Apolipoprotein A-IV (apoA-IV) is a major component of high-density lipoprotein (HDL) and chylomicrons in rats. Similar to apoA-I and apoE, apoA-IV contains repeated units of 22 residues that are organized in amphipathic α-helices and have been implicated in lipid binding. In humans and the majority of animal species, apoA-IV is synthesized primarily by the intestine and, to a lesser extent, by the liver, and is found in plasma, the lymph, and cerebrospinal fluid. Our findings are consistent with a novel function of apoA-IV in the biogenesis of discrete HDL-apoA-IV particles with the participation of ABCA1 and CAT, and may explain previously reported anti-inflammatory and atheroprotective properties of apoA-IV.

Abstract

The objective of this study was to establish the role of apoA-IV, ABCA1, and LCAT in the biogenesis of apoA-IV-containing HDL (HDL-AIV) using different mouse models. Adenovirus-mediated gene transfer of apoA-IV in apoA-I−/− mice did not change plasma lipid levels. apoA-IV, floated in the HDL2/HDL3 region, promoted the formation of spherical HDL particles as determined by electron microscopy, and generated mostly α- and a few β-like HDL subpopulations. Gene transfer of apoA-IV in apoA-I−/− × apoE−/− mice increased plasma cholesterol and triglyceride levels, and 80% of the protein was distributed in the VLDL/IDL/LDL region. This treatment likewise generated α- and β-like HDL subpopulations. Spherical and α-migrating HDL particles were not detectable following gene transfer of apoA-IV in ABCA1−/− or CAT−/− mice. Coexpression of apoA-IV and LCAT in apoA-I−/− mice restored the formation of HDL-AIV. Lipid-free apoA-IV and reconstituted HDL-AIV promoted ABCA1 and scavenger receptor BI (SR-BI)-mediated cholesterol efflux, respectively, as efficiently as apoA-I and apoE. Our findings are consistent with a novel function of apoA-IV in the biogenesis of discrete HDL-AIV particles with the participation of ABCA1 and CAT, and may explain previously reported anti-inflammatory and atheroprotective properties of apoA-IV.

ApoA-IV promotes the biogenesis of apoA-IV-containing HDL particles with the participation of ABCA1 and LCAT.


Supplementary key words: apolipoprotein A-IV • lipoproteins • genetically altered mice • lecithin:cholesterol acyltransferase • ATP binding cassette transporter A1

Supplemental Material can be found at: http://www.jlr.org/content/suppl/2012/11/06/jlr.M030114.DC1.html
has structural (2, 3) and several functional similarities with apoA-I and apoE. Thus lipid-free apoA-IV promotes cholesterol efflux from cells (20–22), and rHDL-A-IV particles activate LCAT (23). Apo-AIV was also shown to bind saturated to cell surface sites (21, 24), as well as to hepatic cell membranes (25), to potentiate the apoCII-mediated activation of lipoprotein lipase (26) and the activity of cholesteryl ester transfer protein (27). Furthermore, apo-AIV was reported to have antioxidant (28) and anti-inflammatory (16, 29) properties, and similarly to apoA-I (30), and apoE (31), may also play some role in the development of Alzheimer’s disease (32). A difference between apoA-IV and apoA-I or apoE exists on the contribution of the C-terminal domain of these proteins to the solubilization of dimyristoyl-α-phosphatidyl-choline (DMPC) phospholipids (33, 34). In the case of apoA-I and apoE, deletion of the C-terminal domain drastically reduced the ability of the truncated forms to solubilize DMPC phospholipids and to associate with preformed HDL (35, 36). In the case of apoA-IV, deletion of the 44 C-terminal residues increased its ability to solubilize DMPC phospholipids (34). Subsequent studies showed that deletion of the C-terminal residues 333–343 strongly increased the rate of association of truncated apoA-IV with DMPC phospholipids, and this enhancement required residues 11–20 of the truncated apoA-IV (37). The reduced capacity of the full-length apoA-IV to associate with phospholipids was attributed to intramolecular interactions of C- and N-terminal regions that contain residues F334 and F335, and W12 and F15, respectively (33). In cell culture studies, lipid secretion and the size of secreted lipoprotein particles increased dramatically with the deletion of the 344–354 region that contains three EQQQ motifs and one EIVQ motif in human apoA-IV (38). Increased lipid secretion was also observed in newborn swine, where apoA-IV lacks the EQQQ sequences, suggesting that these sequences modulate chylomycin packaging and secretion (38).

