aggregation and release experiments were performed in a Chronolog Lumiaggregometer (Coulter Electronics, Luton, UK) using luciferine-luciferase bioluminescence reaction [16]. $[Ca^{2+}]_i$ was measured using fura-2 as previously described [17].

2.5. *Others*

Protein concentrations of lipoproteins were measured according to the method of Lowry [18] with minor modifications. The concentrations of cholesterol and DMPC were measured by enzymatic methods using a TC-555 Kit (Kyowa Medics, Tokyo) and a PL- Kit (Nihon Shoji, Osaka), respectively. The total cholesterol content of the platelet plasma membrane was measured with chloroform-methanol extracted supernatant [19]. Released lactic dehydrogenase (LDH) activity in the supernatant was measured as described previously [16]. Other experimental conditions are given in the figure legends.

3. RESULTS AND DISCUSSION

We reported previously [7] that HDL_2 inhibited collagen- and thrombin-induced platelet aggregation more potently than HDL_3. These results confirm the earlier results of Desai et al. [8], who suggested that the more potent inhibition of ADP-induced aggregation by HDL_2 than that by HDL_3 was derived from the difference in apoE content.

ApoE-rich HDL (apoE/apoA-I = 0.65) from normal donors prepared by heparin-Sepharose column chromatography (Fig. 1A), inhibited platelet aggregation and ATP release induced by ADP (Fig. 1B), collagen (Fig. 1C), or thrombin (Fig. 1D) in a dose-dependent fashion. Submaximal inhibitory effects were observed with 30-min preincubation. ApoE-poor HDL fractions (flow-through fractions) had little inhibitory effect, even at concentrations of 0.5 mg protein/ml (data not shown).

We examined the effects of apoE-rich HDL fraction (apoE/apoA-I = 0.9) from a hyperalphalipoproteinemic patient with plasma cholesteryl ester transfer protein deficiency on platelet aggregation. This fraction also inhibited collagen- and thrombin-induced platelet aggregation in a dose-dependent fashion (Fig. 2). HDL from normal donors at concentrations up to 0.2 mg protein/ml showed little inhibition (data not shown). These results suggest that apoE may have a critical role in the potent inhibition of platelet function by HDL_2, as suggested by Desai et al. [8].

In order to study the inhibitory effect of apoE on apoE-rich HDL which also contains other apolipoproteins, i.e. apoA-I, we prepared liposomes consisting of only apoE and DMPC, which had disc forms like nascent HDL (Fig. 3A). ApoE·DMPC inhibited collagen-

