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Rapid Reversal of Endothelial Dysfunction in Hypercholesterolemic Apolipoprotein E-Null Mice by Recombinant Apolipoprotein A-I_{Milano}-Phospholipid Complex

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OBJECTIVES In this study, we examined whether a reconstituted high-density lipoprotein (HDL) utilizing recombinant apolipoprotein A-I_{Milano} (apo A-I_M)/phospholipid complex (PC) could restore normal endothelial function in hypercholesterolemic apolipoprotein (apo) E-null mice.

BACKGROUND We have previously shown antiatherosclerotic and vasculoprotective effects of recombinant apo A-I_M.

METHODS A perfused vessel preparation was used to examine vascular responses in control wild-type, untreated, and treated apo E-null mice. Aortic tissue cholesterol content and platelet aggregation were also measured.

RESULTS Endothelium-dependent vasodilator responses to acetycholine were significantly inhibited in untreated apo E-null mice compared with control wild-type mice ($p < 0.001$). Treatment of the mice for five weeks with once every-other-day intravenous bolus injections of apo A-I_M/PC restored endothelium-dependent dilation in a dose-dependent manner ($p < 0.01$ at 80 mg/kg dose). The improvement in endothelial function was associated with a reduction in aortic cholesterol content and reduced platelet aggregability and occurred despite severe and persistent hypercholesterolemia. Neither treatment with free protein nor phospholipid carrier alone produced any significant effects. We performed additional experiments in vitro in isolated rabbit carotid arteries to compare the effects on lysophosphatidylcholine (LPC)-induced endothelial dysfunction. Treatment with apo A-I_M/PC prevented impairment of endothelium-dependent vasodilator responses to acetylcholine to a greater degree than either wild-type apo A-I or plasma-derived HDL.

CONCLUSIONS Our results indicate a rapid improvement in endothelial dysfunction with recombinant apo A-I_M/PC that is associated with mobilization of tissue cholesterol. Taken together with previously established antiatherosclerotic and antithrombotic effects, these findings suggest significant vasculoprotective effects with apo A-I_M/PC therapy. (J Am Coll Cardiol 2004; 44:1311–9) © 2004 by the American College of Cardiology Foundation

Several lines of evidence suggest a protective effect of high-density lipoprotein (HDL) and apolipoprotein (apo) A-I against atherosclerotic vascular disease. Epidemiologic studies have demonstrated an inverse relationship between plasma HDL or apo A-I levels and atherosclerosis (1). More directly, attenuation of atherosclerosis by intravenous homologous HDL has been demonstrated in cholesterol-fed rabbits (2). Furthermore, overexpression of the human apo A-I gene in apo E-null mice prevents atherosclerotic plaque formation (3). The protective effects of HDL have been primarily attributed to its effect on reverse cholesterol transport activity (4). However, evidence showing direct association of HDL in humans in ameliorating abnormal vascular reactivity in atherosclerosis (5,6) and ex vivo platelet thrombus formation (7) supports the notion that improvement in endothelial function and thrombogenicity might also contribute to the beneficial effects of HDL.

Recently, we and others have focused our investigations on a naturally occurring molecular variant of apo A-I, apo A-I_{Milano} (apo A-I_M) characterized by a cysteine-for-arginine substitution and formation of apo A-I_M/A-I_M dimers (8). These latter are characterized by a prolonged circulation time in plasma and a more effective cholesterol efflux capacity, thereby conferring protection from cardiovascular disease despite markedly reduced HDL levels in human carriers of this mutation (9,10). A number of animal studies from our laboratory and others have demonstrated antiatherogenic (11–14), antiproliferative (11,15), antirestenotic (16), antiplatelet, and antithrombotic (17) properties of apo A-I_M. Most recently, a pilot clinical trial of recombinant apo A-I_M demonstrated significant and rapid regression of atherosclerosis in human coronary arteries (18).

In this study, we examined the effects of treatment with recombinant apo A-I_M/phospholipid complex (PC) on endothelium-dependent vasodilator responses in a murine model of atherosclerosis, the apo E-null mice. We hypothesized that treatment with apo A-I_M/PC will attenuate

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Abbreviations and Acronyms

apo	= apolipoprotein
apo A-I$_M$	= apo A-I$_{Milano}$
EDNO	= endothelium-derived nitric oxide
HDL	= high-density lipoprotein
LPC	= L-α-phosphatidylcholine palmitoyl
PC	= phospholipid complex
WT	= wild-type

impairment of endothelium-dependent vasodilator responses in apo E-null mice.

To compare the protective effects of apo A-I$_M$ on endothelium-dependent vasodilator responses with apo A-I wild-type (apo A-I$_{WT}$) or plasma-derived HDL, we performed an additional set of experiments in isolated rabbit carotid arteries in which endothelial dysfunction was induced by incubation with lysophosphatidylcholine (LPC); LPC has been identified as the principal mediator of the inhibitory effects of oxidized low-density lipoprotein on endothelium-dependent vasomotor reactivity (19). We hypothesized that LPC-induced inhibition of endothelium-dependent vasodilation would be prevented by treatment with HDL, and that reconstituted HDL using recombinant apo A-I$_M$ would exert greater protective effects compared with apo A-I$_{WT}$ or plasma-derived HDL.

METHODS

Study protocol. Two sets of experiments were performed as shown in Figure 1. In the first set, recombinant apo A-I$_M$ was administered intravenously in hypercholesterolemic apo E-null mice and descending thoracic aortas harvested for vascular reactivity studies. In the second set, carotid arteries were harvested from New Zealand White rabbits fed a normal diet (2 to 3 kg), endothelial dysfunction was induced in vitro with incubation with LPC, and effects of treatment agents on LPC-induced impairment of endothelium-dependent vasodilator responses were examined.

Three groups of mice (Jackson Laboratory, Bar Harbor, Maine) were studied: 1) 5-week-old WT C57BL/6J strain mice fed a standard rodent chow for 25 weeks (WT, n = 5) were used as normal control; 2) 5-week-old untreated apo E-null mice (C57BL/6J strain, 18 to 20 g), fed an atherogenic diet for 25 weeks (ApoE, n = 6) were used as hypercholesterolemic control; 3) 5-week-old apo E-null mice fed an atherogenic diet for 25 weeks and treated with two doses of apo A-I$_M$ complexed with the phospholipid carrier, DPPC (1,2-dipalmitoyl-sn-3-phosphoglycerocholine) in a protein-to-phospholipid ratio of 1:2.7 by weight (12) and dissolved in 0.5 ml saline—20 mg/kg/dose (ApoE + apo A-I$_M$/DPPC 20, n = 9) and 80 mg/kg/dose (ApoE + apo A-I$_M$/DPPC 80, n = 10), or 80 mg/kg/dose free apoA-I$_M$ without DPPC (apo A-I$_M$ 80, n = 5), or 210 mg/kg/dose DPPC alone (n = 5). Each mouse received 18 intravenous injections through the tail vein on alternate days from 25 to 30 weeks.