Studies with transgenic mice showed that overexpression of apoA-IV in the intestine did not affect the intestinal absorption of cholesterol and triglycerides and fat-soluble vitamins or the clearance of chylomircons. It also did not cause weight gain and did not alter feeding behavior in transgenic mice as compared with control mice (15). Similar conclusions regarding lipid absorption and weight gain were reached by the study of apoA-IV-deficient mice (14). Previous studies had implicated apoA-IV as a satiety factor (13). The transgenic mice expressing the mouse apoA-IV gene mostly in the intestine had reduced levels of atherosclerotic lesions in response to atherogenic diets (19). The lipid profiles of these mice were similar, but not identical to those of the control wild-type (WT) mice (15). Plasma isolated from the mouse apoA-IV-transgenic mice had increased endogenous cholesterol esterification rates, and their HDL, isolated following fat feeding, promoted more efficiently cholesterol efflux from cholesterol-loaded human monocytes, as compared with HDL obtained from WT mice (19). Reduced atherosclerotic lesions were also observed in transgenic mice expressing human apoA-IV mainly in the intestine in an apoE-deficient background. Injection of lipopolysaccharide into these human apoA-IV-transgenic mice in an apoE-deficient background resulted in fewer atherosclerotic lesions than in apoE-deficient mice. The protective effect of apoA-IV in this case was attributed to its antioxidant properties (17) and the stronger Th1 response of the lymphocytes in the presence of apoA-IV. Lymphocytes isolated from human apoA-IV × apoE−/− transgenic mice produced lower levels of proinflammatory cytokines as compared with apoE−/− mice (29). The anti-inflammatory properties of apoA-IV were also manifested by intraperitoneal injection of the recombinant protein in WT and apoA-IV-deficient mice. This treatment delayed the onset and reduced severity of the inflammation associated with experimentally induced colitis (16). Reduced atherosclerosis was also observed in transgenic mice overexpressing the apoA-IV gene in the liver of either normal or apoE-deficient mice under the control of the hepatic control region of the apoE/apoC-I gene cluster (18).

The origin and the metabolic fate and the physiological significance of apoA-IV that resides on the HDL particle are not fully understood. Here we show that apoA-IV participates in the biogenesis of apoA-IV-containing HDL (HDL-A-IV) particles using the same pathway that is utilized by apoA-I and apoE. The HDL-A-IV particles formed may explain, at least partially, the previously reported anti-inflammatory and atheroprotective functions of apoA-IV.

### EXPERIMENTAL PROCEDURES

#### Materials

Materials not mentioned in the experimental procedures have been obtained from sources described previously (39).

#### Methods

**Generation of an adenovirus expressing the human apoA-IV.** The apoA-IV cDNA was generated by RT-PCR of human mRNA using 5′ and 3′ primers contained restriction sites for BglII and EcoRV, respectively. The apoA-IV cDNA was digested with BglII and EcoRV and cloned into the corresponding sites of the pAdTrack-CMV vector. The recombinant adenoviruses were constructed and purified using the Ad-Easy-1 system where the adenovirus construct is generated in bacteria BJ-5183 (Agilent Technologies; Santa Clara, CA) as described (39). Correct clones were propagated in RecA DH5α cells (Invitrogen; Carlsbad, CA). The recombinant adenoviral vectors were linearized with PaelI and used to transfect 911 cells. Following large-scale infection of HEK293 cell cultures with virus-containing cell lysates, the recombinant adenoviruses were purified by two consecutive Caesium chloride ultracentrifugation steps, dialyzed, and titrated (39).

**Cholesterol efflux measurements.** ATP-binding cassette transporter (ABC) A1-mediated cholesterol efflux measurements by lipid-free apoA-IV using HEK293/EBNA cells was performed as described (39). Net efflux was calculated by subtracting the efflux obtained in the untransfected cells from that of the ABCA1-transfected cells (40). Scavenger receptor BI (SR-BI)-mediated cholesterol efflux by reconstituted HDL-A-IV (rHDL-A-IV) using CHO ldlA[mSR-B1] cells was performed as described (39, 41, 42). Net efflux was calculated by subtracting the efflux...
obtained in the parent IdlA CHO cells from that of IdlA[mSR-BI] CHO cells.

