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HDL_3 ACTIVATES PHOSPHOLIPASE D IN NORMAL BUT NOT IN GLYCOPROTEIN IIb/IIIa-DEFICIENT PLATELETS

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SUMMARY: Glycoprotein IIb/IIIa has been proposed as the platelet receptor for high density lipoproteins (HDL_3). We characterized the HDL_3-induced second messenger response in normal and glycoprotein IIb/IIIa-deficient platelets. In normal platelets physiological concentrations of HDL_3 induced the time-dependent generation of phosphatidic acid in the absence of phosphoinositide turnover. The rise in phosphatidic acid preceded that of diacyglycerol which was inconsistent with phospholipase C/diacylglycerol kinase pathway being the source of phosphatidic acid and suggested the involvement of phospholipase D. In the presence of butanol, HDL_3 stimulated the accumulation of phosphatidylbutanol, an unequivocal indicator of phospholipase D activity. No increase in phosphatidic acid, diacylglycerol, and phosphatidylbutanol was observed upon addition of HDL_3 to glycoprotein IIb/IIIa-deficient platelets. We conclude that phosphatidic acid is generated in HDL_3-stimulated platelets by phospholipase D and that glycoprotein IIb/IIIa is the receptor involved in this process.

Numerous epidemiological studies have provided evidence for a negative correlation between cardiovascular disease and plasma levels of HDL cholesterol [1]. It has been proposed that HDL is antiatherogenic by virtue of its potential to promote the efflux of cholesterol from the artery wall. However, an increasing body of evidence suggests that HDL may also be protective through an inhibitory effect on platelet function [2,3]. A low incidence of coronary heart disease and a significantly longer bleeding time has been observed in a population with a high concentra-

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Abbreviations: HDL, high density lipoproteins; DAG, diacylglycerol; PA, phosphatidic acid; PBut, phosphatidylbutanol; PIP_2, phosphatidylinositol 4,5-bisphosphate; PIP, phosphatidylinositol 4-monophosphate; PC, phosphatidylcholine; PLC, phospholipase C; PLD, phospholipase D; Gp IIb/IIIa, glycoprotein IIb/IIIa.

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tion of HDL [4]. Hypersensitivity of platelets, by contrast, was reported in probands with hypoalphalipoproteinemia [5]. Moreover, *in vitro* studies demonstrated an inhibitory effect of HDL on the thrombin- and collagen-induced aggregation and dense granule secretion [6,7].

The inhibitory effect of HDL on platelet aggregation may be mediated by reversible binding of HDL to high-affinity binding sites and the subsequent induction of specific cellular responses [8,9,10]. Evidence for the formation of phosphatidylcholine (PC)-derived diacylglycerol (DAG) upon stimulation of platelets has previously been presented [11,12]. Recently, Glycoprotein IIb/IIIa (Gp IIb/IIIa) has been proposed as the platelet receptor for high density lipoproteins (HDL_3) [13]. However, it is not clear at present whether binding of HDL_3 to Gp IIb/IIIa is related to any physiological response. To test this possibility, we characterized the HDL_3-induced second messenger response in normal and Gp IIb/IIIa-deficient platelets.

MATERIALS AND METHODS

Blood donors. For experiments with normal platelets blood was obtained from healthy human volunteers. All blood donors denied having taken any medication for at least two weeks before sampling. Gp IIb/IIIa-deficient platelets were obtained from patient A.M. with type I Glanzmann thrombasthenia. The history revealed life-long easy bruising and serious bleeding episodes which made several platelet transfusions necessary. The patient had a normal platelet count and normal platelet morphology. Bleeding times were markedly prolonged. The patient's platelets did not respond to ADP, collagen, thrombin, adrenalin, and arachidonic acid but displayed normal ristocetin-induced agglutination. No clot reaction was observed with the patient's blood.

HDL_3 isolation and characterization. HDL_3 was isolated from human serum by the standard procedure of differential flotation [14] except that an additional washing step at a density of 1.125 g/cm^3 was included. The density range for HDL_3 was 1.125-1.210 g/cm^3. The protein content of HDL_3 was determined according to Lowry. The apolipoprotein E content was determined by standard ELISA and did not exceed 0.3% of total protein.

