Apolipoprotein E Inhibits Platelet Aggregation through the L-Arginine: Nitric Oxide Pathway

IMPLICATIONS FOR VASCULAR DISEASE

(Received for publication, July 23, 1996, and in revised form, October 12, 1996)

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We have previously reported that plasma apolipoprotein (apo) E-containing high density lipoprotein particles have a potent anti-platelet action, apparently by occupying saturable binding sites in the cell surface. Here we show that purified apoE (10–50 µg/ml), complexed with phospholipid vesicles (dimyristoylphosphatidylcholine, DMPC), suppresses platelet aggregation induced by ADP, epinephrine, or collagen. This effect was not due to sequestration of cholesterol from platelet membranes; apoE:DMPC chemically modified with cyclohexanedione (cyclohexanedione-apoE:DMPC) did not inhibit aggregation but nevertheless removed similar amounts of cholesterol as untreated complexes, about 2% during the aggregation period. Rather we found that apoE influenced intracellular platelet signaling. Thus, apoE:DMPC markedly increased cGMP in ADP-stimulated platelets which correlated with the resulting inhibition of aggregation (\( r = 0.85; p < 0.01, n = 10 \)), whereas cyclohexanedione-apoE:DMPC vesicles had no effect. One important cellular mechanism for up-regulation of cGMP is through stimulation of nitric oxide (NO) synthase, the NO generated by conversion of L-arginine to L-citrulline, binds to and activates guanylate cyclase. This signal transduction pathway was implicated by the finding that NO synthase inhibitors of distinct structural and functional types all reversed the anti-platelet action of apoE, whereas a selective inhibitor of soluble guanylate cyclase, 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (100 nm), had a similar reversing action. Direct confirmation that apoE stimulates NO synthase was obtained by use of \( \text{L-}[^{3} \text{H}] \text{arginine; platelets pretreated with apoE:DMPC produced markedly more L-}[^{3} \text{H}] \text{citrulline (0.71 ± 0.1 pmol/l/10}^9 \text{ platelets} \) than controls (0.18 ± 0.03; \( p < 0.05 \)). In addition, hemoglobin which avidly binds NO also suppressed the anti-aggregatory effect, indicating that apoE stimulated sufficient production of NO by platelets for extracellular release to occur. We conclude that apoE inhibits platelet aggregation through the L-arginine:NO signal transduction pathway.

### Experimental Procedures

**Materials**—N\(^{1}\)-Nitro-L-arginine methyl ester (L-NNAME), N\(^{1}\)-monomethyl-L-arginine (L-NMMA), S-nitroso-L-glutathione (GSNO), and 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ) were obtained from Alexis Corp. Ltd. (Nottingham, UK). Diphenyleneiodonium chloride (DPI), 3-isobutyl-1-methylxanthine (IBMX), and 2-ethyl-isothiourea (Ethyl-ITU) were supplied by Calbiochem-Novabiochem Ltd. (Nottingham, UK). Other chemicals were from Sigma (Dorset, UK).

**ApoE:DMPC Complexes**—ApoE was purified from triglyceride-rich lipoproteins by heparin-Sepharose affinity chromatography (11) and was incorporated into small, unilamellar vesicles of dimyristoylphosphatidylcholine (DMPC) liposomes at a 3.75:1 phospholipid:protein ratio. Protein and DMPC concentrations were measured using commercially available methods (Bradford reagent (Bio-Rad Laboratories, Hemel Hempstead, UK) and phospholipid B kit (Wako, Neuss, Germany)).

**Human apolipoprotein E (apoE)** is a 299-residue protein of molecular mass 34 kDa found in the surface of circulating triglyceride-rich lipoproteins (very low density lipoprotein and chylomicrons, or their remnants) and certain HDL particles (1). Its major function is to mediate hepatic clearance of lipoproteins through interaction with two receptors, the low density lipoprotein or B,E receptor and an apoE-specific receptor, most probably the low density lipoprotein receptor-related protein (2). When the apoE polypeptide is dysfunctional or absent severe hyperlipidemia and atherosclerosis in humans or animal models ensues (1, 3–5). Although apoE is synthesized predominantly by the liver, macrophages also secrete apoE; this appears important for facilitating local cholesterol redistribution, for reverse cholesterol transport, and for restricting development of atherosclerotic lesions (6). Indeed, atherosclerosis in apoE-deficient (apoE\(^{-/-}\)) mice can be prevented by transplantation of normal murine bone marrow cells (5), by macrophage-specific expression of the human apoE transgene (7), or by adenovirus-mediated gene replacement (8).

