

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

Prediman K. Shah, MD; Juliana Yano, BS; Odette Reyes, BS; Kuang-Yuh Chyu, MD, PhD; Sanjay Kaul, MD; Charles L. Bisgaier, PhD; Sandra Drake, PhD; Bojan Cercek, MD, PhD

Background—Repeated doses of recombinant apolipoprotein A-I_{Milano} phospholipid complex (apoA-I_m) reduce atherosclerosis and favorably change plaque composition in rabbits and mice. In this study, we tested whether a single high dose of recombinant apoA-I_m could rapidly mobilize tissue cholesterol and reduce plaque lipid and macrophage content in apoE-deficient mice.

Methods and Results—High cholesterol-fed, 26-week-old apoE-deficient mice received a single intravenous injection of saline (n=16), 1080 mg/kg dipalmitoylphosphatidylcholine (DPPC; n=14), or 400 mg/kg of recombinant apoA-I_m complexed with DPPC (1:2.7 weight ratio; n=18). Blood was sampled before and 1 and 48 hours after injection, and aortic root plaques were evaluated for lipid content and macrophage content after oil-red O and immunostaining, respectively. One hour after injection, the plasma cholesterol efflux-promoting capacity was nearly 2-fold higher in recombinant apoA-I_m-treated mice compared with saline and DPPC-treated mice ($P<0.01$). Compared with baseline values, serum free cholesterol, an index of tissue cholesterol mobilization, increased 1.6-fold by 1 hour after recombinant apoA-I_m injection, and it remained significantly elevated at 48 hours ($P<0.01$). Mice receiving recombinant apoA-I_m had 40% to 50% lower lipid content ($P<0.01$) and 29% to 36% lower macrophage content ($P<0.05$) in their plaques compared with the saline- and DPPC-treated mice, respectively.

Conclusions—A single high dose of recombinant apoA-I_m rapidly mobilizes tissue cholesterol and reduces plaque lipid and macrophage content in apoE-deficient mice. These findings suggest that this strategy could rapidly change plaque composition toward a more stable phenotype. (*Circulation*. 2001;103:3047-3050.)

Key Words: apolipoproteins ■ cholesterol ■ atherosclerosis

Apolipoprotein A-I_{Milano} (apoA-I_m) is a naturally occurring mutant of apoA-I, with a cysteine to arginine substitution at position 173, that is associated with freedom from vascular disease and longevity in its carriers, despite markedly reduced HDL and elevated triglyceride levels.¹ We have previously demonstrated that repeated administration of recombinant apoA-I_{Milano} complexed with dipalmitoylphosphatidylcholine (DPPC) significantly reduces neointimal lesions in the balloon-injured ileofemoral arteries of cholesterol-fed rabbits and reduces atherosclerosis progression, plaque lipid content, and inflammation in apoE-deficient mice.^{2,3} Similar results have also been reported by Soma et al⁴ using a periaortic carotid injury model in cholesterol-fed rabbits. In the present study, we assessed whether a large single dose of recombinant apoA-I_m could rapidly mobilize tissue chole-

sterol and reduce lipid and macrophage contents in aortic atherosclerotic plaques in apoE-deficient mice.

Methods

ApoE-deficient mice (C57BL/6J strain; age, 5 weeks; weight, 18 to 20 g) obtained from Jackson Laboratory (Bar Harbor, Maine) were fed a high-fat, high-cholesterol (atherogenic) diet containing 21% (wt/wt) fat and 0.15% cholesterol throughout the duration of the experiment. At 26 weeks of age, mice were given a single intravenous injection of 0.5 mL of saline (n=16), 1080 mg/kg of DPPC hydrated in saline (n=14), or 400 mg/kg of recombinant apoA-I_m complexed with DPPC in a protein-to-phospholipid ratio of 1:2.7 by weight and dissolved in 0.5 mL saline (n=18) through the tail vein. 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.

Received March 16, 2001; revision received May 1, 2001; accepted May 1, 2001.

From the Atherosclerosis Research Center, the Division of Cardiology, Cedars-Sinai Medical Center and UCLA School of Medicine, Los Angeles, Calif and Esperion Therapeutics Inc, Ann Arbor, Mich (C.L.B., S.D.).

Drs Bisgaier and Drake are employees of Esperion Therapeutics, Inc, a commercial enterprise involved in developing products to prevent and treat cardiovascular diseases, that makes the apoA-I_{Milano}/DPPC described in the article.

Correspondence to Prediman K. Shah, MD, Cedars-Sinai Medical Center, Room # 5347, 8700 Beverly Boulevard, Los Angeles, CA 90048. E-mail shahp@cshs.org

© 2001 American Heart Association, Inc.