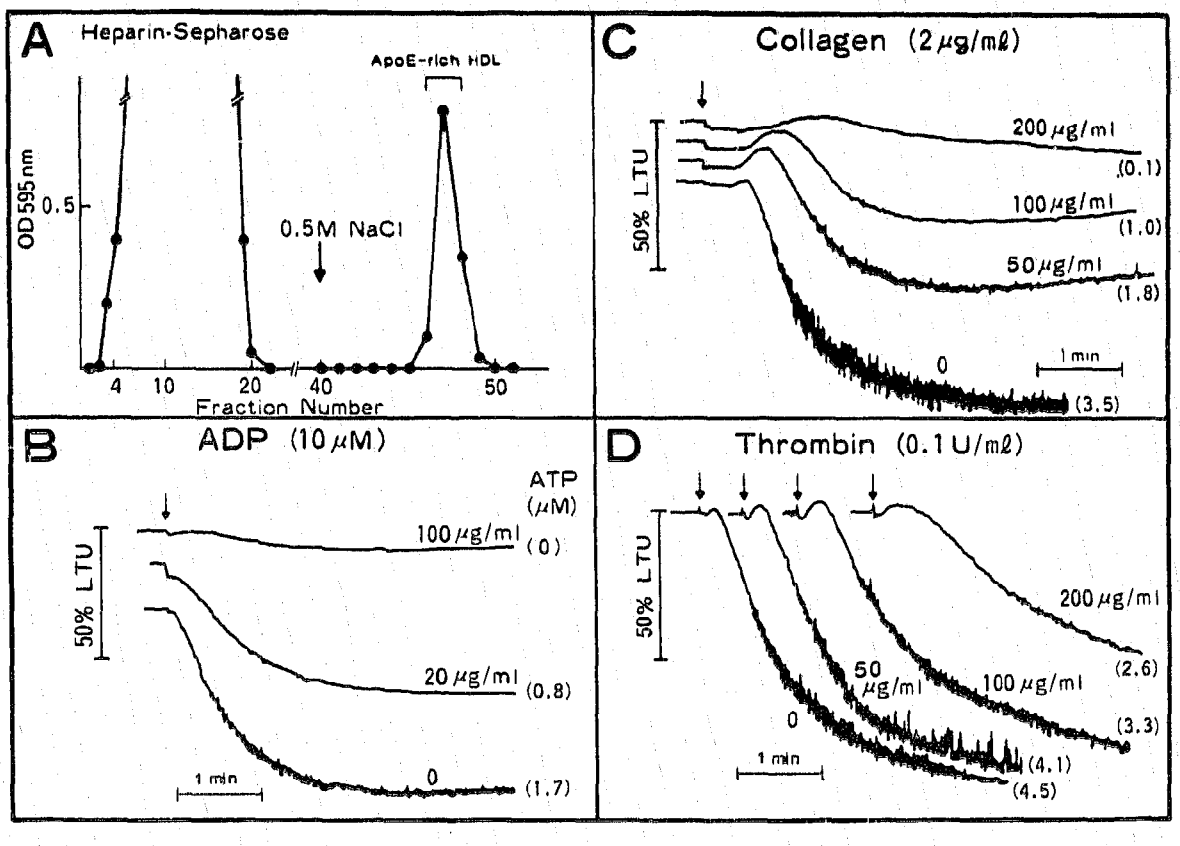

Fig. 1. Inhibition of platelet aggregation and ATP release by apoE-rich HDL. (A) Elution profile of heparin-Sepharose column. The concentration of protein in each fraction was measured by the method of Bradford [30]. (B,C,D) Inhibition of ADP- (B), collagen- (C), or thrombin- (D) induced platelet aggregation and ATP release by apoE-rich HDL. The values in parentheses are those of ATP released (μM). Agonists were added to washed platelets ($30 \times 10^4/\mu l$) preincubated with several protein concentrations of apoE-rich HDL (shown in figures) for 30 min at 37°C. LTU, light transmission unit.

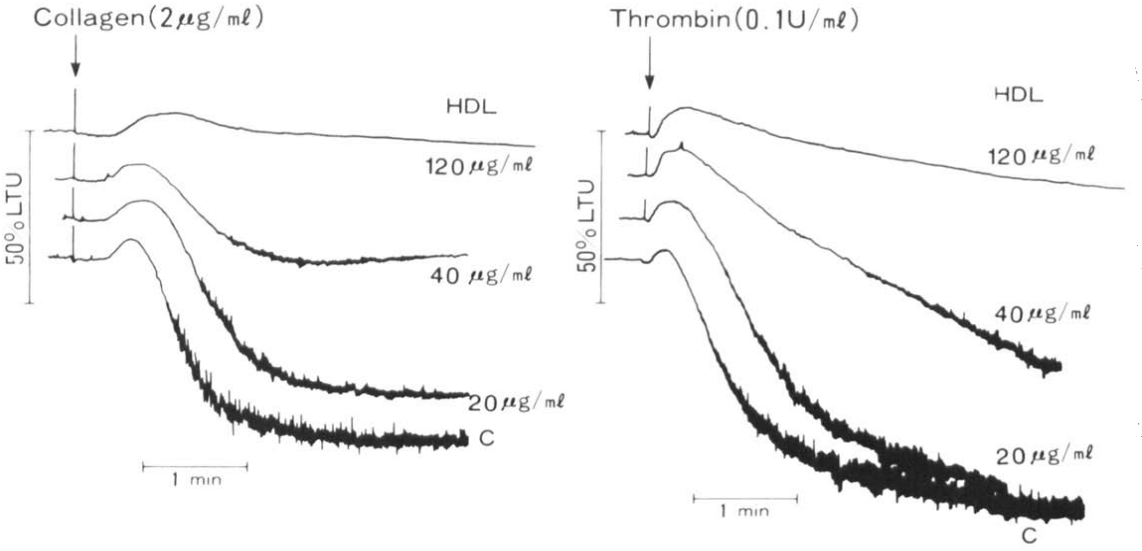

Fig. 2. Inhibition of collagen- (A) or thrombin- (B) induced platelet aggregation by the HDL fraction from a hyperalphalipoproteinemic patient. Agonists were added to washed platelets ($30 \times 10^4/\mu l$) preincubated with several protein concentrations of HDL for 10 min at 37°C. LTU, light transmission unit.