Recently, we proposed an additional anti-atherogenic role for apoE. We found that HDL-E, the minor apoE-containing subclass of bulk plasma HDL was a powerful inhibitor of agonist-induced platelet aggregation, apparently through interaction with saturable binding sites in the platelet surface membrane (9). Indirect evidence implicated apoE as the active constituent; chemically modifying apoE blocked binding and its anti-platelet action (9), whereas abnormal apoE-enriched HDL from patients with hepatic cirrhosis had a highly potent anti-aggregatory effect that correlated with apoE content (\( r = 0.70, p < 0.001 \)) (10). In the present study, we infer that apoE exerts its anti-platelet effect by enhancing production of endogenous nitric oxide (NO); apoE markedly elevated platelet NO synthase activity and intraplatelet levels of cGMP, whereas NO synthase inhibitors restricted its inhibitory action.

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Stabilized PRP (9). Platelets were used for cholesterol depletion studies immediately after correction for the cGMP and cAMP content of platelet-poor plasma. All samples were centrifuged (12,000 g, 3 min) at 37 °C. At defined time intervals up to 10 min, a portion (100 μl) was removed and rapidly centrifuged (12,000 × g, 30 s), and the [3H]cholesterol released into the supernatant (80 μl) was measured by liquid scintillation counting. In addition, at zero time an aliquot of the total platelet suspension (100 μl) was dissolved in 1 M NaOH, neutralized, and was counted similarly.

Preparation of [3H]Cholesterol-labeled Platelets—A [3H]cholesterol/albumin emulsion was prepared as before (13). Platelets were pelleted from prostacyclin-stabilized PRP, resuspended in the [3H]cholesterol/albumin emulsion for 1 h, and treated again with prostacyclin (300 nM). The mixture was then diluted 50-fold with Tyrode's buffer, centrifuged at 750 × g for 20 min, and the platelet pellet resuspended in buffer. The platelets were used for cholesterol depletion studies immediately after recovering from the prostacyclin effects.

Cholesterol Removal—Aliquots of [3H]cholesterol-labeled platelet suspensions (600 μl, 3 × 10^8 cells/ml) were incubated in the aggregometer with buffer, apoE-DMPC, or chD-apoE-DMPC at 37 °C. At defined time intervals up to 10 min, a portion (100 μl) was removed and rapidly centrifuged (12,000 × g, 30 s), and the [3H]cholesterol released into the supernatant (80 μl) was measured by liquid scintillation counting. In addition, at zero time an aliquot of the total platelet suspension (100 μl) was dissolved in 1 M NaOH, neutralized, and was counted similarly.

Cyclic Nucleotide Assays—Intraplatelet cGMP and cAMP concentrations were measured in PRP with and without a 10-min preincubation at 20 °C with the phosphodiesterase inhibitor, 3-isobutyl-1-methylxanthine (IBMX) (1 mM). Aggregation was terminated after 3 min by addition of 100 μl of 1% trichloroacetic acid; the samples were then neutralized with 1.08 M K2PO4 (80 μl), centrifuged (2000 × g for 15 min at 4 °C), and after acetylation were assayed for cGMP and cAMP contents by commercial radioimmunooassay kits (Amersham Int. Plc.). All samples were corrected for the cGMP and cAMP contents of platelet-poor plasma.