Circulation is available at <http://www.circulationaha.org>

Effects of a Single Dose of Saline, DPPC, or Recombinant ApoA-I_m on Lipoproteins, Efflux Capacity, Plaque Lipid, and Macrophage Content in ApoE-Deficient Mice

Variables	Saline	DPPC	Recombinant ApoA-I _m
Before treatment	(n=12)	(n=12)	(n=11)
Total cholesterol, mg/dL	818±126	729±199	696±312
Free cholesterol, mg/dL	368±55	316±82	300±115
Esterified cholesterol, mg/dL	450±88	412±131	395±199
ApoA-I _m levels, μg/dL	0	0	0
1 Hour after treatment			
Total cholesterol, mg/dL	703±131	993±352	1377±345*
Free cholesterol, mg/dL	322±76	495±182†	742±178*
Esterified cholesterol, mg/dL	381±76	497±176	635±184*
ApoA-I _m levels, μg/dL	0	0	4767±1503
48 Hours after treatment			
Total cholesterol, mg/dL	621±158	656±190	1085±263*
Free cholesterol, mg/dL	300±81	365±112	812±168*
Esterified cholesterol, mg/dL	321±87	291±86	272±125
ApoA-I _m levels, μg/dL	0	0	552±136
Cholesterol efflux-promoting capacity at 1 hr, % efflux during 4 hours of incubation	(n=12) 25±3	(n=12) 18±2§	(n=12) 40±3‡
Plaque lipid content at 48 hr, % of plaque area	(n=16) 19.6±6.3	(n=14) 18.1±4.7	(n=18) 10.1±4.2*
Plaque macrophage content at 48 hr, % of plaque area	(n=12) 10.4±3.4	(n=9) 9.3±5.8	(n=12) 6.4±2.0§

Values are mean±SD.

* $P<0.01$ vs saline and DPPC; † $P<0.05$ vs saline; ‡ $P<0.001$ vs saline and DPPC; § $P<0.01$ vs saline.

Blood samples were taken before and 1 and 48 hours after injection in EDTA-treated microtainer tubes (Becton Dickinson) and stored at -70°C until analysis. Mice were euthanized 48 hours after injection. Total and free cholesterol levels were measured by enzymatic techniques, and fractional analysis of lipoproteins was performed by online high-performance gel-filtration chromatography. Cholesterol efflux-promoting capacity was determined in the serum 1 hour after injection using the technique described by de La Llera Moya et al.⁵ The circulating levels of apoA-I_m were determined by ELISA, as previously described³

Tissue Preparation and Histological Analysis

After anesthesia with enflurane, mice were euthanized, and their hearts and aortas were perfusion-fixed and harvested as described previously.³ The lipid and macrophage contents of aortic root plaques was measured as described in detail elsewhere.³

Statistical Analysis

Data are presented as mean±SD. For group comparisons, ANOVA was followed by Tukey's test, with $P\leq 0.05$ considered significant.

Results

Changes in Cholesterol Efflux-Promoting Capacity

One hour after injection, the cholesterol efflux-promoting capacity of the plasma was nearly 2-fold higher in mice receiving recombinant apoA-I_m compared with mice receiving saline or DPPC alone (Table).

Circulating Cholesterol and ApoA-I_m Levels

Circulating cholesterol levels before treatment were similar in the 3 groups. One hour after injection, the total, free, and esterified cholesterol levels increased significantly in mice

receiving recombinant apoA-I_m, and they remained elevated at 48 hours. There was no significant change in these levels in mice receiving saline alone. There was a modest increase in total and free cholesterol levels at 1 hour after administration of DPPC, with a return to baseline levels by 48 hours (Table). High-performance gel-filtration chromatography showed that 1 hour after the administration of recombinant apoA-I_m, the increase in free cholesterol was associated with the HDL fraction, but by 48 hours, it was associated with LDL and VLDL fractions (Figure, A). ApoA-I_m levels averaged 477 mg/dL at 1 hour but dropped to 55 mg/dL at 48 hours.

