Original Research

Synthetic dimyristoylphosphatidylcholine liposomes assimilating into high-density lipoprotein promote regression of atherosclerotic lesions in cholesterol-fed rabbits

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Abstract

We have reported recently that enrichment of high-density lipoprotein (HDL) with phosphatidylcholine (PC) liposomes is effective in solubilizing cholesterol from isolated human atherosclerotic plaques. In the present study, we investigated the *in vivo* effect of enrichment of HDL with PC on regression of diet-induced atherosclerosis in rabbits. As part of the study, a preliminary *in vitro* study on blood collected from the cholesterol-fed rabbits was performed to assess the capacity of the HDL density ($d > 1.063$ g/mL) plasma fraction from cholesterol-fed rabbits to assimilate multilamellar liposomes of synthetic dimyristoylphosphatidylcholine (DMPC). This was compared with the capacities of egg- and soy-PC liposomes to be assimilated into the HDL density plasma fraction. The capacity of the HDL density fraction to absorb PC from DMPC liposomes (11.5 mg/mL) was more than 10 times greater than egg or soy liposomes. Therefore, DMPC liposomes were chosen to infuse into cholesterol-fed rabbits. Cholesterol-fed rabbits infused weekly with DMPC liposomes (300 mg/kg body weight) for five weeks had significantly decreased aortic cholesterol contents ($P < 0.05$) compared with saline-infused cholesterol-fed controls. Atherosclerotic plaque volume, as measured by a type of new magnetic resonance imaging analysis, also decreased significantly ($P < 0.05$) after DMPC treatment. The present findings suggest that the enrichment of HDL with PC via intravenous infusion of synthetic DMPC liposomes could be a potential therapeutic approach for atherosclerotic plaque regression.

Keywords: atherosclerotic lesions, dimyristoylphosphatidylcholine, high-density lipoprotein, liposomes, plaque regression

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Introduction

Numerous epidemiological and clinical studies indicate that high plasma high-density lipoprotein (HDL) levels have a protective effect against the development of coronary heart disease (CHD).[1–3] This protective effect is usually attributed to the role of HDL in removing excess cholesterol from atherosclerotic lesions and transporting it to the liver, a process called reverse cholesterol transport (RCT).[4,5] In experimental animals, increased HDL levels induced by over-expression of the apolipoprotein (apo) A-I gene or induced by intravenous infusion of native and recombinant HDL or apo A-I were shown to cause regression or suppression of atherosclerosis.[6–10] In humans, a pharmacological increase in plasma HDL level in patients with established CHD reduces the occurrence of subsequent cardiovascular events.[11] These observations suggest that increasing HDL or apo A-I could be a major therapeutic strategy for treating atherosclerotic disease.

The HDLs are a family of lipoproteins containing apo A-I and phospholipids (PLs) as the principal constituents. It is generally believed that the antiatherogenic function of HDL is mediated by apo A-I because apo A-I is necessary to initiate RCT by three pathways. The first is by recruiting PLs and free cholesterol (FC) from cell membranes via ATP-binding cassette AI (ABCAI) transporter.[12] The second mechanism functions by providing a nidus for the formation, via pre-β-migrating HDL, of α-migrating spheroidal HDLs. These serve as acceptors of cholesterol released

from cell membranes via scavenger receptor BI (SR-BI) and ATP-binding cassette GI (ABCGI) transporter.[13,14] The third pathway is by activating lecithin cholesterol acyltransferase (LCAT), an enzyme that promotes net cholesterol efflux by generating a concentration gradient of FC between cell membranes and plasma via esterification of FC on HDL.[4] Although the PL moiety, rather than the apo A-I moiety, is the actual HDL component that binds cholesterol, the relative contribution of PL and apo A-I moieties to the antiatherogenic functions of HDL has not been fully evaluated.

The ability of HDL to promote cholesterol efflux from cultured cells *in vitro* correlates better with HDL-PL content than apo A-I or cholesterol content.[15,16] Furthermore, this efflux can be increased with enrichment of HDL with PLs, but not after enrichment with apo A-I.[17,18] In humans, HDL-PL levels are inversely related to the severity of angiographically defined coronary artery disease.[19] In experimental animals, an increase in plasma PL levels via infusion of various natural phosphatidylcholine (PC) liposomes promotes the mobilization of tissue cholesterol and regression of atherosclerosis.[20–23] Although the intestinal breakdown of dimyristoylphosphatidylcholine (DMPC) was not addressed, a recent study[24] reported that the oral feeding of DMPC, but not the feeding of egg PC or soy PC, prevented the development or caused the regression of atherosclerotic lesions in apo E-null mice. This suggests that the biological properties of synthetic DMPC may differ from those of natural egg or soy PC, or, that the effect of PCs may differ among species. Studies using reconstituted HDL composed of various apolipoproteins of HDL and PC with differing fatty acyl chain composition showed that the potency of reconstituted HDL in promoting cholesterol efflux from cultured cells and regression of atherosclerotic lesions *in vivo* was altered by compositional changes in PLs but not by the substitution of apolipoproteins on the reconstituted HDL.[10,25–27] The above observations raise the possibility that the PL moiety of HDL could have a significant impact on the antiatherogenic function of HDL.

We reported recently that fresh plasma or isolated native HDL and its lipid-free apolipoproteins (apo HDL) were not effective in releasing cholesterol from insoluble cholesterol-rich atherosclerotic plaques isolated from human atherosclerotic aortas.[28] However, the HDL or apo HDL became effective when PL content of HDL was increased or when apo HDL was associated with PC via solubilization of multilamellar DMPC liposomes. We further found that DMPC-rich HDL and apo HDL–DMPC complexes, but not native HDL or apo HDL, allowed the solubilization of cholesterol crystals in atherosclerotic plaques by transferring DMPC onto atherosclerotic plaques *in vitro*.[28]

In the present study, we further investigated *in vivo* the effect of intravenous infusion of DMPC liposomes on the regression of diet-induced atherosclerosis in rabbits. Although infusion of several natural PCs has been shown previously to regress atherosclerotic lesions,[20–22] we have chosen the synthetic DMPC as a source of PC liposomes because DMPC liposomes, unlike other natural PC liposomes, have an unique property of rapidly assimilating onto HDL by completely dissolving their vesicular structures upon their exposure to plasma,[29,30] and the effect of intravenous infusion of pure DMPC on the regression of atherosclerosis has not been examined previously.

Materials and methods

Materials

1,2-Dimyristoyl-sn-glycero-3-phophocholine (DMPC, >99% purity), egg PC and soy PC were purchased from Avanti Polar Lipids, Inc (Alabaster, AL, USA). DMPC liposomes were freshly and aseptically prepared by hydrating DMPC powder in sterile phosphate-buffered saline (PBS). Briefly, DMPC was weighed into sterile glass tubes and hydrated by mixing with sterile PBS (pH 7.4). The hydrated DMPC in PBS was then sonicated three times for 60 s in an ice bath. The final concentration of DMPC was adjusted to 200 mg/mL solution. Enzymatic kits for total cholesterol (TC), FC, PLs and triglycerides (TGs) were purchased from Wako Diagnostics (Richmond, VA, USA). Male New Zealand White rabbits were purchased from Myrtle's Rabbitry (Thompson, TN, USA).