NO Synthase Activity—Washed platelets (10^6 cells) were incubated with or without apoE-DMPC (50 μg of protein/3 × 10^8 cells) for 10 min at 37 °C in a final volume of 1 ml, and the reaction was stopped by addition of 100 μl of 1% homogenization buffer (250 mM Tris-HCl, pH 7.4, containing 10 mM EDTA and 10 mM EGTA). The cells were pelleted in a microcentrifuge for 30 s, resuspended in 100 μl of 1% homogenization buffer, and lysed by two cycles of freezing in liquid nitrogen and thawing on ice. NO synthase activity was measured by the conversion of l-[15N]arginine to l-[15N]citrulline using the NOSdetect assay kit (Stratagene) and expressed as pmol/l h per platelet. Briefly, 25 μl of platelet extract was incubated with 25 μl of substrate buffer (50 mM Tris-HCl, pH 7.4, containing 1 mM NADPH, 6 μM tetrahydrobiopterin, 2 μM flavin adenine dinucleotide, 2 μM flavin adenine mononucleotide, 0.2 μM calmodulin, 1.2 mM CaCl2, and l-[15N]arginine (200,000 dpm)) for 1 h at 37 °C, terminating the reaction by addition of 400 μl of stop buffer (50 mM HEPES, pH 5.5, containing 5 mM EDTA) and 100 μl of cationic resin (Dowex AF 50-X8). The mixture was transferred to a spin filter, microcentrifuged for 30 s, and the l-[15N]citrulline in the eluate was measured by liquid scintillation counting. Nonenzymic formation of citrulline was controlled for by addition of the specific NO synthase inhibitor, l-NAME (1 mM), to a parallel set of tubes.

Results—Results are expressed as means ± S.E., and statistical differences were determined by Student’s two-tailed paired t test.

Inhibition of Platelet Aggregation by ApoE-DMPC—Aqueous solutions of apoE had no effect on agonist-induced aggregation using either PRP or washed platelets but were highly potent anti-platelet agents when complexed with phospholipid (DMPC) vesicles. Thus, ADP-induced aggregation was inhibited in a dose-dependent manner using a physiological range (10–50 μg/ml) of apoE (Fig. 1), and similar findings were seen with epinephrine and collagen as agonists. By contrast, little inhibition was noted with thrombin as the aggregating agent, unless either very high amounts of apoE were added (500 μg/ml) or the incubations were prolonged (up to 30 min).

Ability of ApoE-DMPC to Remove Cholesterol from Platelet Membranes—As cholesterol-deficient platelets respond poorly to agonists (14), we investigated whether the inhibitory action of apoE-DMPC might reflect extraction of cholesterol from platelet membranes. Incubation of washed [3H]cholesterol-labeled platelets with apoE-DMPC and CHD-apoE-DMPC (both 50 μg of protein/ml) released similar amounts of cholesterol as a function of time, corresponding to <1% after our standard 30-s preincubation period and only about 2% after a further 3 min when aggregation studies would be completed (Fig. 2A). However, CHD-apoE-DMPC was an ineffective inhibitor of platelet aggregation (Fig. 2B) compared with its unmodified control (–2 ± 5% versus 63 ± 4% inhibition, respectively, p < 0.001), implying that an alternative mechanism to cholesterol sequestration must explain the anti-platelet effect of apoE.

Effects of ApoE-DMPC on Intraplatelet cGMP and cAMP Levels—Because attenuation of platelet responsiveness is frequently accomplished by changes in intraplatelet cGMP or cAMP levels (15), we measured the influence of apoE on these

FIG. 1. Inhibition of ADP-induced platelet aggregation by apoE-DMPC complexes. Washed platelets (3 × 10^7/ml) were preincubated with apoE-DMPC (●), free apoE (■), or DMPC vesicles alone (▲) for 30 s at 37 °C. The extent of aggregation was measured 3 min after addition of a predetermined threshold concentration of ADP and is expressed as a percentage of controls with buffer alone. Points for apoE-DMPC are the mean percentage inhibition of aggregation (± S.E.) for three different preparations tested on three separate platelet suspensions; other points represent at least two independent measurements.