Lipid Content in Aortic Sinus Plaque

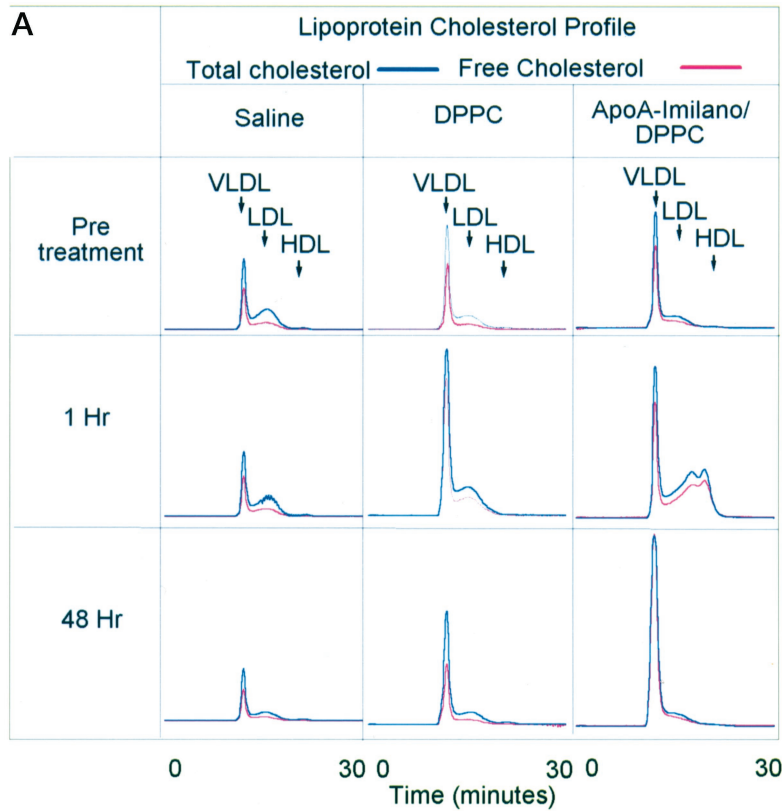
The lipid content in the aortic sinus plaque was 40% to 50% less in mice receiving recombinant apoA-I_m compared with mice receiving saline alone or DPPC alone ($P<0.001$; Table and Figure, B).

Macrophage Content in Aortic Sinus Plaque

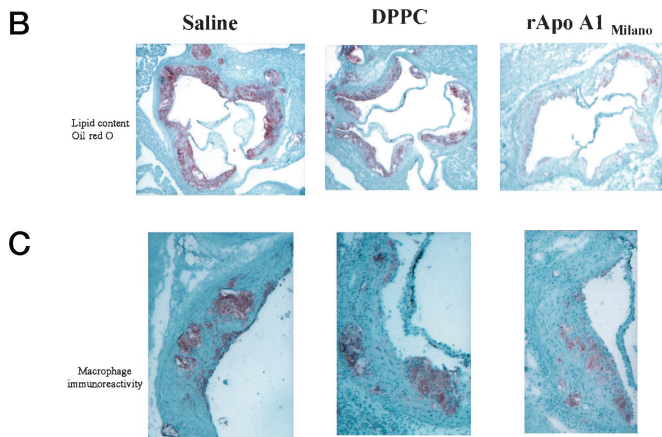
The macrophage content was 29% to 36% less in mice receiving recombinant apoA-I_m compared with mice receiving saline ($P<0.05$; Table and Figure, C).

Discussion

This study demonstrates that a single large dose of recombinant apoA-I_m rapidly increases the cholesterol efflux-promoting capacity, mobilizes tissue cholesterol, and reduces plaque lipid and macrophage contents within 48 hours in apoE-deficient mice. Because of the close similarity of lesions at 26



Representative examples of lipoprotein gel-filtration profile (A) and cross-sections of aortic root are shown to demonstrate the effects of a single injection of saline, DPPC, and recombinant apoA-I_m on plaque lipid content (B) and macrophage immunoreactivity (C). See text for details.



weeks to those of advanced human atherosclerosis, we chose to evaluate the effects of recombinant apoA-I_m in this model⁶

Stimulation of Tissue Cholesterol Mobilization by ApoA-I_m

The favorable effects of HDL and apoA-I have been attributed, in part, to the promotion of reverse cholesterol transport.⁷ One of the first steps in reverse cholesterol transport is the mobilization of free cholesterol from tissues, including the arterial wall, by nascent phospholipid-rich but cholesterol-poor apoA-I-containing lipoproteins.⁷ According to a recent model, apoA-I acts as an acceptor and the phospholipid component of HDL acts as a sink for the mobilized cholesterol.⁸ Thus, the complex of recombinant apoA-I_m and DPPC simulates a nascent HDL-like particle with the ability to mobilize cholesterol from peripheral tissues. A rapid increase

in circulating free cholesterol levels within 1 hour of the administration of recombinant apoA-I_m, which was largely HDL-associated, is consistent with the stimulation of cholesterol mobilization from peripheral tissues into the circulating blood. These observations are further supported by our in vitro findings showing a significantly higher cholesterol efflux-promoting capacity in the plasma of mice receiving recombinant apoA-I_m compared with mice receiving saline or DPPC alone. The association of increased cholesterol levels with the LDL and VLDL fraction observed at 48 hours after recombinant apoA-I_m injection may be related to the transfer of mobilized cholesterol to these lipoproteins or enhanced uptake and secretion of cholesterol from the liver.