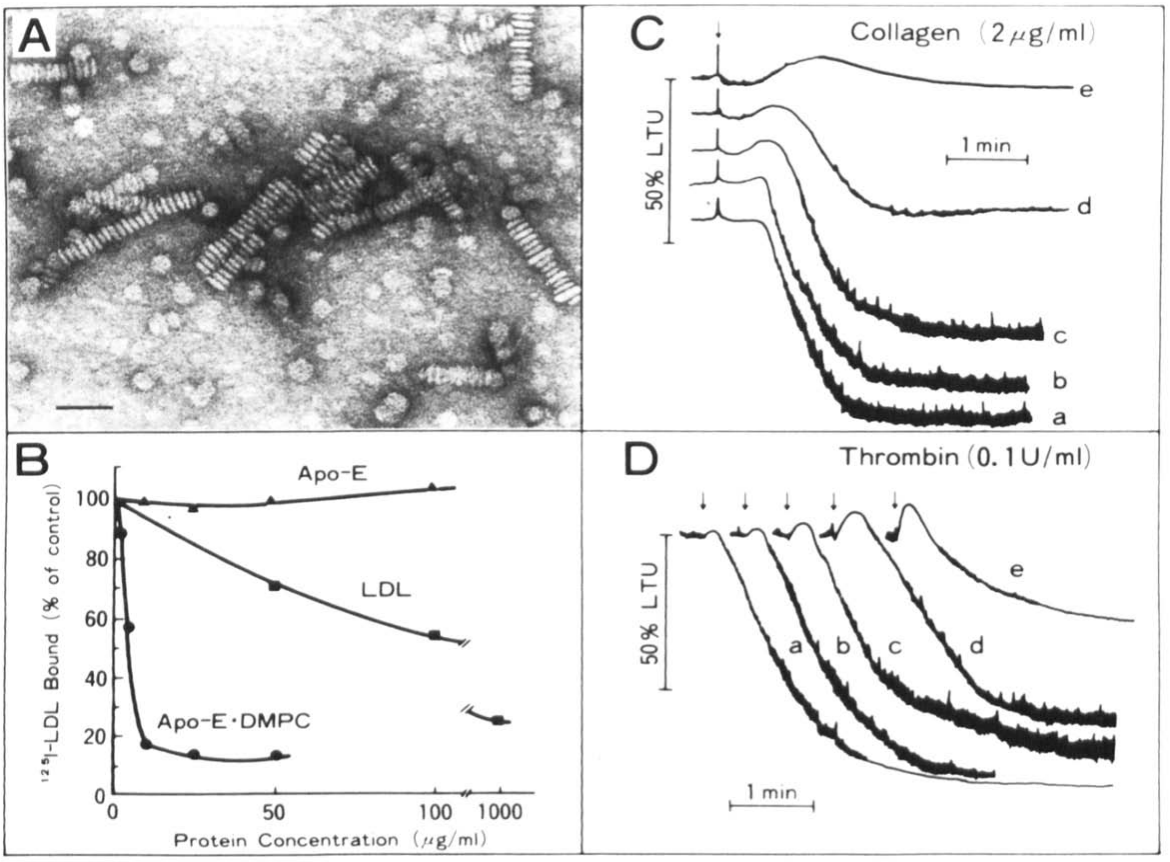

Fig. 3. Inhibition of [^{125}I]LDL binding to platelets and platelet aggregation by apoE·DMPC. (A) Electron micrography of negatively stained apoE·DMPC. (B) Inhibition of [^{125}I]LDL binding by cold LDL (■), apoE·DMPC (●) or apoE alone (▲). (C,D) Inhibition of collagen- (C), or thrombin- (D) induced platelet aggregation by several protein concentrations of HDL. (a) control; (b) apoA-I·DMPC, 100 μg/ml; (c,d,e) apoE·DMPC, 25 μg/ml, 100 μg/ml and 200 μg/ml, respectively. Experimental conditions were the same as in Fig. 1. LTU, light transmission unit.