Before euthanasia, mice were anesthetized with enflurane

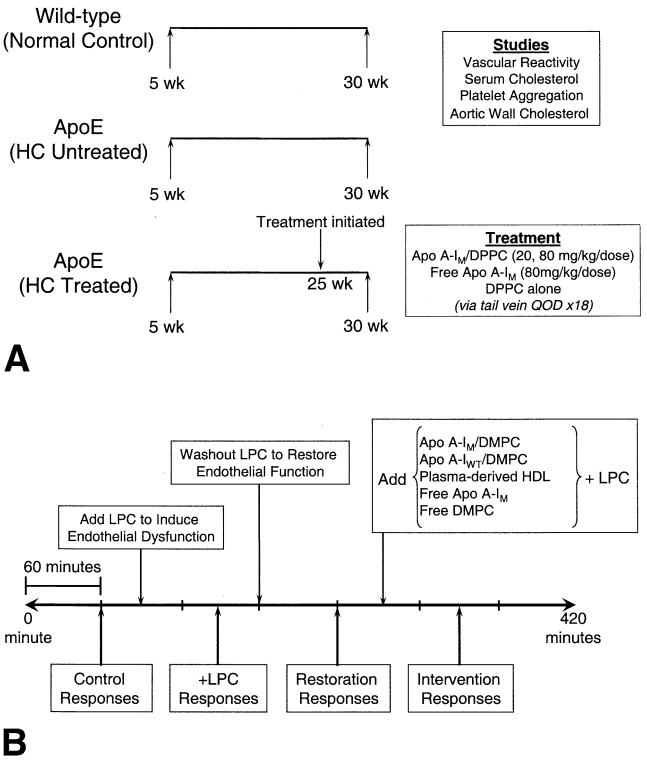

Figure 1. Schema of the experimental protocols are shown. In the mice experimental protocol **(A)**, three groups of mice were studied: normal wild-type control, untreated hypercholesterolemic (HC) apolipoprotein E-null mice (ApoE), and treated HC ApoE-null mice. The types of studies and treatment interventions are shown. See text for details of rabbit experiments **(B)**. Apo A-I$_M$ = apolipoprotein A-I$_{Milano}$; Apo A-I$_{WT}$ = apolipoprotein A-I wilt-type; DMPC = dimyristylphosphatidylcholine; DPPC = 1,2-dipalmitoyl-sn-3-phosphoglycerocholine; HDL = high-density lipoprotein; LDL = low-density lipoprotein; LPC = lysophosphatidylcholine.

inhalation, and 100 to 300 μl of blood was obtained from the retro-orbital plexus in heparin-coated capillaries (Fisher Scientific, Hampton, New Hampshire) and collected for platelet aggregation, serum total cholesterol, and serum apo A-I$_M$ measurements. Serum cholesterol was measured by enzymatic technique, and the apo A-I$_M$ levels were determined by ELISA as described previously (12). After anesthesia, aortas were harvested and the proximal portion of descending thoracic aorta used for vascular reactivity studies and the distal portion used for aortic tissue cholesterol content. Treated mice were euthanized <12 h after the last injection of apo A-I$_M$. All research involving these animals was approved by the institutional animal care and use committee and conformed to the Guiding Principles in the Care and Use of Laboratory Animals established by the council of the American Physiology Society.

Isolated perfused artery preparation for vascular reactivity studies. Whole mounted pressurized vessels with constant perfusion were used to study vascular responses (20). The arteries were isolated, removed, and immediately placed in cold (5°C to 10°C) oxygenated modified Krebs solution (NaCI, 118.3 mM; KC1, 4.7 mM; CaCI2, 2.5 mM; MgSO4.7H20, 1.2mM; NaHCO3, 25.0 mM; KH2PO4,

1.2 mM; dextrose, 11.0 mM). Loose adventitial connective tissue was removed, and 1.0- to 1.5-cm long segments were mounted between two plastic cannulae and placed in an organ bath. Arteries were perfused intraluminally and superfused abluminally with oxygenated modified Krebs solution by means of a peristaltic pump (Rainin Instrument Col. Inc., Woburn, Massachusetts) at a constant rate of 3.0 ml/min. Pressure at the downstream end of the vessel was maintained at 80 mm Hg throughout the experimental protocol. Arterial segments were observed with ×40 magnification, and images were displayed with a video camera attached to a microscope. Outer diameter of arteries was recorded continuously with an optical system utilizing computer-assisted edge detection and digital analysis of the video image. This preparation has been described in detail previously (20).

Drugs. Recombinant apo A-I$_M$ and the phospholipid carriers 1,2-dipalmitoyl-sn-3-phosphoglycerocholine (DPPC) and dimyristylphosphatidylcholine (DMPC) were supplied by Pharmacia-Upjohn (Stockholm, Sweden). The recombinant apo A-I$_M$/DPPC preparation used in the mice studies has been described previously (12,13). For rabbit studies, DMPC was complexed with both recombinant apo A-I$_M$ and apo A-I$_{WT}$ in a 1:3 molar ratio; apo A-I$_{WT}$ was generously provided by Swiss Red Cross (Bern, Switzerland). Plasma HDL was graciously provided by Mohammad Nawab, PhD (UCLA, Los Angeles, California); HDL was isolated from blood of normal volunteers by sequential ultracentrifugation according to the method of Havel et al. (21) and the protein content determined by the method of Lowry et al. (22). Freshly prepared HDL (final concentration of 5 mg/ml) was kept in dark under nitrogen at 4°C and used within three weeks.

Phenylephrine hydrochloride, serotonin creatinine sulfate (5-HT), acetylcholine hydrochloride, sodium nitroprusside, and L-α-phosphatidylcholine palmitoyl (LPC) were purchased from Sigma Chemicals Co. (St. Louis, Missouri). Thromboxane A_2 mimetic, U46,619, was obtained from Biomol Research Laboratories, Inc. (Plymouth Meeting, Pennsylvania). Phenylephrine, acetylcholine, and sodium nitroprusside were dissolved in deionized water; serotonin was dissolved in 0.1 N HC1 with 0.1% ascorbic acid; U46,619 was dissolved in ethanol-0.1 M NaHCO3 (1:3, vol/vol) to make a stock solution. Each agent was then diluted in modified Krebs solution, aerated with a mixture of 95% O_2 and 5% CO_2 immediately before use; LPC was dissolved in chloroform:methanol 1:1 and sonicated for 10 min before addition to baths. To avoid inactivation of sodium nitroprusside by light, the drug and the stock solution were kept in the dark, and perfusion tubing was covered by aluminum foil.