Experimental protocol

Male New Zealand White rabbits, eight weeks old (4–5 lbs), were housed in stainless-steel cages, and all studies were performed with the approval of the University of Illinois Institutional Animal Care and Use Committee. Animals were maintained in a temperature-controlled room with a 12 h dark/light cycle. Initially, rabbits ($n = 18$) were fed *ad libitum* an atherogenic diet containing 2% cholesterol and 6% peanut oil (Teklad diet 91314) for eight weeks to develop atherosclerosis (Figure 1). Thereafter, the diet was replaced with a normal rabbit chow. Among the rabbits, five pairs of rabbits with matching levels of plasma cholesterol were then selected and divided into two groups for treatment either with saline ($n = 5$) or with DMPC ($n = 5$). At week 13, each rabbit in the DMPC-treated group was injected intravenously once weekly with DMPC liposomes (300 mg/kg body weight in total volume of 5–7 mL) for five weeks (a total of 5 injections), using a butterfly catheter in the ear vein, while the control rabbits were injected with the same volume of normal saline. Both groups of rabbits were euthanized a week after the last injection of DMPC or saline. Another group of rabbits ($n = 5$) that was fed only a normal rabbit chow throughout the study served as negative controls and was euthanized at the same time as the infused rabbits for sample collection as for the other two groups.

Analysis of plasma lipids and lipoprotein cholesterol

Prior to euthanasia and after a 12-h fast, blood was drawn from the marginal ear veins of rabbits and placed into tubes containing 0.1% ethylenediaminetetraacetic acid. Concentrations of plasma TC, PLs and TGs were measured by colorimetric methods using enzymatic assay kits obtained from Waco Diagnostics (Richmond, VA, USA).

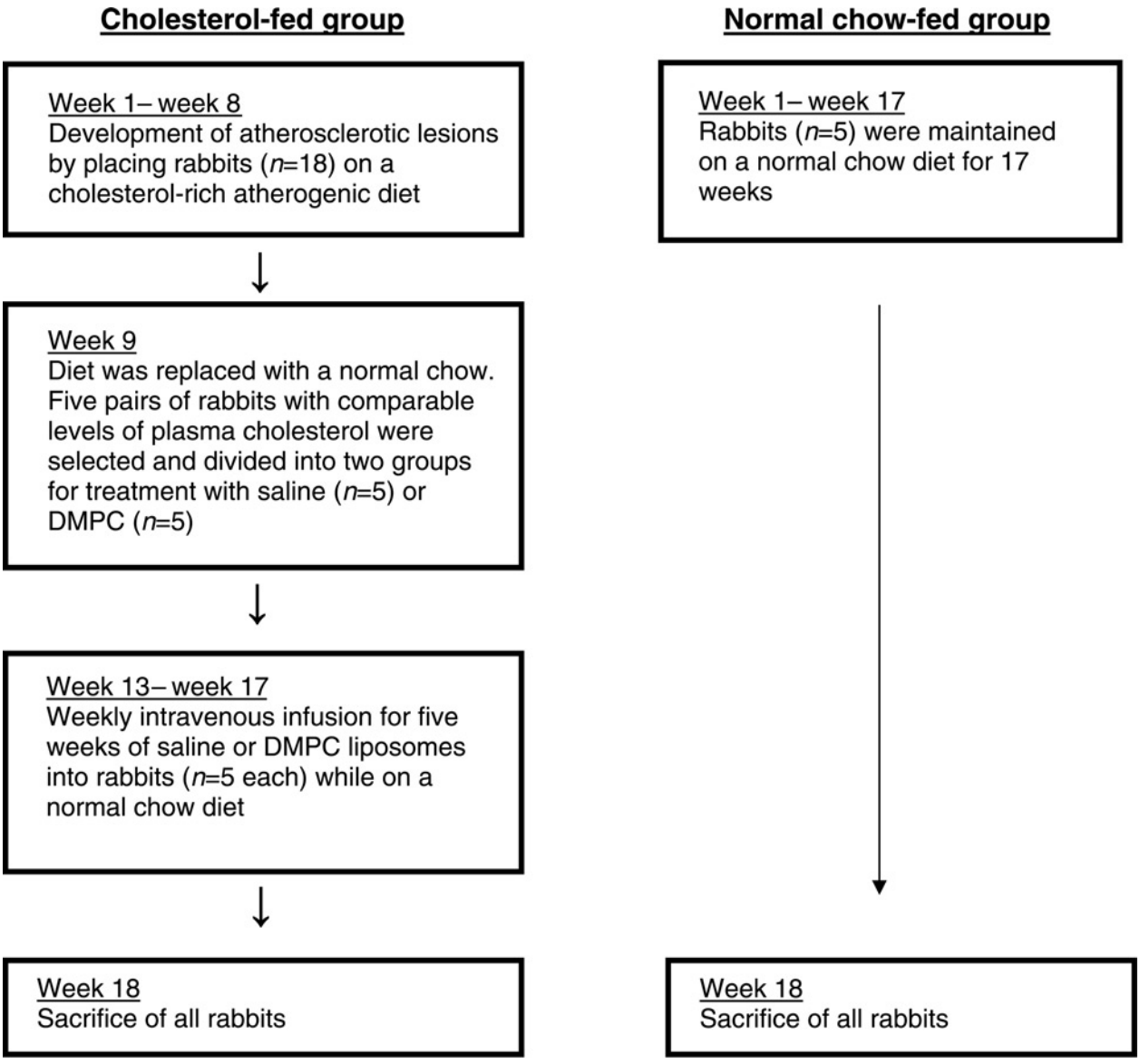

Figure 1 Experimental design for the *in vivo* study to investigate the effects of DMPC liposomal infusion on regression of atherosclerosis in rabbits. DMPC, dimyristoylphosphatidylcholine

The levels and distribution of cholesterol among very-low-density lipoprotein (VLDL), low-density lipoprotein (LDL) and HDL density fractions in pooled plasma of the five rabbits were measured following fractionation of the major class of lipoproteins in plasma by the density gradient ultracentrifugation method[31] and subsequent measurement of cholesterol levels on each density gradient fraction using the enzymatic cholesterol assay reagent.