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cyclic nucleotides during ADP-induced aggregation of PRP. The basal levels of platelet cGMP and cAMP (3.7 ± 1.1 and 11.7 ± 1.9 pmol/10⁹ platelets, respectively) were not significantly altered by incubation with 50 μg of protein/ml of apoEDMPC vesicles (1.9 ± 0.7 and 9.5 ± 1.5 pmol/10⁹ platelets, respectively). However, with threshold concentrations of ADP, the same apoEDMPC complexes produced marked dose-dependent increases in both cGMP (33.9 ± 3.2 versus 13.6 ± 1.5 pmol/10⁹ platelets at 50 μg of protein/ml of apoEDMPC; p < 0.001, n = 3) and cAMP (23.5 ± 3.3 versus 7.4 ± 1.1 pmol/10⁹ platelets at 50 μg of protein/ml of apoEDMPC, p < 0.001, n = 3) (Fig. 3); these correlated with the observed concomitant inhibition of aggregation after 3 min (r = 0.85 and 0.81 for cGMP and cAMP, respectively; both p < 0.01, n = 10). No changes in cyclic nucleotides were noted with CHD-apoEDMPC (Fig. 3A), free apoE or DMPC alone.

Effects of IBMX and ODQ on ApoEDMPC-treated Platelets—Levels of cGMP and cAMP are controlled directly by the activities of their synthesizing enzymes, guanylate cyclase and adenylyl cyclase, respectively, and catabolizing enzymes, cGMP and cAMP phosphodiesterases (16, 17). When platelets were preincubated with the general phosphodiesterase inhibitor, IBMX (1 mm) (18), basal cAMP levels were increased 2-fold (14.8 ± 3.0 versus 7.4 ± 1.1 pmol/10⁹ platelets) as a consequence of inhibition of platelet cAMP phosphodiesterase. However, addition of apoEDMPC vesicles to the system did not invoke a further rise in cAMP (14.7 ± 3.4 versus 14.8 ± 3.0 pmol/10⁹ platelets at 50 μg of protein/ml of apoEDMPC; Fig. 3B), implying that apoE had no effect on adenylyl cyclase. By contrast, apoEDMPC still elicited a dose-dependent rise in cGMP levels in the presence of IBMX (10.8 ± 0.8 versus 2.6 ± 1.1 pmol/10⁹ platelets at 50 μg of protein/ml; Fig. 3A), indicating an apoE-induced increase in synthesis of this cyclic nucleotide rather than a decrease in its catabolism. Further support for this pre-eminent role of cGMP was obtained by use of ODQ, a potent and specific inhibitor of soluble guanylate cyclase (19, 20). This reagent impaired, as expected, the anti-aggregatory action of the NO donor, GSNO, but also effectively reversed the anti-platelet effect of 50 μg of protein/ml of apoEDMPC vesicles (7.5 ± 8.9 versus 68.7 ± 4.4% inhibition; p < 0.001, n = 3) (Fig. 4).

Effects of NO Synthase Inhibitors on the Aggregation of ApoEDMPC-treated Platelets—One important cellular mechanism for up-regulation of cGMP is through stimulation of NO synthase (21, 22); this enzyme acts on L-arginine to produce NO which then binds to, and hence activates, soluble heme-containing guanylate cyclase, its physiological target (23, 24). When we preincubated platelets with L-NMMA or L-NAME, chemical analogs of L-arginine and competitive inhibitors of NO synthase (21, 22), the anti-platelet action of apoEDMPC vesicles was essentially blocked (Fig. 5). These inhibitory reactions were enantiomer-specific since D-NMMA was ineffective. Moreover, two additional inhibitors of NO synthase, Ethyl ITU (25, 26) and DPI (27, 29), also reversed the anti-platelet effect of apoE (Fig. 5B) without discernible effect themselves on aggregation. Finally, hemoglobin, a competitor for NO binding (29), suppressed the anti-aggregatory effect of apoEDMPC (Fig. 5A).