Vascular reactivity protocol. Vessels were perfused at flow rate of 3 ml/min and gradually stretched longitudinally to the approximate in situ length and exposed to repeated constrictor (phenylephrine, 1 μM) and dilator (acetylcholine, 1 μM) stimuli until reproducible responses were obtained (usually 60 min). Vascular responses to agonists were recorded for about 5 to 7 min until steady-state responses were observed. Between each series of interventions, the preparation was washed by changing the perfusate solution several times with modified Krebs solution, followed by an equilibration period of 15 to 20 min. Dilator responses to intraluminal acetylcholine (endothelium-dependent agonist) and sodium nitroprusside (endothelium-independent agonist) were examined in arteries preconstricted with abluminal 1 μM 5-HT (EC_{80}) in mice, or 1 μM phenylephrine (EC_{80}) in rabbit, and expressed as percent of preconstriction (20). Constrictor responses were measured in quiescent arteries and expressed as percent change from baseline diameter (20). Baseline diameter of perfused mice aorta and rabbit carotid arteries was 1.4 ± 0.1 mm and 2.9 ± 0.1 mm (mean ± SEM), respectively.

The experimental protocol for rabbit carotid arteries is shown in Figure 1B and composed of initially performing concentration- and time-response experiments to determine the optimal concentration and incubation time to induce reversible endothelial dysfunction. After measurement of dilator responses at baseline (*control responses*), 50 μM LPC was added to the intraluminal perfusate and the arteries incubated for 45 to 60 min in a closed-loop system. Dilator responses were then measured to confirm the presence of endothelial dysfunction (*+LPC responses*); LPC was then washed out by perfusing 0.1% albumin dissolved in modified Krebs solution for 30 to 45 min and dilator responses repeated (*restoration responses*). Finally, LPC was added to the intraluminal perfusate in presence of the following agents—apo A-I$_M$ /DPPC (1 mg/ml), apo A-I$_{WT}$/DPPC (1 mg/ml), plasma-derived HDL (1 mg/ml), free apo A-I$_M$ (1 mg/ml), and free DPPC (300 μg /ml), and dilator responses measured (*intervention responses*). Each experiment took an average of 7 h to complete.

Aortic tissue cholesterol content. Aortic tissue cholesterol content was determined in the distal half of the descending thoracic aorta using an assay that has been previously reported (23). Briefly, aortic tissue was homogenized, and the lipid content was extracted by treatment with chloroform and methanol followed by centrifugation. The extracted lipid was dissolved in ethanol and measured via a cholesterol assay kit (Sigma) utilizing absorbance spectrophotometry, and the amount quantified as μg/mg tissue.

Platelet aggregation. Citrated whole blood samples (100 μl) were collected from mice and diluted with an equal volume of isotonic saline. Collagen-induced (2 μg/ml) whole blood platelet aggregation was measured by impedance aggregometry (Chronolog, Havertown, Pennsylvania). Platelet aggregation was measured as the maximal change in impedance in ohms at 6 min after the addition of collagen.

Statistical analysis. Data are presented as mean ± SEM. Statistical comparisons were performed by analysis of variance, followed by pairwise comparisons by the two-tailed *t* test with the Bonferroni correction. A value of $p \leq 0.05$ was considered statistically significant.