Assimilation of synthetic DMPC, egg and soy PC liposomes into HDL density ($d > 1.063$ g/mL) plasma fraction from cholesterol-fed rabbits

The blood plasma obtained from cholesterol-fed rabbits ($n = 10$) selected for saline/DMPC treatments were pooled and the density of pooled plasma was adjusted to $d = 1.063$ g/mL by adding solid KBr. The HDL density ($d > 1.063$ g/mL) plasma fraction containing HDL and lipid-free plasma proteins devoid of VLDL and LDL was prepared by ultracentrifugation of density-adjusted, cholesterol-fed rabbit plasma ($d = 1.063$ g/mL) at 50,000 rpm for 18 h in a swing-rotor (Beckman SW 55). Multilamellar DMPC, egg PC or soy PC liposomes were prepared following the hydration of the respective dried PC powders by adding Tris-buffered saline (TBS) (0.01 mol/L Tris-0.15 mol/L NaCl, pH 7.4). Multilamellar liposomes of DMPC, egg PC or soy PC were pelleted by centrifuging the PC suspension at 12,000 rpm for 10 min. The pellets were washed 3× with TBS. The HDL ($d > 1.063$ g/mL) plasma fraction was then incubated, in duplicate, with an excess amount of multilamellar liposomes – (>20 mg/mL) of DMPC, egg PC or soy PC – at 37°C. The amount of multilamellar liposomes solubilized by the HDL density plasma fractions was measured after 1, 2, 4, 8 and 16 h incubation of the mixtures. Briefly, the density of the incubated samples was adjusted to $d = 1.15$ g/mL by adding $d = 1.30$ g/mL KBr solution, and

then the PC liposomes that solubilized by the HDL density plasma fraction were separated from non-disrupted multilamellar liposomes by centrifugiation at 12,000 rpm for 15 min using a microcentrifuge. After removing non-disrupted multilamellar liposomes floating at the top of the centrifuged tube by aspiration, the level of solubilized PLs in the clear infranate fraction was measured by an enzymatic PL assay kit. As a control, solubilization of multilamellar liposomes following incubation with TBS was also measured.

Distribution of DMPC among lipoprotein fractions

To determine the distribution of DMPC among lipoproteins, 1 mL of pooled plasma from cholesterol-fed rabbits was incubated at 37°C for four hours with an excess amount of multilamellar DMPC (>50 mg) containing a trace amount of 9,10, ^{3}H-DMPC (New England Nuclear, Boston, MA, USA). Following centrifugal removal of non-disrupted multilamellar DMPC liposomes, plasma containing solubilized DMPC liposomes was subjected to a density gradient ultracentrifugation to separate major lipoprotein fractions. Distribution of ^{3}H-DMPC radioactivity among VLDL, LDL and HDL density fractions was then measured.

Preparation of aortas

Rabbits were euthanized with an intravenous overdose of sodium pentobarbital (130 mg/kg body weight). The intrathoracic aorta from semilunar valve to diaphragm was then dissected, gently washed and photographed by a digital camera. To determine the area percentage of atherosclerotic lesions, the photographic images of aortas were analyzed using Image-Pro Plus 4.5.1.22 software (Media Cybernetics Inc, Silver Spring, MD, USA), and then the percentage of the aortic surface covered by atherosclerotic lesions was calculated. The aortas were immediately frozen at −20°C until magnetic resonance imaging (MRI) was performed.

MRI measurement

For MRI measurements, individual aortas were thawed and inserted into the glass tubing (8 mm i.d.) mimicking the round-form of aorta. Each aorta from DMPC- ($n = 5$) and saline-treated ($n = 5$) rabbits was placed inside a vertical imaging scanner (Oxford Instruments, Abington, UK) equipped with a Unity/Inova console (Varian, Palo Alto, CA, USA). Images were acquired for 10 min at room temperature. T2-weighted coronal two-dimensional (2-D) sections were acquired first in the region of thoracic aorta in the total length of 4 cm using a spin–echo (SE) multi-slice pulse sequence. Then, 20 weighted T2 (SE) images were acquired in transversal sections in sequence slice by slice (2 mm thickness) in the total length of 4 cm section of the thoracic aorta from each one of saline- ($n = 5$) and DMPC-infused ($n = 5$) rabbits using the following set of imaging parameters: the slice thickness was 2.0 mm with no gap between the 20 slices. Spin echo parameters were echo time (TE) = 30 ms, repetition time (TR) = 3000 ms, number of transients/averages/(NT) = 1, spectral width (SW) = 50 kHz, field of view (FOV) = 0.8×0.8 cm^2, matrix size = 256×256, in-plain resolution = 30×30 μm^2 and acquisition time = 10 min. The total involvement of atherosclerotic lesions was also measured by compressing 20 MRI images into a single image by a newly computerized MRI method developed in the Biomedical Imaging Center of the Beckman Institute for Advancement of Science and Technology.

Analysis of aortic tissue cholesterol level

After non-invasive MRI examination, segments of the aortic arch and thoracic aortas from each animal were cut, weighed and homogenized. The total lipids were then extracted with chloroform/methanol (2:1, v/v) according to the method of Folch *et al.*[32] The lipid extracts were dried by evaporating solvent under the stream of nitrogen, and the dried extracts were dissolved with isopropyl alcohol containing 100 g Triton X-100/L.[33] The concentrations of TC and FC were then measured colorimetrically using Wako enzymatic assay kits.

Statistical analysis

Values are presented as mean ± standard deviation. The paired Student's *t*-test was applied to compare the levels of cholesterol in plasma and aortic tissues and the atherosclerotic plaque areas and volumes of aortas obtained from saline- ($n = 5$) and DMPC-infused ($n = 5$) cholesterol-fed rabbits. Values of $P < 0.05$ were judged as significant.

Results

Effect of cholesterol feeding and DMPC infusion on cholesterol levels

The placement of rabbits on an atherogenic diet for eight weeks caused a marked (70-fold) elevation of plasma TC (Table 1). The elevated plasma TC levels in cholesterol-fed rabbits were lowered by >60% following a change to the normal rabbit chow diet afterwards. The levels of plasma TC in these saline- or DMPC-infused cholesterol-fed rabbits were still much greater (25×) than those of the negative control rabbits fed only normal rabbit chow. Plasma

Table 1 Plasma cholesterol levels

Treatment	Plasma cholesterol (mg/dL)
Chow diet for 17 weeks ($n = 5$)	40.2 ± 6.1
Atherogenic diet for eight weeks ($n = 18$)	2918 ± 489
Saline infusion ($n = 5$)*	1159 ± 690
DMPC infusion ($n = 5$)*	972 ± 442

DMPC, dimyristoylphosphatidylcholine

*Rabbits were fed an atherogenic diet for eight weeks. Thereafter, rabbits were placed on a normal chow diet until the end of the study. Among rabbits, five pairs of those with comparable levels of plasma cholesterol were selected and divided into two groups. At week 13, animals were intravenously injected weekly with saline or DMPC liposomes for a period of five weeks and blood samples were taken at termination on week 18

cholesterol levels of rabbits infused with DMPC were somewhat higher than those of rabbits infused with saline (1115 versus 971 mg/dL), but the differences were not statistically significant. The levels of plasma TGs and PLs at the termination of the study were about the same between saline- and DMPC-treated groups (110 ± 12 versus 104 ± 26 mg/dL in TGs and 357 ± 201 versus 398 ± 224 mg/dL in PLs, respectively).