Stimulation of Platelet NO Synthase Activity by ApoEDMPC Vesicles—Platelet NO synthase is the constitutive, strictly Ca²⁺-dependent form of the enzyme which generates only trace amounts of NO (21, 22, 30). Indeed, a highly specialized, noncommercial porphyrinic microsensor was required to detect the transient release of low pmol amounts of NO following agonist activation, whereas basal synthesis and release of NO by human platelets was not detected (30). We monitored, therefore, release of NO by measuring the stoichiometric production of L-[¹⁴C]citrulline from L-[¹⁴C]arginine. When platelets were incubated with apoEDMPC vesicles for 10 min, NO synthase activity was markedly increased as judged by a 4-fold rise in conversion of L-[¹⁴C]arginine to L-[¹⁴C]citrulline (0.71 ± 0.17 pmol of citrulline produced per h per 10⁹ apoEDMPC-treated platelets versus 0.18 ± 0.03 in untreated platelets; p < 0.05, n = 4) (Fig. 6). This was considered specific NO synthase activity since L-NAME (1 mm) negated any increase.
DISCUSSION

This study substantiates our previous work implicating apoE as the active anti-platelet constituent of HDL-E. The metabolic activity of apoE is sensitive to its lipid environment; purified apoE does not interact with the low density lipoprotein receptor (12), whereas the apoE in surfaces of very low density lipoprotein and chylomicron is also inactive unless these lipoproteins are from hypertriglyceridemic subjects (31) or have undergone substantial lipolysis to form remnant particles (32). Similarly, apoE in free solution did not inhibit platelet aggregation, although apoE-phospholipid complexes, which mimic the form secreted by macrophages (1), were potent inhibitors; presumably the DMPC allowed the apoE polypeptide to assume an appropriate orientation and conformation for biological activity (12). Recently, apoE-DMPC complexes were reported to inhibit collagen- or thrombin-induced aggregation (33), apparently by avidly extracting cholesterol from platelet plasma membranes and hence impairing arachidonate release and conversion to thromboxane A2 (35). However, sequestration of cholesterol in our experiments was much lower, 1% versus 10%, reflecting our shorter preincubation times (30 s versus 30 min), an amount insufficient to be anti-aggregatory with ADP as agonist (13). Indeed, we showed this explanation to be implausible by use of chemically modified apoE; CHD-apoE-DMPC failed to suppress platelet aggregation but still removed the same amount of platelet cholesterol as inhibitory apoE-DMPC.

Calcium is central to control of platelet reactivity, interacting with diverse second messengers through a myriad of complex, but tightly regulated, signaling pathways (35). Two important control elements for suppression of platelet activation are the cyclic nucleotides, cAMP and cGMP, and agents that increase their intraplatelet levels exert anti-aggregatory effects both in vitro and in vivo. For example, prostacyclin and adenosine limit platelet activation by raising cAMP (36, 37), while NO or NO-donating compounds are similarly restrictive by increasing cGMP (19, 38). Although apoE induced increases in both cAMP and cGMP, additional experiments implicated a specific stimulation of guanylate cyclase activity and a rise in cGMP as prerequisites for the anti-platelet action of apoE. Thus, ODQ, a potent and selective inhibitor of soluble guany-
late cyclohexyl (19, 20), was able to reverse the anti-aggregatory action of apoE. Similarly, studies with the general phosphodiesterase inhibitor, IBMX, supported a primary role for cGMP since this reagent abolished the apoE DHPC-induced rise in cAMP but not the dose-dependent increase in cGMP. Interestingly, these findings were consistent with second messenger “cross-talk” (15), namely that the increase in cGMP invoked by apoE in the absence of IBMX may cause inhibition of cAMP phosphodiesterase (39, 40), permitting levels of cAMP to rise (Fig. 3B and Fig. 7).