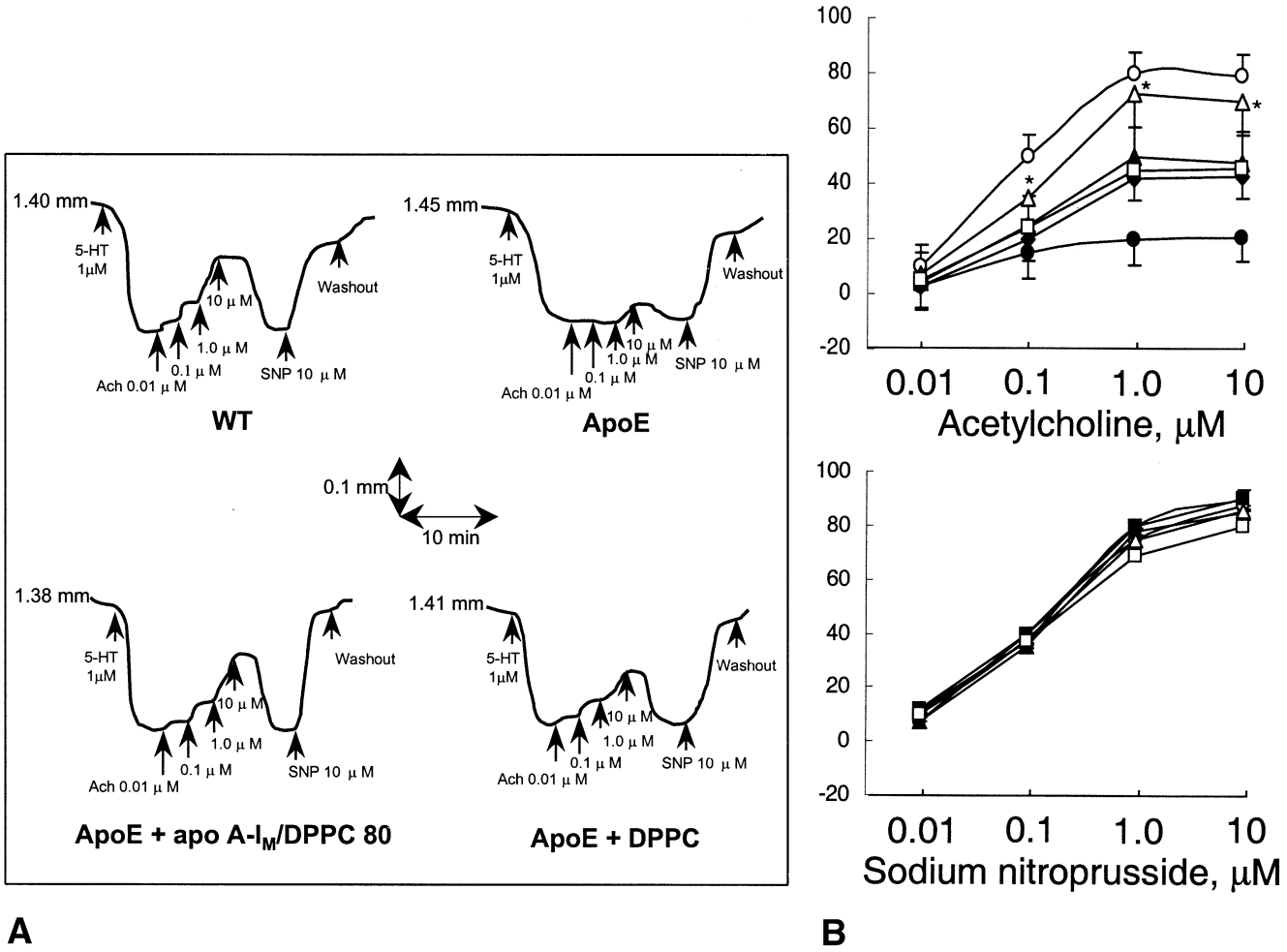

Figure 2. (A) Original tracings showing vasomotor responses in isolated descending thoracic arteries from wild-type mice (WT), untreated apo E-null mice (ApoE), and ApoE mice treated with high-dose (80 mg/kg/dose) apo A-I$_{Milano}$/DPPC complex (ApoE + apo A-I$_M$/DPPC 80) and DPPC alone (ApoE + DPPC). Dilator responses to intraluminal perfusion of endothelium-dependent agonist, acetylcholine (Ach), and endothelium-independent agonist, sodium nitroprusside (SNP) were examined in arteries preconstricted with serotonin (5-HT, 1 μM) administered abluminally. **(B) Line plots** showing concentration response to Ach **(top panel)** and sodium nitroprusside **(bottom panel)**. Values (mean ± SEM) are percent change from a preconstricted diameter of 1.12 ± 0.02 mm. *$p < 0.001$ vs. ApoE, analysis of variance. **Open circles** = wild-type (n = 5); **open triangles** = ApoE + apo A-I$_M$/DPPC 80 (n = 10); **solid triangles** = ApoE + apo A-I$_M$/DPPC 20 (n = 10); **open squares** = ApoE + apo A-I$_M$ 80 (n = 5); **solid diamonds** = ApoE + DPPC (n = 5); **solid circles** = ApoE (n = 6).

RESULTS

Mice studies. VASODILATOR RESPONSES. Representative examples of concentration-response curves to acetylcholine and sodium nitroprusside in the three groups of mice are shown in Figure 2A and the data quantified in Figure 2B. Acetylcholine produced a concentration-dependent dilation of aorta in WT mice. Vasodilator responses to acetylcholine were significantly inhibited in untreated ApoE mice ($p < 0.001$. vs. WT). Treatment with apo A-I$_M$/DPPC complex produced a dose-dependent improvement in endothelium-dependent vasodilator responses, which was significant at an 80-mg/kg dose (Fig. 2B). Treatment with both free apo A-I$_M$ or DPPC alone tended to improve dilator responses, but the effects were not significant.

Dilator responses to endothelium-independent agonist sodium nitroprusside were not significantly different in the untreated ApoE mice compared with the WT mice (Fig. 2B). None of the treatment interventions had any significant effects on dilator responses to sodium nitroprusside (Fig. 2B).

VASOCONSTRICTOR RESPONSES. Maximal constrictor responses to 5-HT (10 μM) did not differ significantly between the groups (16 ± 2% in WT, 15 ± 1% in ApoE, 17 ± 2% in ApoE + apo A-I$_M$/DPPC 80, 16 ± 1% in ApoE + apo A-I$_M$ 80, and 15 ± 2% in ApoE + DPPC). Likewise, maximal constrictor responses to U46,619 (1 μM) were not significantly different (19 ± 2% in WT, 20 ± 1% in ApoE, 18 ± 2% in ApoE + apo A-I$_M$/DPPC 80, 19 ± 1% in ApoE + apo A-I$_M$ 80, and 20 ± 2% in ApoE + DPPC). These data indicate that altered vasomotor responses in hypercholesterolemic apo E-null mice are confined to impaired endothelium-dependent dilator responses and that treatment had no impact on smooth-muscle vasoconstrictor activity.

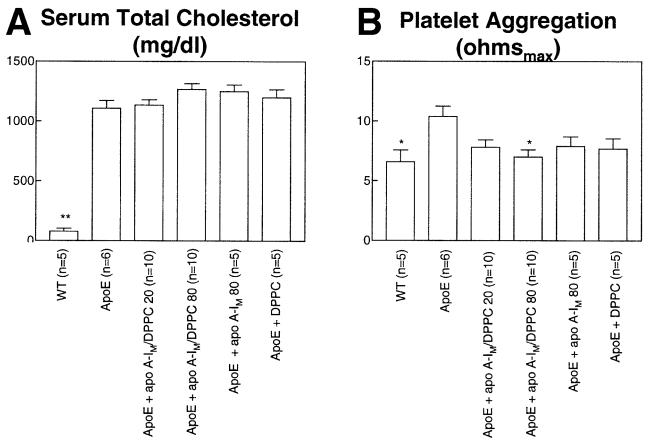

Figure 3. Bar graphs showing effects on serum total cholesterol **(A)** and collagen (2 μg/ml)-induced whole blood platelet aggregation **(B)**. Platelet aggregation was measured by impedance aggregometry (Chronolog, Havertown, Pennsylvania) and data expressed as the maximal change in impedance in ohms (ohms$_{max}$) at 6 min after the addition of collagen. Values are mean ± SEM. *p < 0.05; **p < 0.001 vs. apolipoprotein E (ApoE), analysis of variance. DPPC = 1,2-dipalmitoyl-sn-3-phosphoglycerocholine; WT = wild-type.

SERUM CHOLESTEROL AND APO A-I$_M$ LEVELS. Serum cholesterol levels were significantly elevated in ApoE compared with WT control mice. However, the cholesterol levels did not differ between the untreated and treated ApoE mice (Fig. 3A). At the time of euthanasia (12 h after last injection), apo A-I$_M$ was detectable in all mice receiving apo A-I$_M$/DPPC complex (276 ± 105 μg/ml, n = 8), but in none of the controls. Antibodies against apo A-I$_M$ were detected in the sera of mice receiving apo A-I$_M$ treatment but in none of the control mice.

PLATELET AGGREGATION. Platelet aggregation was slightly, but significantly, increased in untreated ApoE mice compared with WT mice (p < 0.05). Treatment with apo A-I$_M$/DPPC produced a significant inhibition of platelet aggregation at 80 mg/kg dose (p < 0.05). None of the other interventions produced significant effects on platelet aggregation (Fig. 3B).

AORTIC TISSUE CHOLESTEROL CONTENT. In the untreated ApoE mice, the aortic tissue cholesterol content increased nearly eight-fold compared with WT mice (Fig. 4). Treatment with apo A-I$_M$/DPPC produced a dose-dependent reduction in aortic cholesterol content that achieved statistical significance at the higher dose. Treatment with free apo A-I$_M$ or DPPC alone produced modest, but nonsignificant, reductions in aortic tissue cholesterol levels (Fig. 4). There was a significant inverse correlation between aortic tissue cholesterol content and dilator responses to acetylcholine (Pearson r = −0.88, p < 0.05).