Figure 2 shows the level and distribution of cholesterol among VLDL, LDL and HDL density fractions in pooled plasma obtained at the end of the study. Compared with chow-fed rabbits, the cholesterol-fed rabbits with saline or DMPC infusion had much higher levels of cholesterol in the VLDL–LDL density regions and lower levels of cholesterol in the HDL density region (Figure 2, inset). It is evident from Figure 2 that compared with saline infusion, the DMPC infusion had little or no effect on the levels of cholesterol associated with HDL density fraction, but caused an increase in levels of cholesterol in the intermediate-density lipoprotein (IDL) density region by shifting the LDL peak into the less dense IDL peak.

In vitro assimilation of multilamellar liposomes composed of PC from various sources

Figure 3a shows that the HDL density fraction from 1 mL of cholesterol-fed rabbit plasma solubilized 11.4, 12.7, 13.4, 14.1 and 16.1 mg of multilamellar DMPC liposomes after 1, 2, 4, 8 and 16 h of incubation, respectively. Under the same conditions, the extent of solubilization of soy or egg PC liposomes by the same HDL density plasma fraction of cholesterol-fed rabbit plasma were 10.1× and 15.5× lower than that of DMPC liposomes (Figure 3a). Further study examining the distribution of DMPC among lipoprotein fractions following incubation of cholesterol-fed rabbit plasma with multilamellar DMPC liposomes containing trace amount of ^{3}H-labeled DMPC revealed that solubilized DMPC was associated primarily with particles within the HDL density region but some with particles in the VLDL and LDL peaks (Figure 3b).

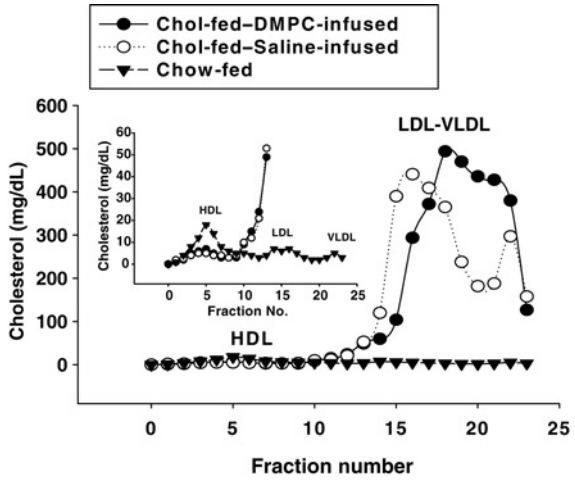

Figure 2 Density gradient profiles of plasma cholesterol showing the level and distribution of cholesterol among VLDL, LDL and HDL density regions of pooled plasma obtained from chow-fed rabbits and cholesterol-fed rabbits infused with saline or DMPC. Lipoprotein cholesterol profile (top) was magnified further to show the level of cholesterol in the HDL density region (inset). An aliquot (0.5 mL) of plasma from each rabbit in the same treatment group ($n = 5$) was pooled, and then lipoproteins in each pooled plasma were separated by density gradient ultracentrifugation. Distribution of cholesterol among VLDL, LDL and HDL density fractions was determined by measuring the cholesterol level in the density gradient fraction collected from the bottom of the centrifuged tubes. DMPC, dimyristoylphosphatidylcholine

Effect of DMPC infusion on aortic atherosclerotic lesions

Figure 4 shows the photographs of aortas obtained from chow-fed rabbits (panel a), cholesterol-fed rabbits with saline infusion (panel b) or cholesterol-fed rabbits with DMPC liposome infusion (panel c). In chow-fed rabbits (panel a), the aortas had smooth, semi-shiny intimal surfaces and no sign of atherosclerotic lesions were observed. In cholesterol-fed animals, however, extensive atherosclerotic lesions (yellowish dull irregular plaques) were visible on the intimal surfaces of the aortic arch and thoracic aorta (panels b and c). The atherosclerotic lesions in DMPC-infused cholesterol-fed rabbits (panel b) subjectively appeared to be shrunk compared with those from saline-infused cholesterol-fed rabbits (panel c). Figure 5 shows the results of tissue quantification of cholesterol

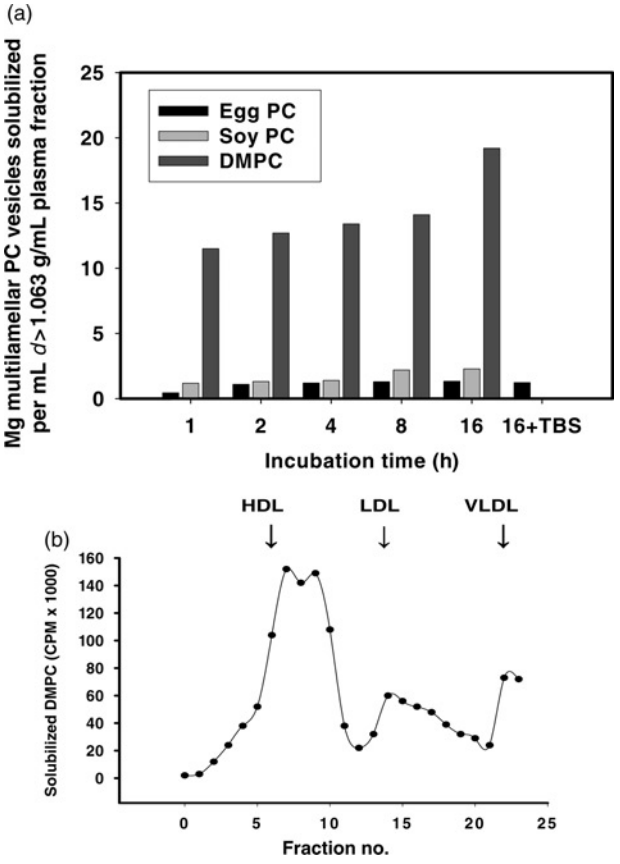

Figure 3 *In vitro* potency of HDL density plasma ($d > 1.063$ g/m) fraction to solubilize multilamellar vesicles of DMPC, egg PC or soy PC (a), and distribution of ^{3}H-labeld DMPC among lipoprotein fractions following incubation with the pooled plasma from the cholesterol-fed rabbits ($n = 10$) (b). (see details in Materials and methods). DMPC, dimyristoylphosphatidylcholine

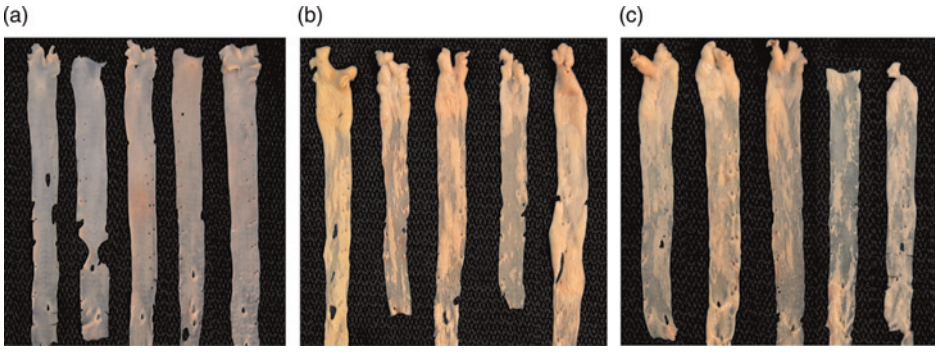

Figure 4 Photographs of rabbit aortas obtained from normal chow-fed rabbits (panel a), cholesterol-fed rabbits infused with saline (panel b) and cholesterol-fed rabbits infused with DMPC liposomes (panel c). The aortas from chow-fed rabbits (panel a) were shiny and smooth, showing no visible sign of atherosclerotic lesions. Aortas from cholesterol-fed animals had extensive atherosclerotic lesions (yellowish dull irregular plaques) in the aortic arch and thoracic aorta (panel b and c). The atherosclerotic lesions in DMPC-infused cholesterol-fed rabbits (panel c) subjectively appeared to be shrunk compared with those lesions in saline-treated cholesterol-fed rabbits (panel b). DMPC, dimyristoylphosphatidylcholine