Platelet isolation and labeling. Following centrifugation at 150g for 15 min platelet rich plasma was mixed 2:9 with citrate solution containing 2.2 g/dl trinatrium-citrate, 0.8 g/dl citric acid and 2.4 g/dl D-glucose. Prostaglandin I_2 and apyrase were added to the final concentration of 500 ng/ml and 0.3 U/ml, respectively and platelet rich plasma was centrifuged for 20 min at 900g. The platelet pellet was resuspended in a buffer containing 137 mM NaCl, 2.7 mM KCl, 0.32 mM NaH_2PO_4, 5.6 mM glucose, 11.9 mM $NaHCO_3$, 10 mM HEPES, 1 mM $CaCl_2$ and 0.35% BSA. The platelets were then adjusted to the concentration of 10^9 cells/ml and incubated for 60 min at 30°C with the desired amount of radioactivity: 0.1 mCi/ml of orthophosphoric acid, 2.5 µCi/ml of [^{14}C]palmitic acid, or 2.5 µCi/ml [^{14}C]PC. After incubation platelets were washed, adjusted to the final concentration of $5x10^8$/ml and used for further experiments.

Stimulation with HDL_3 and lipid analysis. Labeled platelets ($5x10^8$) were stimulated with 1.0 mg/ml of HDL_3 unless otherwise indicated. After the desired time the 0.5 ml aliquots were withdrawn and added to 1.5 ml of ice-cold $CHCl_3/CH_3OH$ (1:2). Lipids were extracted according to the procedure described by Bligh and Dyer [15], dried under nitrogen, and resuspended in 0,2 ml of hexane. Double one-dimensional thin layer chromatography was performed according to Gruchalla et al. [16]. The radioactive spots corresponding to PA, DAG and PBut were identified by comigration with cold standards, cut out, and the radioactivity was measured by liquid scintillation counting.

Flow cytometry. Flow cytometry was performed as described previously [17]. Briefly, fixed platelets were washed twice in sodium citrate (3.8%, v/v) phosphate buffered saline (PBS) and sodium citrate-PBS containing 10% (v/v)of rabbit serum. Washed platelets were diluted with PBS to 50,000 platelets/µl and 200µl aliquots were stained with mouse monoclonal anti-Gp IIb/IIIa antibodies P2 (Immunotech, Marseille, France) or with mouse monoclonal anti-Gp IIIa antibodies RUU-PL7F12 (Beckton-Dickinson, Heidelberg, FRG). For fluorescence labeling, 100 µl of fluorescein isothiocyanate-labeled sheep anti-mouse $F(ab)_2$ were added for further 30 min incubation in sodium citrate-PBS and resuspended in Isoton-II solution. Immediately after staining the fluorescence levels of 10,000 platelets were measured with a FACS-Scan instrument (Beckton-Dickinson, Heidelberg, FRG).

RESULTS

HDL_3 stimulates the formation of DAG and PA in the absence of phosphoinositide turnover.

The effect of HDL_3 on phosphoinositide turnover and PA formation was assessed in [^{32}P]phosphoric acid labeled platelets. Fig. 1a shows the time-course of the radioactivity associated with phosphoinositides and PA. In three independent experiments, the addition of HDL_3 induced no significant change in the radioactivity associated with phospatidylinositol 4,5-bisphosphate (PIP_2) or phosphatidylinositol 4-monophosphate (PIP). PA, by contrast, increased up to 140-170% of basal levels with a maximum at 30 s, followed by a decrease at 60 s. These