Rabbit studies. As shown in Figure 5, LPC produced a concentration- and time-dependent inhibition of dilator responses to acetylcholine. An optimal concentration of 50 μM LPC produced endothelial dysfunction that was short-lived and reversed within 45 min of washout. Irreversible damage was observed at a concentration of 100 μM LPC. These findings are consistent with previous observations that submicellar concentrations of LPC (<50 μM) induce reversible endothelial dysfunction, but irreversibly damage the endothelium at supramicellar concentrations (24).

As shown in Figure 6, vasodilator responses to acetylcholine, but not sodium nitroprusside, were significantly inhibited in arteries incubated with LPC compared with control.

Treatment of arteries with apo A-I$_M$/DMPC complex enhanced the dilator response to acetylcholine up to levels statistically indistinguishable from the response seen in control (−LPC), thereby implying a significant attenuation of LPC-induced impairment of endothelium-dependent vasodilation; Apo A-I$_{WT}$/DMPC complex as well as plasma-derived HDL produced a nonsignificant attenuation. Neither free apo A-I$_M$ nor DMPC alone had any significant effects on LPC-induced endothelial dysfunction. Similar to previous observations (17), dilator responses in arteries with intact endothelial function (−LPC) were unaffected by treatment (data not shown). Quiescent diameter of carotid arteries was not significantly affected by incubation with LPC alone. Also, vasoconstrictor responses to phenylephrine were unaffected by incubation with LPC or coincubation with LPC plus treatment agents (data not

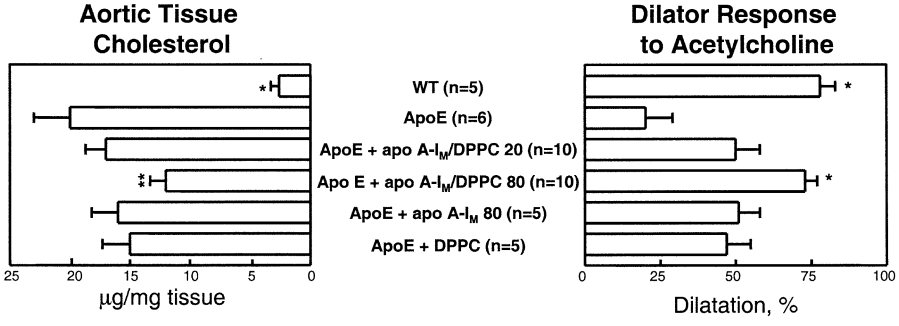

Figure 4. Bar graphs showing effects on aortic tissue cholesterol content **(left panel)** and acetylcholine (1 μM)-induced maximal vasodilator responses **(right panel)**. Values are mean ± SEM. *p < 0.001 vs. apolipoprotein E (ApoE); **p < 0.05 vs. ApoE, analysis of variance. A significant inverse correlation between aortic tissue cholesterol content and maximal vasodilator responses to acetylcholine was observed (Pearson's r = −0.88, p < 0.05). DPPC = 1,2-dipalmitoyl-sn-3-phosphoglycerocholine; WT = wild-type.

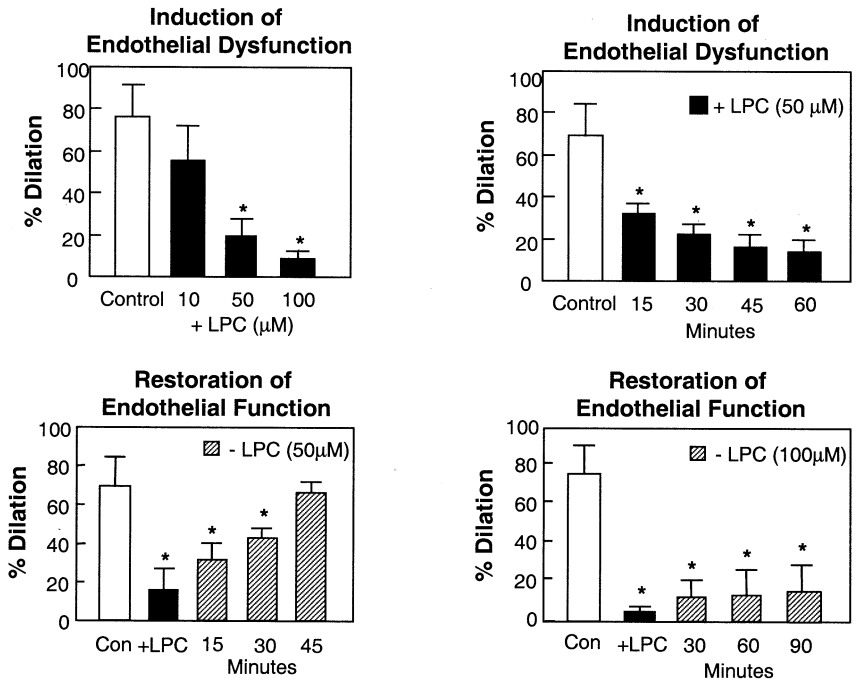

Figure 5. Bar graphs showing concentration-dependent **(upper left)** and time-dependent **(upper right)** effects of incubation of rabbit carotid arteries with lysophosphatidylcholine (LPC) on dilator responses to acetylcholine (1 μM). The **bottom panels** show restoration of dilator responses after washout of 50 μM LPC **(left panel)** and 100 μM LPC **(right panel)**. Values (mean ± SEM, n = 4 to 5) are percent change from a phenylephrine (1 μM)-induced preconstricted diameter of 2.0 ± 0.1 mm. *$p < 0.001$ vs. control; analysis of variance.

shown). These data suggest negligible effects of LPC on basal vascular tone in this vascular reactivity model.

DISCUSSION

The major finding in this study is that administration of recombinant apo A-I$_M$ complexed with phospholipid markedly prevented impairment of endothelium-dependent vasodilator response in a murine model of hypercholesterolemia and atherosclerosis. The improvement in endothelial function with apo A-I$_M$ correlated with a reduction in aortic tissue cholesterol content, and was associated with reduced platelet aggregability. These effects were evident despite persistent and severe circulating hypercholesterolemia. Neither free protein nor phospholipid alone produced significant effects, thereby suggesting the critical role of the apolipoprotein-PC in mediating the protective effects of reconstituted HDL. Finally, based on rabbit carotid artery experiments, the protective effects of apo A-I$_M$ on endothelium-dependent vasodilation were more pronounced than apo A-I$_{WT}$ or plasma-derived HDL.