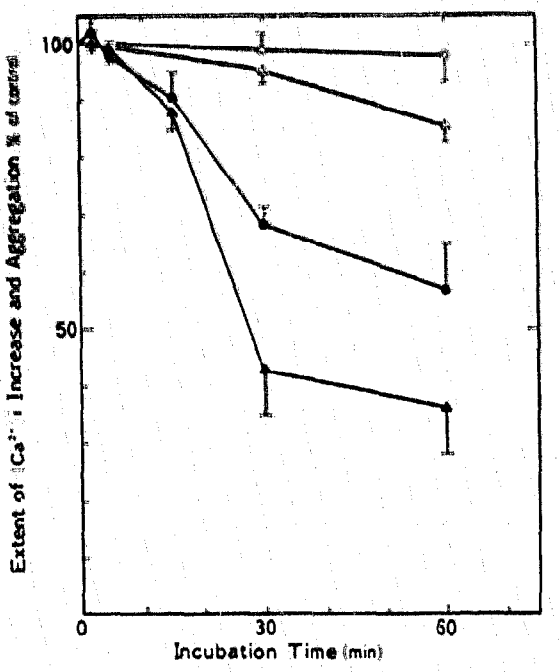

Fig. 4. Inhibition of thrombin-induced platelet aggregation and the increase in $[Ca^{2+}]_i$ by apoE·DMPC. Washed platelets ($30 \times 10^4/\mu l$) were preincubated at 37°C in the presence or absence of apoE·DMPC (100 μg/ml) for various incubation periods, and then thrombin (0.1 U/ml) was added. The extent of $[Ca^{2+}]_i$ increase (○,●) and maximal slope (aggregation/min) (△, ▲) were calculated as percent of control. The values are means ± S.D. of 3 experiments. apoA-I·DMPC (○, △); apoE·DMPC (●, ▲).

and thrombin-induced platelet aggregation in a dose-dependent fashion (Fig. 3C,D). In contrast, few inhibitory effects were seen with apoA-I·DMPC up to 0.1 mg/ml. ApoE·DMPC potently inhibited [^{125}I]LDL binding to platelets (Fig. 3B). Scatchard analysis revealed that the affinity was about 18 times that of native LDL (unpublished observation). Neither DMPC (data not shown) nor apoE alone (Fig. 3B) showed any inhibition, suggesting that apoE acquires the facility of inhibition by forming an active complex with DMPC. Thus, platelet-type LDL receptor [7,20] may share the capacity of binding to both apoB and apoE, like the classical LDL receptor of fibroblasts [21].

Why does apoE·DMPC have inhibitory properties? To obtain insight into the mechanism of this inhibition, the effects of apoE·DMPC on $[Ca^{2+}]_i$ increase and aggregation by thrombin (0.1 U/ml) were examined for various incubation times. Both the increase in $[Ca^{2+}]_i$ and the aggregation were inhibited by apoE·DMPC (100 μg/ml) in a time-dependent fashion (Fig. 4). No inhibition was seen with a shorter incubation time, i.e. 5 min (Fig. 4). Thus apoE·DMPC seems to inhibit the thrombin-induced receptor-mediated signal transduction, by affecting the plasma membrane in a time-dependent fashion.

We measured the amount of cholesterol in the supernatant during incubation of washed platelets with apoE·DMPC. ApoE·DMPC released the cholesterol in the supernatant in a dose- and time-dependent fashion