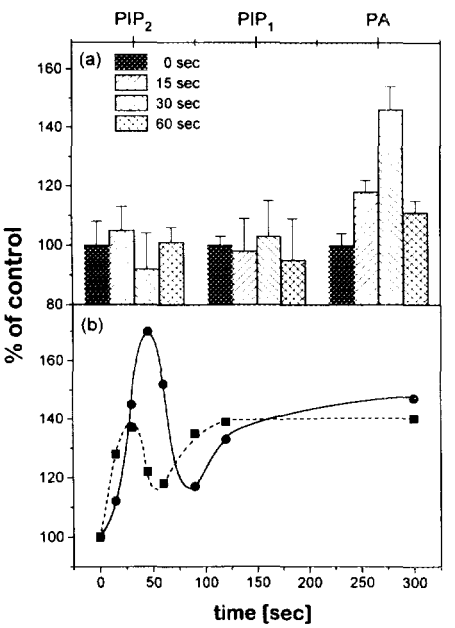

Figure 1. Effect of HDL_3 on [^{32}P]PA, [^{32}P]PIP, [^{32}P]PIP_2, [^{14}C]PA and [^{14}C]DAG levels in human platelets. (a) Washed platelets prelabeled with [^{32}P]ortho-phosphoric acid (0.1 mCi/10^9 cells) were stimulated with HDL_3 for the indicated times. Reactions were stopped by addition of acidified chloroform/methanol. Lipid extracts were analysed for [^{32}P]PA, [^{32}P]PIP_2, and [^{32}P]PIP by thin layer chromatography. Phospholipid radioactivity in unstimulated samples did not change over the time-course of the experiment. Data are means from three or four determinations. (b) Washed platelets prelabeled with [^{14}C]palmitic acid (2.5 µCi/10^9 cells) were stimulated with HDL_3 (1 mg/ml). After the indicated time aliquots of the cell suspension were withdrawn, extracted and analyzed for [^{14}C]PA (■) and [^{14}C]DAG (●). Data are representative of seven experiments.

data suggested that HDL_3 stimulates the formation of PA from sources other than phosphoinositides.

To further characterize the HDL_3 induced formation of PA and DAG the platelets were labeled with [^{14}C]palmitic acid. With this treatment approximately 70% of the total radioactivity was incorporated into PC. Stimulation of [^{14}C]palmitic acid-labeled platelets with HDL_3 resulted in a biphasic accumulation of [^{14}C]PA (Fig. 1b). The early phase peaked rapidly at 20-30 s (to 135-150% of basal levels). The second phase of HDL_3-stimulated PA generation reached a maximum at 90-120s (to 135-145% of basal levels) and remained above control values for at least 5 min. The HDL_3-induced PA production preceded DAG production. The [^{14}C]DAG maximum was reached after 45-55 s rising up to 160-170% of control values. A second increase in [^{14}C]DAG reached its maximum at 120-300 s. DAG remained above control values for at least 5 min.

In order to confirm PC as a source for DAG and PA, the same experiments were performed in [^{14}C]PC labeled platelets. With this treatment virtually all of the radioactivity was found in the [^{14}C]PC fraction. No essential differences in the kinetics of PA and DAG formation were observed between platelets labeled with [^{14}C]palmitic acid and those labeled with [^{14}C]PC (data not shown).

HDL_3-induced PA formation occurs by means of PC-specific PLD.

The observation that the HDL_3-induced increase of PA preceded the increase of DAG in [^{14}C]palmitic acid- or [^{14}C]PC-labeled platelets strongly suggested that the generation of PA primarily occurred by PLD and not via a coupled PLC/DAG kinase pathway. To confirm activation of PLD, [^{14}C]palmitic acid-labeled platelets were stimulated with HDL_3 in the presence of 0.4% butanol. In these experiments we exploited the ability of PLD to catalyse a transphosphatidylation reaction in the presence of butanol resulting in the formation of PBut instead of PA. Since the production of phosphatidylalcohols is mediated exclusively by PLD, synthesis of PBut is an unequivocal marker of PLD activation [18]. As shown in Fig. 2, the HDL_3-induced formation of [^{14}C]PA was decreased by 30-45% in the presence of butanol. The reduction of [^{14}C]PA formation was associated with an accumulation of [^{14}C]PBut reaching a maximum 120 s after stimulation. These data clearly demonstrated the HDL_3-induced activation of PC-specific PLD.

The HDL_3-stimulated activation of PLD is abolished in glycoprotein IIb-IIIa-deficient platelets.