content from aortic arches (a) and thoracic aortas (b) of chow-fed, saline-infused cholesterol-fed or DMPC-infused cholesterol-fed rabbits. The contents of TC, FC and cholesterylester (CE) in aortic arch and thoracic aortas of saline-infused cholesterol-fed rabbits were 19.7 ×, 17.6× and 22.7× higher and 11.2 ×, 8.4× and 14.0 × higher than those of chow-fed rabbits, respectively. Compared with saline-infused cholesterol-fed rabbits, the contents of TC and FC were significantly lower ($P < 0.05$) in both the aortic arch and thoracic aorta of DMPC-infused cholesterol-fed rabbits (Figure 5a and b); the CE content of both aortic arch and thoracic aortas from DMPC-infused rabbits was somewhat lower in DMPC-infused rabbits than in saline-infused rabbits, but the differences were not statistically significant. When the surface areas of aortas from saline- and DMPC-treated rabbits were measured by the computer using Image-Pro-Plus 4.5.1.22 software, the total surface areas covered with atherosclerotic lesions were similar between saline-treated ($82.8 \pm 15.3\%$) and DMPC-treated ($81.2 \pm 18.3\%$) groups.

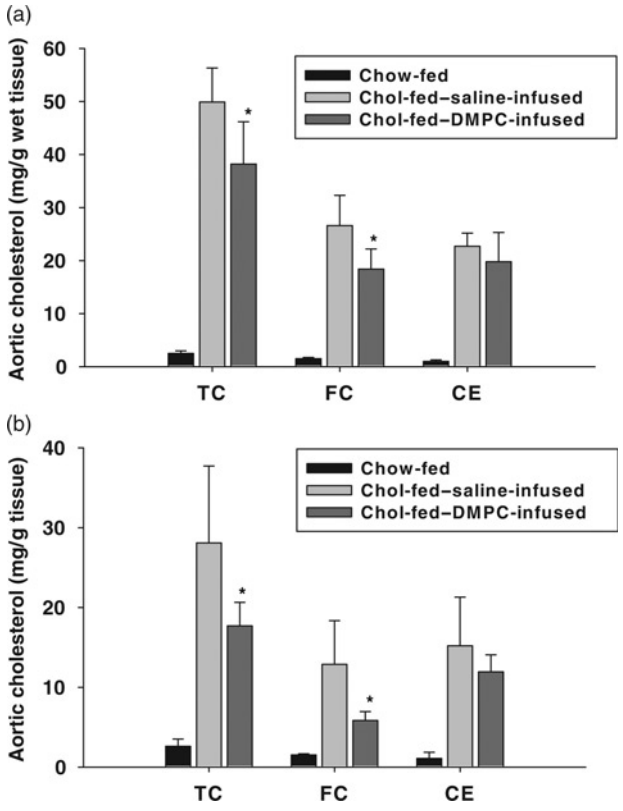

Figure 5 Cholesterol contents of aortic arch (a) and thoracic aortas (b) from chow-fed, saline-infused or DMPC-infused cholesterol-fed rabbits. All values are mean ± SD ($n = 5$). $^{*}P < 0.05$ versus saline-infused. DMPC, dimyristoylphosphatidylcholine; TC, total cholesterol; FC, free cholesterol; CE, cholesteryl ester

To further examine whether DMPC-mediated decreases in cholesterol concentrations in aortic tissue are related to a reduction in the volume of atherosclerotic lesions, the thoracic aorta was analyzed by MRI. Figure 6A shows representative transverse 2-D MRI images of 20 continuous cross-sections (2 mm slices) of a thoracic aorta from saline-treated (top) and DMPC-treated (bottom) cholesterol-fed rabbits. The MRI signal acquisition with no gap between slices provided detailed information about the distribution of atherosclerotic lesions in the aortas. It is evident from the MRI images of this particular rabbit that, overall, the thickness (volume) of atherosclerotic lesions in the DMPC-infused rabbit aorta was lower than its saline-treated counterpart (Figure 6A). Figure 6B-left, shows a representative single compressed image, which was obtained by summarizing the 20 MRI images of thoracic aorta from a DMPC-treated (a) or saline-infused (b) rabbit. The single compressed image further illustrates the regression of atherosclerotic lesions in rabbits infused with DMPC compared with saline-infused rabbits. The mean total plaque volume, as determined from the compressed MRI images, was significantly lower in aortas of DMPC-infused rabbits ($n = 5$) than in aortas of saline-infused rabbits ($n = 5$) (Figure 6B-right). The MRI intensities of aortic lesions correlated significantly with aortic cholesterol content ($r = 0.95$, $P < 0.01$, data not shown).

Discussion

We have demonstrated by chemical and MRI analysis of aortas from cholesterol-fed rabbits that five weekly