Although we have not analyzed all of the potential tissues from which cholesterol mobilization is stimulated by recombinant apoA-I_m, our findings of a significant reduction in lipid

content in aortic atheromatous lesions suggests that at least part of the mobilized cholesterol is originating from the vessel wall. Recently, a bolus dose of human HDL was shown to stimulate cholesterol efflux from the heart, skeletal muscle, lung, spleen, and liver but not from the brain in normal mice.⁹ Accumulation of lipid and its subsequent modification within atherosclerotic plaques is thought to play a major role in the recruitment and retention of inflammatory cells in atherosclerosis, and thus a reduction in the plaque lipid may explain the significant decrease in macrophage immunoreactivity after recombinant apoA-I_m administration.¹⁰

It is likely that most of the mobilized cholesterol is delivered to the liver because the liver plays an important role in eliminating cholesterol through biliary sterol excretion. Enhanced cholesterol mobilization in normal subjects and increased fecal sterol excretion in hyperlipidemic patients after HDL administration has recently been demonstrated.^{11,12}

In the present study, DPPC alone had a small effect, although phospholipid-liposomes have been shown to regress fatty streaks in rabbits.¹³ 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 apoE-deficient mice in a manner consistent with the proposed model of HDL-mediated cholesterol efflux.⁸

Potential Limitations

Additional studies are required to determine the precise sources and the fate of the mobilized cholesterol and to compare the effects with wild-type apoA-I.

Potential Clinical Implications

Although statin therapy favorably alters plaque composition, such changes occur after weeks and months of therapy.^{14,15} By rapidly mobilizing vessel wall lipids, recombinant apoA-I_m has the potential to stabilize plaques in the short-term.¹⁴

Acknowledgments

This study was supported in part by the United Hostesses Charities and the Ralph M. Parson's Foundation. The recombinant apoA-I_m-DPPC complex was a generous gift from Guido Franceschini, PhD,

of the University of Milan. The assistance of Jenny Zhu and Helen Xu is acknowledged.

References

- 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.
- 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;90:1935-1941.
- 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;97:780-785.
- Soma MR, Donetti E, Parolini C, et al. Recombinant apolipoprotein A-I Milano dimer inhibits carotid intimal thickening induced by perivascular manipulation in rabbits. *Circ Res*. 1995;76:405-411.
- de la Llera Moya M, Atger V, Paul JL, et al. A cell culture system for screening human serum for ability to promote cellular cholesterol efflux: relations between serum components and efflux, esterification, and transfer. *Arterioscler Thromb Vasc Biol*. 1994;14:1056-1065.
- Qiao JH, Xie PZ, Fishbein MC, et al. Pathology of atheromatous lesions in inbred and genetically engineered mice. *Arterioscler Thromb Vasc Biol*. 1994;14:1480-1497.
- Franceschini G, Maderna P, Sirtori CR. Reverse cholesterol transport: physiology and pharmacology. *Atherosclerosis*. 1991;88:99-107.
- Rodrigueza WV, Williams KJ, Rothblatt GH, et al. Rempdelling and shuttling: mechanisms for the synergistic effects between different acceptor particles in the mobilization of cellular cholesterol. *Arterioscler Thromb Vasc Biol*. 1997;17:383-393.
- Alam K, Meidell RS, Spady DK. Effects of upregulating individual steps in the reverse cholesterol transport pathway on reverse cholesterol transport in normolipidemic mice. *J Biol Chem*. 2001;276:15641-15649.
- Nawab M, Berliner JA, Watson AD, et al. The yin and yang of oxidation in the development of the fatty streak. *Arterioscler Thromb Vasc Biol*. 1996;16:831-842.
- Nanjee MN, Doran JE, Lerch PG, et al. Acute effects of intravenous infusion of Apo A-I/phosphatidylcholine discs on plasma lipoproteins in humans. *Arterioscler Thromb Vasc Biol*. 1999;19:979-989.
- Eriksson M, Carlson LA, Miettinen TA, et al. Stimulation of fecal sterol excretion after infusion of recombinant proapolipoprotein A-I: potential reverse cholesterol transport in humans. *Circulation*. 1999;100:594-598.
- Rodrigueza WV, Klimuk SK, Pritchard PH, et al. Cholesterol mobilization and regression of atheroma in cholesterol-fed rabbits induced by large unilamellar vesicles. *Biochim Biophys Acta*. 1998;1368:306-320.
- Crisby M, Nordin-Fredriksson G, Shah PK, et al. Pravastatin treatment increases collagen content, and decreases lipid content, inflammation, metalloproteinases, and cell death in human carotid plaques: Implications for plaque stabilization. *Circulation*. 2001;103:926-933.
- Fukumoto Y, Libby P, Rabkin E, et al. Statins alter smooth muscle cell accumulation and collagen content in established atheroma of Watanabe heritable hyperlipidemic rabbits. *Circulation*. 2001;103:993-999.