Animal studies, plasma lipids, fractionation of plasma, two-dimensional gel electrophoresis, electron microscopy, and apoA-IV mRNA analyses. ApoAIV−/− (ApoAImi/Imi) C57BL/6J mice (43) were purchased from Jackson Laboratories (Bar Harbor, ME). Mice deficient for apoA-I and apoE were a gift of Dr. Fayanne Thorngate and Dr. David Williams (44). Mice deficient in ABCA1 (45) (purchased from Jackson Laboratories) were provided by Dr. Mike Filtzerald. Mice deficient for LCAT were a gift of Dr. Santa-Marina Fojo (46). The mice were maintained on a 12 h light/dark cycle and standard rodent chow. All procedures performed on the mice were in accordance with National Institutes of Health guidelines and following an approved IACUC protocol. Mice, 6–8 weeks of age, were injected via the tail vein with 0.5 ml of recombinant adenovirus per animal. Four days postinjection, following a 4 h fast, blood was drawn and the livers were collected for further analyses. The fractionation of plasma by fast-protein liquid chromatography (FPLC) and density gradient ultracentrifugation, the two-dimensional gel electrophoresis of plasma, the cholesterol and triglyceride measurements, the electron microscopy (EM) of the HDL fractions, and the apoA-IV mRNA quantification were performed as described (47). For details, please see the Supplementary Methods.

Statistics
Statistical analyses were performed by two-tailed Student’s-t test with equal variance.

RESULTS
In vitro properties of apoA-IV
We have generated a recombinant adenovirus expressing apoA-IV and used it to study its in vivo and in vitro properties.

ApoA-IV secreted in the culture medium of adenovirus-infected HTB-13 grown on a large scale was purified and used to study its cholesterol efflux potential and its physicochemical properties. As shown in Fig. 1A, the ABCA1-mediated cholesterol efflux to lipid-free apoA-IV, which represents the first step in the biogenesis of HDL, was comparable to that of lipid-free apoA-I and apoE. Similarly the SR-BI-mediated cholesterol efflux of rHDL-A-IV was comparable to those of rHDL containing apoA-I or apoE (Fig. 1B).

Recombinant ApoA-IV had structural and thermodynamic properties that were reminiscent of apoA-I and apoE. Circular dichroism measurements revealed a significant helical content of 41.4%, albeit reduced compared with apoA-I and apoE (48, 49). Upon mixing with egg yolk phosphatidyl-choline, recombinant apoA-IV readily formed HDL-like particles with increased helical content of 46.7% (supplementary Fig. I A, B and Table I). Thermal denaturation of apoA-IV revealed a single limited-cooperativity transition with a Tm of 45.6°C (supplementary Fig. II A). The thermal denaturation of apoA-IV was largely reversible, inasmuch as the protein recovered more than 95% of its secondary structure after cooling (supplementary Fig. II A, B). rHDL-A-IV particles were significantly more stable versus thermal denaturation (Tm = 61.4°C) and exhibited a limited-cooperativity nonreversible transition (supplementary Table I and Fig. II A, B). Chemical denaturation of apoA-IV revealed single-step transition with limited cooperativity that lacked the intermediate described for the thermal denaturation of apoE (49). Chemical denaturation of rHDL-A-IV showed a highly noncooperative transition (supplementary Fig. II C, D). Overall, biophysical analysis of recombinant apoA-IV suggests extensive conformational changes upon lipid binding similar to those described for other apolipoproteins. Furthermore, this analysis suggests that although apoA-IV has structural and thermodynamic properties similar to those of apoA-I and apoE, it still retains a unique structural and thermodynamic profile that may be consistent with distinct functional roles.

Effect of apoA-IV on lipid and lipoprotein profiles and the generation of HDL-A-IV
The changes in the lipid and lipoprotein profiles as a result of hepatic expression of apoA-IV were studied in
particles (Fig. 4J). The findings shown in Fig. 4A, B, E, F, I, and J suggest strongly that apoA-IV participates in the generation of HDL-A-IV particles. The findings shown in Fig. 3A, B and Fig. 4A, B show for the first time that in the absence of both apoE and apoA-I, apoA-IV has increased affinity for triglyceride-rich lipoproteins and that this increased affinity is associated with the induction of hypertriglyceridemia.