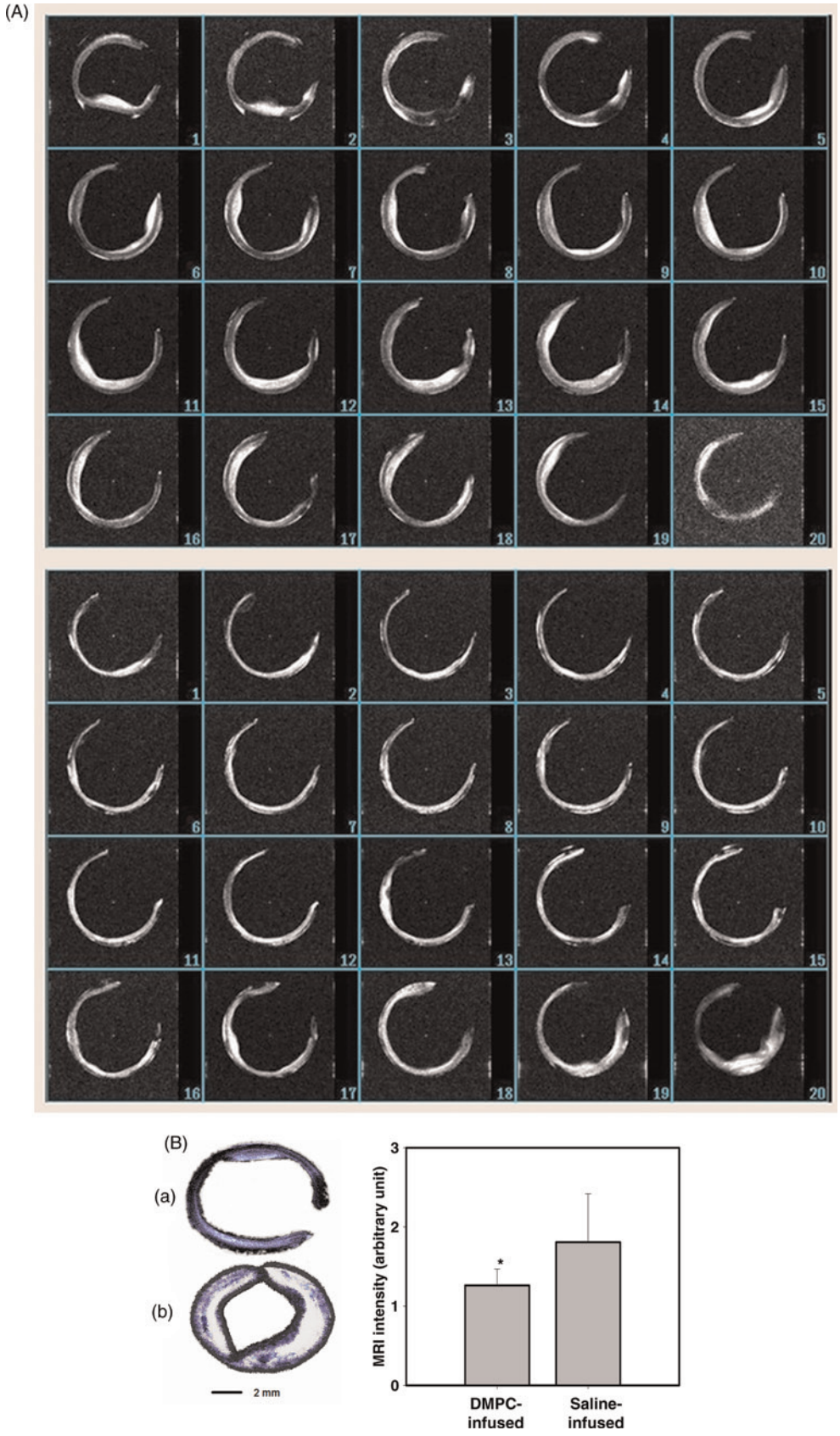

Figure 6 (A) Representative MRI images of 20 cross-sections obtained from the thoracic aorta of a saline-infused (top) or DMPC-infused (bottom) cholesterol-fed rabbit. Twenty MRI images were acquired in sequence slice by slice (2 mm thickness) in the total length of 4 cm section of the thoracic aorta from individual saline-infused ($n = 5$) and DMPC-infused ($n = 5$) cholesterol-fed rabbits. (B-left) A representative single image generated by compressing 20 MRI images (A) of the thoracic aorta of DMPC-infused (a) or saline-infused rabbit (b). The twenty MRI images taken from the thoracic aorta of individual DMPC-infused ($n = 5$) and saline-infused rabbits ($n = 5$) shown in (A) were summarized into a single image by a compressing technique developed at the Biomedical Imaging Center. (B-right) shows the MRI intensity measured from the compressed images of the thoracic aorta of DMPC-infused or saline-infused cholesterol-fed rabbits. The MRI intensity represents the total atherosclerotic plaque volume (mean $\pm$ SD, $n = 5$). $^{*}P < 0.05$ versus saline-infused. DMPC, dimyristoylphosphatidylcholine; MRI, magnetic resonance imaging. (A color version of this figure is available in the online journal)

intravenous infusions of synthetic DMPC liposomes result in a significant reduction in aortic cholesterol content and atherosclerotic plaque volume. These observations are in general agreement with earlier work that demonstrates that infusion of PC liposomes promotes the regression of atherosclerotic lesions.[20–23] In these studies, intravenous infusion of a large dose of unsaturated egg PC liposomes (200–300 mg/kg) into rabbits was shown to cause a marked increase in plasma cholesterol levels, mainly FC. The intravenously injected egg PC liposomes remained as discrete vesicular particles with little or no assimilation of PC vesicles into the endogenous lipoproteins,[22,23] suggesting that the rise in plasma cholesterol after liposomal infusion was associated mostly with incorporation into the vesicular liposomes. The acutely elevated plasma cholesterol, after peaking at 24 h, returned to baseline by five days as vesicles were removed from circulation.[22,23] These *in vivo* observations suggested that infused egg PC liposomes promoted mobilization of cholesterol from the tissue primarily by serving as direct acceptors of cholesterol. Other studies showed that the potency of egg PC liposomes to mobilize tissue cholesterol *in vivo* was affected significantly by sizes and/or residence time of liposomes, where large unilamellar liposomes were more potent in removing cholesterol than rapidly cleared small unilamellar vesicles.[22,34]

William *et al.*[35] and William and Scanu[36] also reported that infusion of natural PC liposomes promotes the regression of atherosclerosis by mobilizing cholesterol from tissues, acting as direct acceptors of cholesterol from peripheral cells. From the results of these studies, it was speculated that PC liposomes may enhance the ability of particles within the HDL density region of plasma to accept cellular cholesterol through compositional and structural modification of the HDL particle itself via incorporation of PC.

It has been shown in this study that the HDL density plasma fraction from cholesterol-fed rabbits can rapidly assimilate large amounts of multilamellar DMPC vesicles (Figure 3a) more efficiently than egg or soy PC liposomes. It is estimated that infusing DMPC liposomes into rabbits (~2.5 kg body weight with estimated plasma volume of 150 mL) at 300 mg DMPC/kg body weight would cause a transient 5 mg/mL increase in plasma PC levels. Since we found that the HDL density fraction in 1 mL of plasma from cholesterol-fed rabbits was able to solubilize 11.5 mg DMPC within one hour of incubation (Figure 3a), it is suggested that all DMPC liposomes infused into cholesterol-fed rabbits would circulate in plasma after complete solubilization of its vesicular structure via assimilation into HDL density plasma fraction. Tall and Green[37] reported previously that incubating human plasma or isolated HDL for four hours with an excess amount of egg PC liposomes resulted in maximal transfer of 0.9 mg egg PC to HDL/mL plasma. In this study, 1 mL of the HDL density plasma fraction from cholesterol-fed rabbits dissolved 1.0 and 1.4 mg of multilamellar egg PC and soy PC liposomes, respectively, after four hours of incubation (Figure 3). This suggests that the major portion (70–80%) of egg PC or soy PC liposomes, when infused into rabbits at a dose of 300 mg/kg body weight, may circulate without losing vesicular structure.