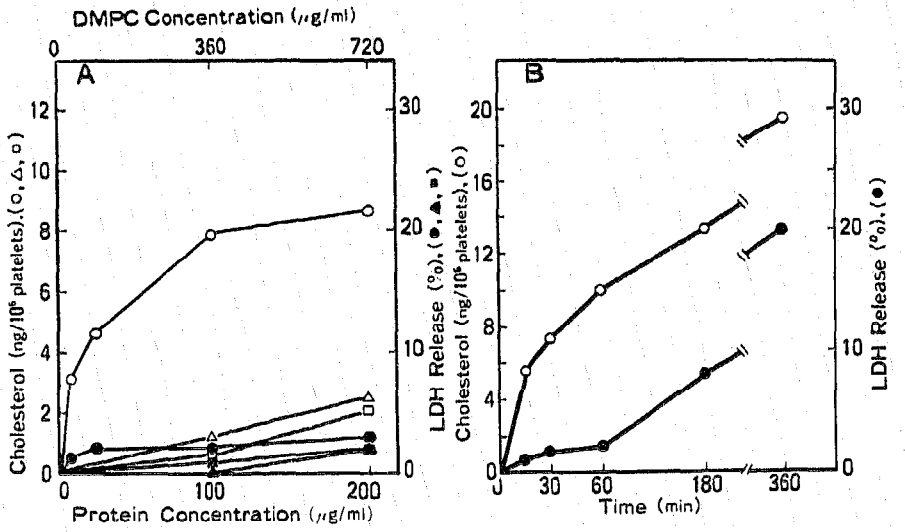

Fig. 5. The release of cholesterol from platelet membrane by apoE·DMPC. A. Cholesterol or LDH release as a function of lipoproteins or DMPC alone. Washed platelets ($30 \times 10^4/\mu l$) were preincubated with apoE·DMPC, ApoA-I·DMPC or DMPC alone for 30 min at 37°C and then put on ice. The supernatant after centrifugation at 3000 rpm for 10 min was determined for cholesterol content or LDH release. Cholesterol release (○, △, □); LDH release (●, ▲, ■); apoE·DMPC (○, ●); apoA-I·DMPC (△, ▲); DMPC alone (□, ■). The concentration of DMPC vesicles, 360 μg/ml, was the same concentration of DMPC that was present in the 100 μg/ml apoE·DMPC. B. Cholesterol or LDH release by apoE·DMPC (100 μg/ml) as a function of incubation time. Experimental conditions are the same as in A.

(Fig. 5A,B). ApoE·DMPC (100 μg/ml) released 10±3% of cholesterol for 30 min of incubation (mean±SD for 3 separate experiments). Little effect was seen with either DMPC alone or apoA-I·DMPC. LDH release was less than 3% in all samples (Fig. 5A). The time dependency of the extent of cholesterol released by apoE·DMPC was parallel with that of the inhibition of the $[Ca^{2+}]_i$ increase and the aggregation induced by thrombin (Figs 4 and 5B). A longer incubation with apoE·DMPC (100 μg/ml), for 6 h, caused the apparent release of LDH (Fig. 5B), suggesting that removal of cholesterol from the plasma membrane leads to fragility of platelet membrane.

In 1975 Shattil et al. [22] showed that platelets incubated with cholesterol-rich liposomes [high cholesterol to phospholipid ratio (C/PL)] had an increased sensitivity to epinephrine and ADP and that their platelets contain higher amounts of cholesterol. Platelets exposed to cholesterol-rich liposomes were found to have increased membrane microviscosity [23] and increased basal levels of adenylate cyclase [24]. These observations in vitro may have a counterpart in human disease since platelets from individuals with type IIa hyperlipoproteinemia have an increase in membrane C/PL [25], and they are more sensitive than normal to aggregating agents [2,3]. More recent studies have indicated that platelets with decreased C/PL showed a decrease in the liberation of arachidonate [26,27], the binding capacities of thrombin [28] and the levels of thromboxane B_2 formation [27,29]. Thus, the cholesterol content of the plasma membrane has been thought to have a crucial role in determining the sensitivities to agonists.

This is the first report showing that DMPC liposomes with apoE release cholesterol from the platelet membrane. We here propose a hypothetical model: that apoE-rich HDL releases cholesterol from the plasma membrane of platelets, resulting in a decreased response to stimuli. However, since apoE·DMPC is a simple model for apoE-rich HDL and lacks other apolipoproteins, i.e. apoA-I as well as lipids like cholesterol and triglyceride, we cannot exclude the possibility that other inhibitory factors may lead to perturbation of the lipid–protein interaction in platelet membranes with different mechanisms, resulting in a decreased platelet function.

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