ABCA1 and LCAT are required for the biogenesis of HDL-A-IV

The next task was to determine the role of ABCA1 and LCAT in the biogenesis of HDL-A-IV. Adenovirus-mediated gene transfer of apoA-IV in apoA-I−/− mice did not significantly alter total plasma lipid levels or the cholesterol and triglyceride FPLC profiles (Fig. 2A, B, Fig. 3A, B, and supplementary Table II). The distribution of apoA-IV to different lipoprotein fractions was determined by density gradient ultracentrifugation of plasma followed by SDS-PAGE of the resulting fractions. This analysis showed that apoA-IV was distributed predominantly to HDL3 and, to a lesser extent, to the HDL2 fraction (Fig. 4A). EM of the HDL fractions showed that hepatic expression of apoA-IV promoted the formation of spherical particles (Fig. 4E). Two-dimensional gel electrophoresis of plasma showed that apoA-IV generated predominantly α-HDL particles with smaller amount of pre-β-like particles (Fig. 4I).

A different picture was obtained by adenovirus mediated gene transfer of apoA-IV in apoA-I−/− × apoE−/− double-deficient mice. Hepatic apoA-IV expression in these mice increased plasma cholesterol to levels greater than those of the uninjected controls and induced hypertriglyceridemia (Fig. 2A, B). FPLC analysis showed that all the cholesterol and triglycerides were found in the VLDL/IDL region (Fig. 3A, B). SDS-PAGE analyses of the lipoprotein fractions separated by density gradient ultracentrifugation of plasma, showed that the observed dyslipidemia was associated with distribution of the majority (80%) of apoA-IV in the VLDL/IDL/LDL region and to a lesser extent to the HDL2/HDL3 region (Fig. 4B). The apoA-IV fractions that float in the VLDL/IDL/LDL region also contain large amounts of apoB-48 (data not shown). EM showed formation of spherical HDL (Fig. 4F) and two-dimensional gel electrophoresis of plasma showed predominantly the formation of α-HDL and a small amount of pre-β-like HDL particles (Fig. 4J). The findings shown in Fig. 4A, B, E, F, I, and J suggest strongly that apoA-IV participates in the generation of HDL-A-IV particles. The findings shown in Fig. 3A, B and Fig. 4A, B show for the first time that in the absence of both apoE and apoA-I, apoA-IV has increased affinity for triglyceride-rich lipoproteins and that this increased affinity is associated with the induction of hypertriglyceridemia.

ABCA1 and LCAT are required for the biogenesis of HDL-A-IV

The next task was to determine the role of ABCA1 and LCAT in the biogenesis of HDL-A-IV. Adenovirus-mediated gene transfer of apoA-IV in ABCA1−/− mice failed to form HDL particles. The density gradient ultracentrifugation did not show the presence of apoA-IV in the HDL region (Fig. 4C), and the EM analysis of the HDL fractions, combined with the two-dimensional gel electrophoresis of plasma, failed to demonstrate formation of HDL particles (Fig. 4G, K).

A similar picture emerged from adenovirus-mediated gene transfer of apoA-IV in LCAT−/− mice. Following gene transfer, apoA-IV was not present in the HDL fractions (Fig. 4D). HDL particles were not detected by EM (Fig. 4H), and the two-dimensional gel electrophoresis of the plasma showed the formation of two types of particles with pre-β-like mobility (Fig. 4L). The relationship of these particles with α-HDL particles formed in apoA-I−/− mice expressing apoA-IV was established by mixing experiments (Fig. 4M).

The role of LCAT in the biogenesis of apoA-IV-containing HDL was also explored by coexpression of apoA-IV and LCAT in apoA-I−/− mice. This treatment increased the plasma HDL cholesterol levels as determined by FPLC
Biogenesis of apoA-IV-containing HDL particles

It dissociates from lipoproteins following ultracentrifugation of plasma (53). This raises the question whether apoA-IV-containing HDL particles originate from the transfer of apoA-IV that is displaced from chylomicrons to the surface of a preformed HDL molecule that contains apoA-I and in some instances other apolipoproteins. An alternative possibility is that HDL-A-IV particles are synthesized de novo by the liver.

Clues pertinent to this question were obtained from studies of transgenic mice expressing the apoA-IV gene under the control of its natural promoter or a heterologous hepatic promoter (15, 17, 19). Transgenic mice carrying the apoA-I gene under the control of the common apoA-I/apoCIII/apoA-IV promoter and enhancer (54) express apoA-IV predominantly in the intestine and to a lesser extend in the liver (15). When the plasma of these transgenic mice was fractionated by gel filtration and Western blotting, A, E, I: Analyses of apoA-I mice. B, F, J: Analyses of apoA-I × apoE mice. C, G, K: Analyses of ABCA1 mice. D, H, L, M: Analyses of LCAT mice.