The potency of various PC liposomes *in vitro* to boost the ability of plasma to promote cholesterol efflux from cultured cells has been shown to closely correlate with the solubilization of the vesicular structures of liposomes by the HDL and other plasma components.[17,18] This suggests that the potency of liposomes to mobilize tissue cholesterol or to regress atherosclerotic lesions *in vivo* could be influenced by the degree of solubilization of the basic vesicular structure of the liposomes by HDL and other plasma components. The findings reported here suggest that DMPC liposomes behave in a manner similar to egg or soy PC liposomes. However, the extent of solubilization of the DMPC vesicles by the HDL density plasma fraction from cholesterol-fed rabbits *in vitro* is much (10–15×) greater than other PCs. The results of this study not only suggest that DMPC liposomes are more efficient at removing cholesterol from plaques than natural (soy or egg) PC liposomes, but also that DMPC may have promising potential as a therapeutic agent to mobilize tissue cholesterol and/or stimulate regression of atherosclerotic lesions. Whether the mechanism of regression is the same as that of other natural PC liposomes is not entirely clear at this point.

With regard to potential for DMPC liposomal action, Hassan *et al.*[38] have shown that the addition of DMPC liposomes to human serum *in vitro* has been shown to increase the PL content of both HDL_2 and HDL_3 and to generate new protein lipid complexes having pre-β electrophoretic mobility (pre-β-HDL) via presumably interaction of DMPC with HDL. Since an increase in PL content of HDL enhances the ability of HDL to promote cholesterol efflux mediated via ABCG1[39] and SR-BI,[40] and the production of pre-β-HDL in plasma enhances the ability of plasma to promote cholesterol efflux via ABCAI,[38] DMPC liposome infusion may accentuate cholesterol efflux from metabolically active viable foam cells via enhancement of all major known specific pathways (ABCAI and ABCGI transporter- and SR-BI receptor-mediated efflux) of cholesterol efflux. We have shown previously that the addition of DMPC liposomes to fresh human plasma or isolated HDL can markedly increase their ability to release cholesterol from red blood cell membranes or isolated human atherosclerotic plaques.[28,41] This observation suggests that the DMPC-mediated increases in PL content of HDL, resulting in a lower HDL FC/PL ratio, could also increase the potency of DMPC-supplemented plasma to promote cholesterol efflux via non-specific aqueous diffusion pathways since cholesterol would diffuse into PL-rich particles from cholesterol-rich atherosclerotic plaques due to chemical gradient potential differences.[42] LCAT also may play an important role by generating a gradient of FC from atherosclerotic lesions to HDL in plasma via esterification of FC on HDL.[4] Since we observed *in vitro* that the addition of DMPC into plasma markedly increased the extent of esterification FC in plasma,[41] DMPC infusion should enhance the extent of cholesterol efflux via an increase in LCAT activity *in vivo.*

In summary, this study suggests that the enrichment of plasma HDL by intravenous infusion with synthetic

DMPC liposomes could be a potential therapeutic approach for atherosclerotic plaque regression.

Author contributions: BHSC and B-HC developed the study concept. BHSC, J-RP and MTN conducted the experiment and interpretation of data. BMO conducted MRI image analysis. MAW performed morphological work and edited the manuscript. B-HC performed lipoprotein analysis and drafted the manuscript.