Since Gp IIb/IIIa was previously shown to bind HDL_3 on human platelets [13], we examined the possibility that this complex is involved in PA and DAG formation brought about by HDL_3. The lack of expression of Gp IIb/IIIa on platelets obtained from donor A.M. was demonstrated by flow cytometry. As shown in Fig. 3, the patient's platelets failed to bind monoclonal antibody RUU-PL7F12 against Gp IIIa and monoclonal antibody P2 against Gp IIb/IIIa complex. The Gp

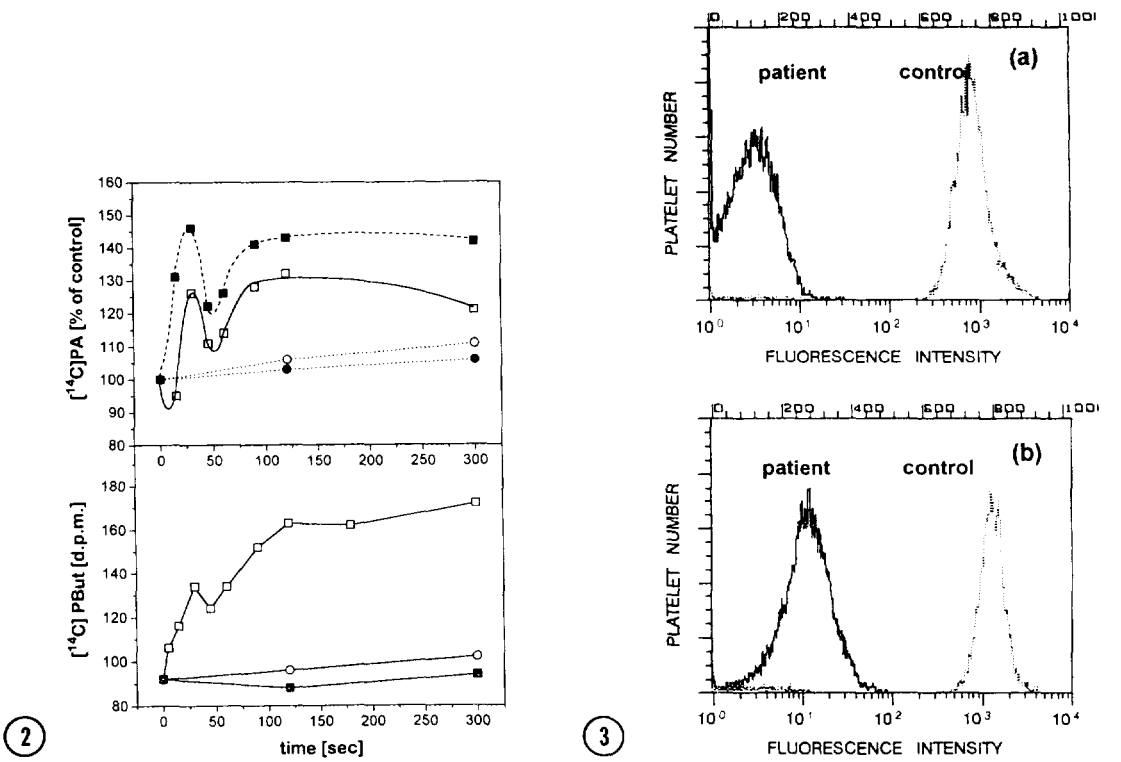

Figure 2. Effect of HDL_3 on [^{14}C]PA and [^{14}C]PBut formation in human platelets in the presence of 0.4% butanol. Washed platelets were prelabeled with [^{14}C]palmitic acid (2.5 μCi/10^9 cells) and incubated for 5 min with butanol (0.4% v/v) or without butanol (control). Platelets were stimulated with HDL_3 (1 mg/ml) and aliquots were withdrawn after the indicated time, extracted and analysed for [^{14}C]PA or [^{14}C]PBut. Upper panel: [^{14}C]PA formation, HDL_3 alone (■); HDL_3 + butanol (□); control (no addition) (●); butanol alone (○). Lower panel: [^{14}C]PBut accumulation, HDL_3 alone (■); HDL_3+butanol (□); butanol alone (○). Data are representative of three separate experiments.

Figure 3. Fluorescence pattern obtained with the monoclonal antibodies against Gp IIIa (a) and against GpIIb/IIIa (b) of control platelets and platelets from patient A.M.