The present findings extend our previously described vasculoprotective effects of recombinant apo A-I$_M$/PC, which include inhibition of neointimal lesions in balloon-injured ileofemoral arteries of hypercholesterolemic rabbits (11), reduced progression and induction of regression of aortic atherosclerosis in hypercholesterolemic apo E-deficient mice (12,13), and inhibition of restenosis after coronary stenting in swine (16). Taken together with the antiproliferative (15) and antithrombotic (17) effects demonstrated by other investigators, these experimental observations illustrate the potential therapeutic benefit of recombinant apo A-I$_M$ in patients with atherothrombotic vascular disease.

Endothelial function is an important regulator of vascular tone, inflammation, and hemostasis. Hypercholesterolemia may contribute to high coronary heart disease risk by inducing endothelial dysfunction, which plays a key role in the pathogenesis of atherosclerosis and its complications. Besides elevated low-density lipoprotein, low HDL has also been implicated in the high coronary heart disease risk associated with hypercholesterolemia. Low plasma HDL concentration has been reported to be an independent predictor of endothelial dysfunction (5,6). Moreover, elevation of plasma HDL levels by drug treatment (25) or infusion of synthetic HDL using apo A-I$_{WT}$/PC complex (26) leads to a significant improvement of impaired endothelial function. Taken together, these findings suggest a protective role of HDL against the development of endothelial dysfunction.

The precise cellular or subcellular mechanism by which apo A-I$_M$/PC restored endothelial function in our study is not fully understood. First, our current and previous data in mice (12,13) indicate that treatment with apo A-I$_M$/PC may accelerate reverse cholesterol transport in vivo as evidenced by depletion of aortic cholesterol/lipid content. Enhancement of reverse cholesterol transport may be sufficient to rapidly ameliorate the major sequelae of hypercholesterolemia including endothelial dysfunction, platelet hyperreactivity, and atherosclerosis.

Second, a direct effect on endothelial function via mod-

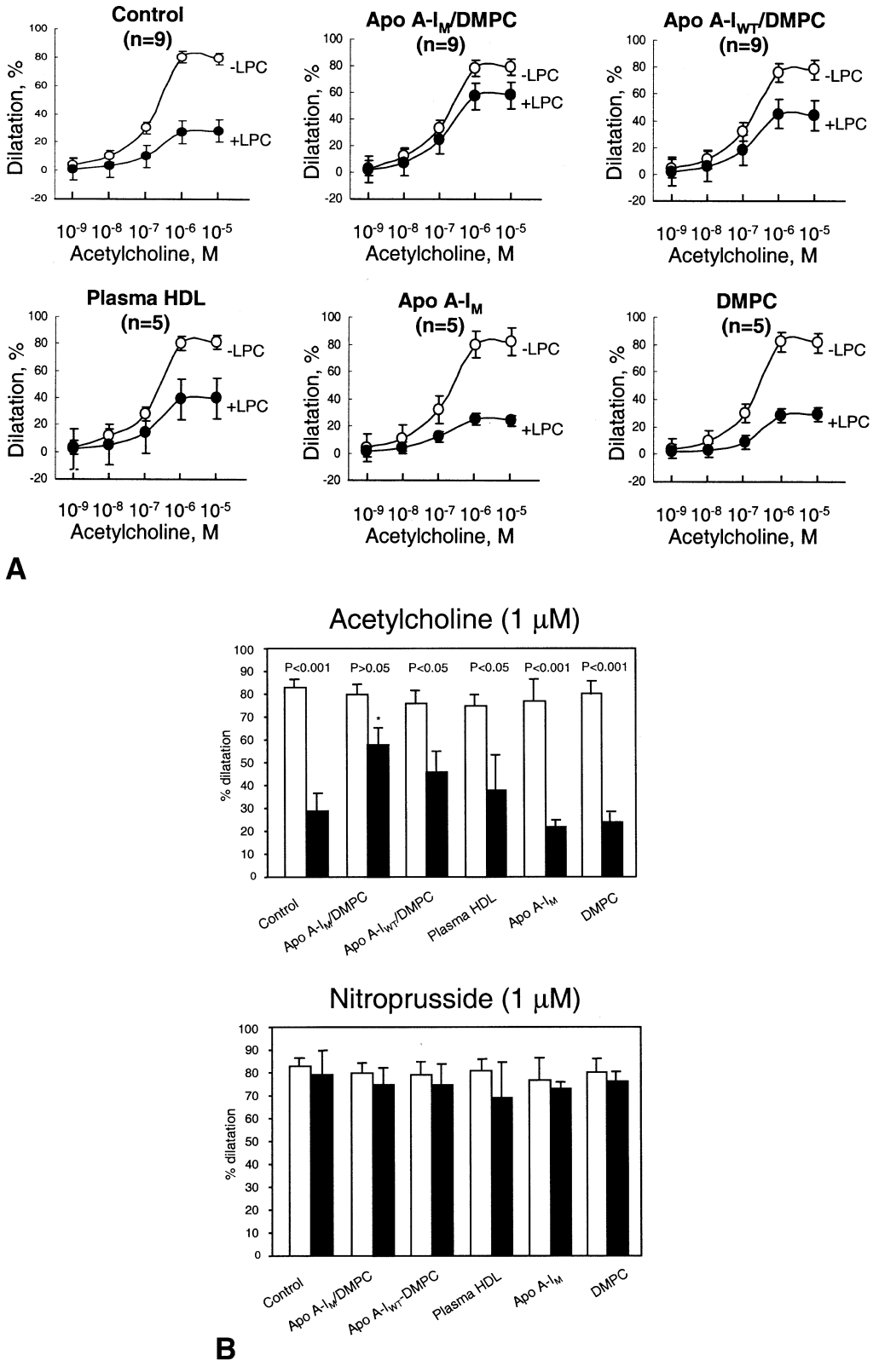

Figure 6. **(A)** Line plots showing concentration response to acetylcholine in arteries incubated with and without lysophosphatidylcholine (LPC) (50 μM) for 30 to 45 min under different treatment conditions—control, reconstituted high-density lipoprotein (HDL) using apo A-I$_{Milano}$ (apo A-I$_M$/DMPC), apo A-I$_{wild\text{-}type}$ (apo A-I$_{WT}$/DMPC), plasma-derived HDL, free apo A-I$_{Milano}$ without DMPC (apo A-I$_M$), and DMPC alone. A concentration of 1 mg/ml based on protein was used for all preparations. The concentration of DMPC was based on a protein:phospholipid 1:3 molar ratio. **(B)** Bar graphs showing maximal dilator response to acetylcholine **(top panel)** and sodium nitroprusside **(bottom panel)** in rabbit carotid arteries under different treatment conditions. Values (mean ± SEM) are percent change from a preconstricted diameter of 1.9 ± 0.1 mm. p values are based on comparisons before and after incubation with LPC. **Open bars** = −LPC (50 μM); **solid bars** = +LPC (50 μM). *p < 0.005 vs. control + LPC.