Several experiments implicated platelet NO synthase in mediating the anti-aggregatory action of apoE through production of NO, a physiological activator of guanylate cyclase. Thus, NO synthase inhibitors of distinct structural and functional types, the amino acid analogs of L-arginine, L-NMMA, and L-NNAME, a nonamino acid analog, ethyl-ITU, and the flavoprotein inhibitor, DPI, all reversed the anti-platelet action of apoE. These chemicals inhibit cellular production of NO by binding to NO synthase to displace either L-arginine or, in the case of DPI, essential cofactors (26). Because all reagents were used at concentrations close to their quoted IC50 values for specific NO synthase inhibition, it seems improbable that they act via diverse NO-independent mechanisms to block the apoE-DHPC effects. Indeed, we demonstrated direct involvement of the L-arginine:NO pathway by measuring platelet NO synthase activity; lysates from platelets pretreated with apoE-DHPC had a 4-fold increased ability to convert L-arginine to L-citruline which could be abolished by L-NNAME. Furthermore, hemoglobin, which strongly inhibits NO activation of guanylate cyclase by forming a hemoglobin-NO adduct, suppressed the anti-platelet action of apoE. As hemoglobin does not penetrate platelets (41), this implies that apoE generated sufficient NO for secretion; presumably binding of NO by hemoglobin in the extracellular medium created a concentration gradient to reduce NO inside platelets (28) and hence allow aggregation.

In summary, as indicated in Fig. 7, we believe that our findings provide clear evidence for the L-arginine: NO signal transduction pathway as the mechanism by which apoE exerts

**Fig. 5.** NO synthase inhibitors prevent the anti-aggregatory action of apoE-DHPC complexes. A, aliquots of PRP (2–3 × 10⁸ cells/ml) were preincubated with 300 μM L-NAME, L-NMMA, D-NMMA, or 100 nM hemoglobin (Hb) for 10 min at 20 °C and then for a further 10 min with 10 μg of protein/ml of apoE-DHPC. Aggregation measurements were carried out in triplicate as described in Fig. 1. The results shown are the means ± S.E. of one experiment but were reproduced in two independent assays. B, aliquots of PRP (2–3 × 10⁸ cells/ml) were preincubated with 20 μg of protein/ml of apoE-DHPC for 9 min at 20 °C and then for a further 1 min at 37 °C with 100 nM DPI or 3 μM Ethyl-ITU. Aggregation measurements were carried out as before and are the means ± S.E. of three independent experiments.

**Fig. 6.** ApoE-DHPC complexes increase intraplatelet NO synthase activity. Washed platelets (10⁸ cells) were incubated with or without apoE-DHPC vesicles (50 μg of protein/3 × 10⁶ cells) for 10 min at 37 °C in a final volume of 1 ml, and cell lysates were prepared as described under “Experimental Procedures.” NO synthase activity was assessed by measuring the conversion of L-[³H]arginine to L-[³H]citruline in the absence and presence of the specific inhibitor L-NAME (1 mM) using a NOSdetect assay kit (Stratagene). Results are expressed as picomoles of L-[³H]citruline produced per h per 10⁹ platelets and are corrected for nonenzymic production of citruline; values are the means ± S.E. of four independent experiments.

late cyclohexyl (19, 20), was able to reverse the anti-aggregatory action of apoE. Similarly, studies with the general phosphodiesterase inhibitor, IBMX, supported a primary role for cGMP since this reagent abolished the apoE-DHPC-induced rise in cAMP but not the dose-dependent increase in cGMP. Interestingly, these findings were consistent with second messenger

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its anti-platelet effect. Less clear are the initial events required to activate this pathway. However, the ability of platelets to bind saturably HDL-E (9) and apoE-DMPC complexes (33) suggests a distinctive apoE receptor in platelet surface membranes, a proposal consistent with the noninhibitory action of CHD-apoE.

Although NO synthesized by the constitutive enzyme NO synthase is now well-recognized as an important regulatory mechanism for platelet hemostasis (21–24), and hence prevention of thrombosis, our finding that apoE substantially augments basal levels of cGMP was unexpected; platelet NO synthase requires Ca\(^{2+}\)-calmodulin for activation, and an agonist such as ADP is essential to supply the initial burst of Ca\(^{2+}\)-calmodulin (Ca\(^{2+}\)-CAM) (47, 52). Some of the NO generated acts on soluble guanylate cyclase to produce inhibitory cGMP, the concomitant rise in cAMP restricts the apoE inhibitory effect, apoprotein E (apoE) may "prime" platelets to help attenuate activation when challenged by agonists or other agents.

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J. Biol. Chem. 1997, 272:89-95.
doi: 10.1074/jbc.272.1.89

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