IIb/IIIa deficiency was further confirmed by Western blotting using polyclonal antibodies against Gp IIb/IIIa complex (gift from E. Plow, La Jolla, CA) and the monoclonal antibody Y2/51 (Dakopatts, Glostrup) against Gp IIIa (data not shown). These data clearly demonstrated that the platelets from donor A.M. are deficient in Gp IIb/IIIa. As shown in Fig. 4, addition of HDL_3 to these platelets caused no increase in the radioactivity associated with PA or DAG. We also did not observe PBut formation, when GpIIb/IIIa-deficient platelets were stimulated with HDL_3 in the presence of 0.4% butanol (not shown).

DISCUSSION

Previously, Nazih et al. reported on the PC-PLC-mediated DAG generation in platelets exposed to HDL_3 [11,12]. However, these authors did not examine other phospholipids as a source for

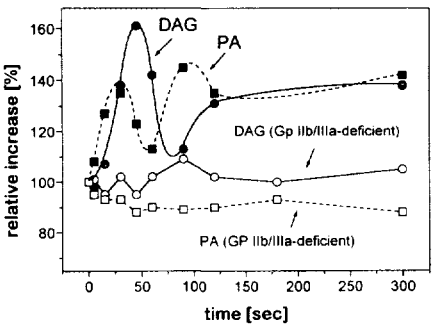

Figure 4. Effect of HDL_3 on [^{14}C]PA and [^{14}C]DAG formation in normal and Gp IIb/IIIa-deficient platelets. Washed platelets were prelabeled with [^{14}C]palmitic acid (2.5 μCi/10^9 cells). After addition of HDL_3 (1 mg/ml) aliquots were withdrawn and analysed for [^{14}C]PA and [^{14}C]DAG. PA formation in control platelets (■); PA formation in Gp IIb/IIIa-deficient platelets (□); DAG formation in control platelets (●); DAG formation in Gp IIb/IIIa-deficient platelets (○). The data are representative of three separate experiments.

DAG and PA. In addition, it was not established whether the HDL_3-induced PC hydrolysis is mediated by PLC or PLD. The activation of PLD in human platelets upon stimulation with various agonists has been demonstrated by several groups [19,20], whereas PC-specific PLC appears to provide a negligible contribution to the second-messenger response with most agonists [21]. We demonstrate here that HDL_3 induces the rapid increase in PA by PC-specific PLD. The HDL_3-induced PC hydrolysis was not accompanied by phosphoinositide breakdown.

Glycoproteins IIb and IIIa are receptors for fibrinogen and play a crucial role in platelet aggregation. Recently, Koller et al. identified Gp IIb and GpIIIa as HDL_3-binding proteins in platelet membranes [13]. However, it was not clear whether binding of HDL_3 to GpIIb/IIIa is related to cell signalling. Our results demonstrate that the generation of PA and DAG is abolished in platelets from a patient with type I Glanzmann thrombasthenia, a condition in which expression of Gp IIb/IIIa does not exceed 1% of the normal level. These findings strongly support the contention that binding of HDL_3 to Gp IIb/IIIa is linked to the activation of PLD in human platelets.

Previously, several groups demonstrated that binding of ligands to Gp IIb/IIIa not only mediates platelet adhesion but is also associated with the activation of cellular processes involved in platelet activation [22]. These include tyrosine phosphorylation of intracellular proteins, synthesis of 3-phosphorylated phosphatidylinositols, liberation of arachidonic acid, and the regulation of platelet membrane calcium flux. For the first time, the present study provides evidence that activation of phospholipases may also be involved in cell signalling through Gp IIb/IIIa.

The relevance of the present results to the pathogenesis of cardiovascular disease remains unclear. The anti-atherogenic role of HDL is generally explained by its ability to transport cholesterol from the peripheral cells to the liver [1]. However, the inhibitory effects of HDL on platelet

function may also contribute to its anti-atherogenic actvity [3]. Recently, we demonstrated that HDL_3 inhibits platelet activation by activation of protein kinase C [23]. It is possible that the anti-thrombotic effect of HDL is related to the Gp IIb/IIIa-dependent PLD activation and PA/DAG formation described in this paper.

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