ulation of production or bioactivity of endothelium-derived factors, such as nitric oxide (EDNO) is also likely. Recent in vitro studies have suggested that HDL can enhance expression (25) and promote activation of eNOS (27), the rate-limiting enzyme for synthesis of EDNO. In vivo studies provide additional support to the concept that HDLs prevent endothelial dysfunction by promoting endothelial nitric oxide production (25). Most recently, intravenous infusion of apo A-I_{WT}/PC in hypercholesterolemic subjects rapidly restored the altered endothelium-dependent vasodilation by increasing nitric oxide bioavailability (26). Lack of effect on dilator responses in rabbit carotid arteries with preserved endothelial function are consistent with previous observations (17) and suggest that the protective effect of HDL appears in large part mediated by reversal of the suppressive effect of oxidized low-density lipoproteins or LPC on EDNO bioavailability.

Third, oxidized low-density lipoproteins are potent inducers of endothelial dysfunction, and there is evidence that the protective effects of HDL on endothelial function may be attributable, in part, to its capacity to neutralize the adverse effects of oxidized low-density lipoprotein. High-density lipoprotein has been shown to inhibit the oxidation of low-density lipoprotein (28,29). High-density lipoprotein can block harmful effects of oxidized low-density lipoprotein on endothelial cells via sequestration of oxidized lipids such as LPC from low-density lipoprotein (30) as well as prevention of its infiltration into vascular tissue (31), thereby limiting the inhibitory effect on endothelial function. The inhibitory effects of HDL have been attributed to its antioxidant effects via the binding of prooxidant transition metals by apo A-I and to paraoxonase and platelet-activating factor acetylhydrolase (32).

Our findings in rabbit arteries incubated with LPC corroborate previous observations (19,30,31) and also provide evidence for the first time for enhanced protective effects of reconstituted HDL using the recombinant apo A-I_M compared with apo A-I_{WT} or plasma-derived HDL. The cysteine-for-arginine substitution at position 173 in the amino acid sequence of recombinant apo A-I_M results, among other changes, in a higher antioxidant effect (33) and kinetic affinity for lipids and an easier dissociation from lipid/protein complexes (34), which might contribute to its increased efficiency for uptake of tissue lipids and cholesterol efflux (12–14), greater inhibitory effect on neointimal lesions in balloon-injured rabbit arteries (11), and augmented prevention of endothelial dysfunction compared with apo A-I_{WT} or HDL.

The fact that protective effects of apo A-I_M/PC on endothelial and platelet function were observed despite the persistence of hypercholesterolemia is concordant with previous observations and indicates that the effects are not mediated by a major reduction in severity of hypercholesterolemia. Consistent with previous observations (11–13), the current findings also confirm that, for optimal vascular biological effects, apo A-I_M complexed with phospholipid is essential compared with free protein or phospholipid alone. However, in the present study, phospholipid alone had a modest, but nonsignificant, effect in reducing aortic tissue cholesterol content and improving endothelium-dependent dilator function in mice, but not in rabbit arteries. Because enhanced reverse cholesterol transport by phospholipid liposomes is facilitated by HDL, modest effects of DPPC alone may have resulted from the low endogenous HDL levels in apo E-null mice in a manner consistent with the proposed model of HDL-mediated cholesterol efflux (35). This is supported by our previous observations of a transient increase in plasma-unesterified cholesterol levels (reflecting mobilization of tissue cholesterol) with DPPC treatment alone (13). The lack of an effect of treatment with phospholipid alone in rabbit arteries is due to the fact that arteries were treated in vitro with DMPC with no potential source of apo A-I available to form an HDL complex. In contrast, studies have shown regression of fatty streaks in rabbits (36) and marked improvement in endothelial function in apo E-null mice treated with high-dose phospholipid-liposomes alone (37). It is likely that difference in properties and dose of the phospholipid-liposomes may account for the contrasting findings.

The inhibitory effects on platelet aggregation in ApoE mice are consistent with previous in vitro observations in rats by Li et al. (17). The underlying mechanism is likely related to alterations in platelet membrane function, including stimulation of NO synthase (38), resulting from lipid fluxes induced by apo A-I_M/PC.

Potential clinical implications. Although lipid modification therapy favorably alters endothelial dysfunction, platelet hyperreactivity, and atherosclerotic plaque burden associated with hypercholesterolemia, such changes occur after months and years of therapy. By rapidly mobilizing vessel wall lipids, recombinant apo A-I_M has the potential to exert these beneficial effects in the short-term, thereby providing a promising therapeutic strategy for acute stabilization of unstable coronary heart disease.

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REFERENCES

1. Gordon DJ, Rifkind BM. High density lipoprotein: the clinical implications of recent studies. N Engl J Med 1989;321:1311–6.
2. Badimon JJ, Badimon L, Fuster V. Regression of atherosclerotic lesions by high-density lipoprotein plasma fraction in the cholesterol-fed rabbit. J Clin Invest 1990;85:1234–41.
3. Plump AS, Scott CJ, Breslow JL. Human apolipoprotein A-I gene expression increases high density lipoprotein and suppresses atherosclerosis in the apolipoprotein E-deficient mouse. Proc Natl Acad Sci USA 1994;91:9607–11.
4. von Eckardstein A, Nofer JR, Assmann G. High density lipoproteins and arteriosclerosis: role of cholesterol efflux and reverse cholesterol transport. Arterioscler Thromb Vasc Biol 2001;21:13–27.
5. Zeiher AM, Schachlinger V, Hohnloser SH, Saurbier B, Just H. Coronary atherosclerotic wall thickening and vascular reactivity in