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REFERENCES

1 Goldon T, Castelli WP, Hjortland M, Kannel WB, Dawber T. High-density lipoproteins as a protective factor against coronary heart disease. *Am J Med* 1977;**62**:707–14
2 Puchois P, Kandossi A, Fiebr O, Fournier JL, Bertland M, Koren E, Fruchard JC. Apolipoprotein A-I-containing lipoproteins in coronary heart disease. *Atherosclerosis* 1987;**68**:35–46
3 Goldon GJ, Probstfeld JL, Garrisom RJ, Neaton JD, Castelli WP, Knoke JD, Jacobs JR, Bangdlwala S, Tyroler A. High density lipoprotein cholesterol and cardiovascular disease. Four prospective American studies. *Circulation* 1989;**79**:8–15
4 Glomset JA. The plasma lecithin:cholesterol acyltransferase. *J Lipid Res* 1968;**9**:155–67
5 Franceschini G, Maderna P, Cirtori CR. Reverse cholesterol transport: physiology and pharmacology. *Atherosclerosis* 1981;**88**:99–107
6 Plump AS, Scott CJ, Breslow JL. Human apolipoprotein A-I gene expression increases high density lipoproteins and suppress atherosclerosis in the apolipoprotein E-deficient mouse. *Proc Natl Acad Sci USA* 1994;**91**:9607–11
7 Badimon JJ, Badimon L, Fuster V. Regression of atherosclerotic lesions by high density lipoprotein plasma fraction in the cholesterol-fed rabbits. *J Clin Invest* 1990;**85**:1234–41
8 Miyazaki A, Sakuma S, Morikawa W, Takiue T, Miake F, Teano T, Sakai M, Hakamata H, Sakamoto TI, Naito M, Ruan Y, Takahashi K, Ohta T, Horiuchi S. Intravenous injection of rabbit apolipoprotein A-I inhibits the progression of atherosclerosis in cholesterol-fed rabbits. *Arterioscler Thromb Vasc Biol* 1995;**15**:1882–8
9 Shah PK, Yano J, Reyes O, Chyu K-Y, Kaul S, Bisgaier CL, Drake S, Cercek B. High dose recombinant apolipoprotein A-I_{Milano} mobilizes tissue cholesterol and rapidly reduces plaque lipid and macrophage content of apolipoprotein E-deficient mice; potential implications for acute plaque stabilization. *Circulation* 2001;**103**:3047–50
10 Nicholls SJ, Cutri B, Worthley SG, Kee P, Rye KA, Bao S, Barter PJ. Impact of short-term administration of high-density lipoproteins and atorvastatin on atherosclerosis. *Arterioscler Thromb Vasc Biol* 2005;**25**:2416–21
11 Robins SJ, Collins D, Wittes JT, Papademetriou V, Deedwania PC, Schaefer EJ, McNamara JR, Kashyap ML, Hershman JM, Wexler LF, Rubins HB, for the VA-HIT Study Group. Relation of gemfibrozil treatment and lipid levels with major coronary events: VA-HIT: a randomized controlled trial. *JAMA* 2001;**285**:1585–91
12 Lawn RM, Wade DP, Garvin MR, Wang X, Schwartz K, Portewr JG, Seilhamer JJ, Vaughan AM, Oram JF. The Tangier disease gene product ABCAI control the cellular apolipoprotein-mediated lipid removal pathway. *J Clin Invest* 1999;**104**:R25–31
13 Ji Y, Jian B, Wang N, Sun Y, Llera-Moya MDL, Phillips MC, Rothblat GH, Swaney JB, Tall AR. Scavenger receptor BI promotes high density lipoprotein-mediated cellular cholesterol efflux. *J Biol Chem* 1997;**272**:20982–5
14 Wang N, Lan W, Chen F, Matuura F, Tall AR. ATP-binding cassette transporter GI and G4 mediate cellular cholesterol efflux to high density lipoproteins. *Proc Natl Acad Sci USA* 2004;**101**:9774–9
15 Fournier N, Llera-Moya MDL, Burkey BF, Swaney JB, Paterniti J, Moatti N, Atger V, Rothblat GH. Role of HDL phospholipid in efflux of cell cholesterol to whole serum: studies with human apo A-I transgenic rats. *J Lipid Res* 1996;**37**:1709–17
16 Fournier N, Atger V, Cogny A, Vedie B, Giral P, Simmon A, Moatti N, Paul JL. Analysis of relationship between triglyceridemia and HDL-phospholipid concentrations: consequence on the efflux capacity of serum in the Fu5AH system. *Atherosclerosis* 2001;**157**:315–23
17 Jian B, Llera-Moya MDL, Royer L, Rothblat GH, Francone O, Swaney JB. Modification of the cholesterol efflux properties of human serum by enrichment with phospholipids. *J Lipid Res* 1997;**38**:734–44
18 Pownall HJ. Detergent-mediated phospholipidation of plasma lipoproteins increases HDL cholesterolphilicity and cholesterol efflux via SR-BI. *Biochemistry* 2006;**45**:11514–22
19 Hsia SL, Duncan R, Schob AH, Chakko SC, Mulingtapang R, He JL, Perz GO. Serum levels of high density lipoprotein phospholipids correlate inversely with severity of angiographically defined coronary artery disease. *Atherosclerosis* 2002;**215**:469–73
20 Adams CMW, Adulla YH, Bayliss OB, Morgan RS. Modification of aortic atheroma and fatty liver in cholesterol-fed rabbits by intravenous injection of saturated and polyunsaturated lecithins. *J Pathol Bacteriol* 1967;**94**:77–87
21 Stafford WW, Day CE. Regression of atherosclerosis effected by intravenous phospholipid. *Artery* 1975;**1**:106–14
22 Rodrigueza WV, Klimuk SK, Prichard PH, Hope MJ. Cholesterol mobilization and regression of atheroma in cholesterol-fed rabbits induced by large unilamellar vesicles. *Biochim Biophys Acta* 1998;**1368**:306–20
23 Rodrigueza WV, Pritchard PH, Hope MJ. Influence of size and composition of cholesterol mobilizing properties of liposomes *in vivo*. *Biochim Biophys Acta* 1993;**1153**:9–19
24 Mohamad N, Hama S, Hough G, Fogelman AM. Oral synthetic phospholipid (DMPC) raises high-density lipoprotein cholesterol levels, improves high-density lipoprotein function, and markedly reduces atherosclerosis in apolipoprotein E–null mice. *Circulation* 2003;**108**:1735–9
25 Davidson WS, Gillotte KL, Lund-Katz S, Johnson WJ, Rothblat GH, Phillips MC. The effect of high density lipoprotein phospholipid acyl chain composition on the efflux of cellular free cholesterol. *J Biol Chem* 1995;**270**:5882–90
26 Davidson WS, Lund-Katz S, Johnson WJ, Anatharamaiah AM, Palgunachari MN, Segrest JP, Rothblat GH, Phillips MC. The influence of apolipoprotein structure on the efflux of cellular free cholesterol to high density lipoproteins. *J Biol Chem* 1994;**269**:22975–7982
27 Malberg FH, Glick J, Lund-Katz S, Rothblat GH. Influence of apolipoproteins AI, AII, and Cs on the metabolism of membrane and lysosomal cholesterol in macrophages. *J Biol Chem* 1991;**266**:19930–7
28 Chung BH, Franklin F, Liang P, Doran S, Cho BHS, Curcio CA. Phosphatidylcholine-rich acceptors, but not native HDL or its apolipoproteins, mobilize cholesterol from cholesterol-rich insoluble components of human atherosclerotic plaques. *Biochim Biophys Acta* 2005;**1733**:76–89
29 Tall AR, Hogan RV, Askinazi L, Small DM. Interaction of plasma high density lipoproteins with dimyristolylecithon multilamellar liposomes. *Biochemistry* 1978;**17**:322–6
30 Pownall HJ, Massey JB, Kusserow SK, Gotto AM Jr. Kinetics of lipid–protein interactions: interaction of apolipoproteins A-I from human high density lipoproteins with phosphatidylcholine. *Biochemistry* 1978;**17**:1183–8
31 Chung BH, Segrest JP, Ray M, Brunzell J, Hokanson D, Krauss RM, Beaudrie K, Cone JT. Single vertical spin density gradient ultracentrifugation. *Method Enzymol* 1986;**128**:181–212
32 Folch J, Lees M, Sloane-Stranley CM. A simple method of isolation and purification of total lipids from animal tissues. *J Biol Chem* 1957;**226**:497–509

33 Kishida T, Nagami H, Ogawa H, Ehihara K. The hypocholesterolemic effect of high amylase corn starch in rats is mediated by an enlarged bile acid pool and increased fecal bail acid excretion, not by fecal fermented products. *J Nutr* 2002;**132**:2519–24
34 Rodrigueza WV, Mazany KD, Essenburg AD, Pape ME, Rea TJ, Bisgaier CL, Williams KJ. Large versus small unilamellar vesicles mediate reverse cholesterol transport in vivo into two distinct hepatic metabolic pools: implications for the treatment of atherosclerosis. *Arterioscler Thromb Vasc Biol* 1997;**17**:2132–9
35 Williams KJ, Werth VP, Wolff JA. Intravenously administered lecithin liposomes: a synthetic antiatherogenic lipid particle. *Perspect Biol and Med* 1984;27:417–31
36 Williams KJ, Scanu AM. Uptake of endogenous cholesterol by a synthetic lipoprotein. *Biochim Biophys Acta* 1986;**875**:183–94
37 Tall AR, Green PHR. Incorporation of phosphatidylcholine into spherical and discoidal lipoproteins during incubation of egg phosphatidylcholine vesicles with isolated high density lipoproteins or with plasma. *J Biol Chem* 1981;**256**:2035–44
38 Hassan HH, Blain S, Boucher B, Denis M, Krimbou L, Genest G. Structural modification of plasma HDL by phopholipids promote efficient ABCAI-mediated cholesterol efflux. *J Lipid Res* 2005;**46**:1457–65
39 Gelissen IC, Harris M, Rye KA, Quinn C, Brown AJ, Kockx M, Cartland S, Packianathan M, Kritharides L, Jessup W. ABCAI and ABCGI synergize to mediate cholesterol export to apo A-I. *Arterioscler Thromb Vasc Biol*. 2006;**26**:534–40
40 Yancey PG, Llera-Moya MDL, Swanarkar S, Monzo P, Klein SM, Connelly MA, Johnson WJ, Rothblat GH. HDL phospholipid composition is a major determinant of the bi-directional flux and net movement of cellular cholesterol mediated by scavenger receptor-BI (SR-BI). *J Biol Chem* 2000;**275**:36569–604
41 Chung BH, Franklin F, Cho BHS, Segrest JP, Hart K, Darnell B. Potencies of lipoproteins in fasting and postprandial plasma to accept additional cholesterol molecules released from cell membranes. *Atheroscler Thromb Vasc Biol* 1998;**18**:1217–30
42 Rothblat GH, Llera-Moya MDL, Atger A, Kellner-Weibel G, William DL, Philips MC. Cell cholesterol efflux: integration of old and new observation provides new insights. *J Lipid Res* 1999;**40**:781–96

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