humans: elevated high-density lipoprotein levels ameliorate abnormal vasoconstriction in early atherosclerosis. Circulation 1994;89:2525–32.
6. Lupattelli G, Marchesi S, Roscini A, et al. Direct association between high-density lipoprotein cholesterol and endothelial function in hyperlipemia. Am J Cardiol 2002;90:648–50.
7. Naqvi TZ, Shah PK, Ivey PA, et al. Evidence that high-density lipoprotein cholesterol is an independent predictor of acute platelet-dependent thrombus formation. Am J Cardiol 1999;84:1011–7.
8. Weisgraber KH, Bersot TP, Mahley RW, Franceschini G, Magani D, Sirtori CR. Isolation and characterization of a cysteine-containing variant of the apoprotein A-1 from high density lipoprotein. J Clin Invest 1980;66:901–7.
9. Franceschini G, Sirtori CR, Capurso A, et al. A-I Milano apoprotein: decreased high-density lipoprotein cholesterol levels with significant lipoprotein modifications and without clinical atherosclerosis in an Italian family. J Clin Invest 1980;66:892–900.
10. Sirtori CR, Calabresi L, Franceschini G, et al. Cardiovascular status of carriers of the apolipoprotein A-I$_{Milano}$ mutant: the Limone sul Garda study. Circulation 2001;103:1949–54.
11. Ameli S, Hultgardh-Nilsson A, Cercek B, et al. Recombinant apolipoprotein A-I Milano reduces intimal thickening after balloon injury in hypercholesterolemic rabbits. Circulation 1994;90:1935–41.
12. Shah PK, Nilsson J, Kaul S, et al. Effects of recombinant apolipoprotein A-I$_{Milano}$ on aortic atherosclerosis in apolipoprotein E-deficient mice. Circulation 1998;97:780–5.
13. Shah PK, Yano J, Reyes O, et al. High-dose recombinant apolipoprotein A-I$_{Milano}$ mobilizes tissue cholesterol and rapidly reduces plaque lipid and macrophage content in apolipoprotein E-deficient mice: potential implications for acute plaque stabilization. Circulation 2001; 103:3047–50.
14. Chiesa G, Monteggia E, Marchesi M, et al. Recombinant apolipoprotein A-I$_{Milano}$ infusion into rabbit carotid artery rapidly removes lipid from fatty streaks. Circ Res 2002;90:974–80.
15. Soma MR, Donetti E, Parolini C, Sirtori CR, Fumagalli R, Franceschini G. Recombinant apolipoprotein A-I$_{Milano}$ dimer inhibits carotid intimal thickening induced by perivascular manipulation in rabbits. Circ Res 1995;76:405–11.
16. Kaul S, Rukshin V, Santos R, et al. Intramural delivery of recombinant apolipoprotein A-I$_{Milano}$/phospholipid complex (ETC-216) inhibits in-stent stenosis in porcine coronary arteries. Circulation 2003;107: 2551–4.
17. Li D, Weng S, Yang B, et al. Inhibition of arterial thrombus formation by Apo A1 Milano. Arterioscler Thromb Vasc Biol 199;19:378–83.
18. Nissen SE, Tsunoda T, Tuzcu EM, et al. Effect of recombinant Apo A-I$_{Milano}$ on coronary atherosclerosis in patients with acute coronary syndromes: a randomized controlled trial. JAMA 2003;290:2322–4.
19. Kugiyama K, Kerns SA, Morrisett JD, et al. Impairment of endothelium-dependent arterial relaxation by lysolecithin in modified low-density lipoproteins. Nature 1990;344:160–2.
20. Kaul S, Waack BJ, Padgett RC, Brooks RM, Heistad DD. Altered vascular responses to platelets from hypercholesterolemic humans. Circ Res 1993;72:737–43.
21. Havel RJ, Eder HA, Bragdon JH. The distribution and chemical composition of ultracentrifugally separated lipoproteins in human serum. J Clin Invest 1955;34:1345–53.
22. Lowry OH, Rosenbrough NJ, Farr AL, Randall RJ. Protein measurement with Folin Phenol reagent. J Biol Chem 1951;193:265.
23. Folch J, Lees M, Slona GH. A simple method for isolation and purification of total lipids from animal tissue. J Biol Chem 1957;226: 497–509.
24. Stoll LL, Oskarsson HJ, Spector AA. Interaction of lysophosphatidylcholine with aortic endothelial cells. Am J Physiol 1992:262: H1853–60.
25. Kuvin JT, Ramet ME, Patel AR, Pandian NG, Mendelsohn ME, Karas RH. A novel mechanism for the beneficial vascular effects of high-density lipoprotein cholesterol: enhanced vasorelaxation and increased endothelial nitric oxide synthase expression. Am Heart J 2002;144:165–72.
26. Spieker LE, Sudano I, Hurlimann D, et al. High-density lipoprotein restores endothelial function in hypercholesterolemic men. Circulation 2002;105:1399–402.
27. Yuhanna IS, Zhu Y, Cox BE, et al. High-density lipoprotein binding to scavenger receptor-BI activates endothelial nitric oxide synthase. Nat Med 2001;7:853–7.
28. Parthasarathy S, Barnett J, Fong LG. High density llipoprotein inhibits the oxidative modification of low-density lipoprotein. Biochem Biophys Acta 1990;1044:275–83.
29. Toikka JO, Ahotupa M, Viikari JS, et al. Constantly low HDL-cholesterol concentration relates to endothelial dysfunction and increased in vivo LDL-oxidation in healthy young men. Atherosclerosis 1999;147:133–8.
30. Matsuda Y, Hirata K, Inoue N, Suematsu M, Kawashima S, Akita H. High density lipoprotein reverses inhibitory effect of oxidized low-density lipoprotein on endothelium-dependent arterial relaxation. Circ Res 1993;72:1103–9.
31. Galle J, Ochlslen M, Schollmeyer P, Wanner C. Oxidized lipoproteins inhibit endothelium-dependent vasodilatation: effects of pressure and high-density lipoprotein. Hypertension 1994;23:556–64.
32. Mackness MI, Durrington PN, Mackness B. How high-density lipoprotein protects against the effects of lipid peroxidation. Curr Opin Lipidol 2000;11:383–8.
33. Bielicki JK, Oda MN. Apolipoprotein A-I$_{Milano}$, and apolipoprotein A-IParis exhibit an antioxidant activity distinct from that of wild-type apolipoprotein A-I. Biochemistry 2002;41:2089–96.
34. Franceschin G, Vecchio G, Gianfranceschi G, Magani D, Sirtori CR. Apolipoprotein A-1 Milano: accelerated binding and dissociation from lipids of a human apolipoprotein variant. J Biol Chem 1985;260: 16321–5.
35. Franceschini G, Maderna P, Sirtori CR. Reverse cholesterol transport: physiology and pharmacology. Atherosclerosis 1991;88:99–107.
36. Rodrigueza WV, Klimuk SK, Pritchard PH, et al. Cholesterol mobilization and regression of atheroma in cholesterol-fed rabbits induced by large unilamellar vesicles. Biochem Biophys Acta 1998; 1368:306–20.
37. Williams KJ, Scalia R, Mazany KD, Rodrigueza WV, Lefer AM. Rapid restoration of normal endothelial functions in genetically hyperlipidemic mice by a synthetic mediator of reverse lipid transport. Arterioscler Thromb Vasc Biol 2000;20:1033.
38. Chen LY, Mehta JL. Inhibitory effect of high-density lipoprotein on platelet function is mediated by increase in nitric oxide synthase activity in platelets. Life Sci 1994;55:1815–21.