Fig. 4. Analyses of plasma of apoA-I+/−, apoA-I+/− × apoE−/−, ABCA1−/−, and LCAT−/− mice infected with the adenovirus expressing the human apoA-IV by density gradient ultracentrifugation and SDS-PAGE, EM, and two-dimensional gel electrophoresis. A–D: SDS-PAGE analysis of density gradient ultracentrifugation fractions. E–H: EM pictures of HDL fractions 6–7 obtained from mice expressing human apoA-IV following density gradient ultracentrifugation of plasma, as indicated. Photomicrographs were taken at 75,000× magnification and enlarged three times. I–M: Analysis of plasma obtained from mice expressing the human apoA-IV following two-dimensional gel electrophoresis and Western blotting.

The overall pathway of the biogenesis and the potential functions of HDL-A-IV are depicted in Fig. 5D.

DISCUSSION

Role of apoA-IV, ABCA1, and LCAT in the biogenesis of HDL-A-IV

Although the functions of the intestinally delivered apoA-IV have been extensively studied during the past 35 years, there is limited information on the physiological significance and the functions of apoA-IV synthesized by the liver. Earlier studies showed that when ApoA-IV is purified from plasma by immunoprecipitation, immunoaffinity, gel filtration, or nondenaturing gradient gel electrophoresis, it is found on the HDL density fraction (50–52), but it

(Fig. 5A). It also promoted the flotation of apoA-IV in the HDL2 and HDL3 density region (Fig. 5B) and generated spherical HDL-A-IV particles (Fig. 5C). The LCAT treatment also increased the concentration of the mouse apoE in the HDL2 fraction (Fig. 5B).

The overall pathway of the biogenesis and the potential functions of HDL-A-IV are depicted in Fig. 5D.

particles observed in these experiments did not originate from apoE, we performed gene transfer experiments in apoA-I and apoE double-deficient mice. These studies also showed the formation of spherical HDL particles and preβ/HDL2-like and α-migrating HDL particles. These findings are consistent with in vivo interactions of lipid-free apoA-IV with ABCA1. As shown in Fig. 1A and documented in previous studies (20), lipid free apoA-IV promotes ABCA1 mediated cholesterol efflux to the same extend as lipid free apoA-I and apoE. The functional interactions of lipid-free apoA-IV with ABCA1 in vivo are expected to lipidate apoA-IV and lead to the generation of nascent HDL-A-IV particles. These particles may subsequently mature to spherical HDL-A-IV that can interact functionally with SR-BI. As shown in Fig. 1B, rHDL-A-IV promotes SR-BI mediated cholesterol efflux to similar extend as rHDL-A-I or rHDL-E (41, 57).

The requirement of ABCA1 and LCAT for the formation of HDL-A-IV was established by adenovirus-mediated gene transfer of apoA-IV in ABCA1- and LCAT-deficient mice, respectively. In these experiments, as expected, deficiency in ABCA1 prevented the formation of nascent or mature HDL-A-IV particles. The absence of LCAT also appears to prevent the formation of nascent or mature HDL-A-IV particles. It is possible that in the absence of LCAT, nascent HDL-A-IV particles formed by initial interactions may contribute in the de novo synthesis of HDL-A-IV particles.

We have shown previously that de novo synthesis of HDL particles containing apoA-I or apoE is initiated by interactions of the lipid-poor apolipoproteins with the ABCA1 lipid transporter. These functional interactions catalyze the transfer of phospholipids and subsequently cholesterol from intracellular membrane pools to lipid-free apoA-I or apoE leading to the formation of minimally lipidated particles which are gradually converted to discoidal particles (39, 47, 55, 56). Subsequent esterification of the cholesterol of the nascent preβ and discoidal particles by LCAT generates the spherical HDL particles present in the plasma that can be visualized by EM (55, 56). In the present study the ability of apoA-IV to promote de novo formation of HDL-A-IV particles was established by adenovirus mediated gene transfer in four different mouse models. To ensure that pro-inflammatory conditions resulting from adenovirus over expression were not reached, we monitored the plasma transaminase levels during the experiments. Gene transfer of apoA-IV in apoA-I−/− mice showed that apoA-IV expressed in the liver was distributed in the HDL fraction of plasma. EM showed the presence of spherical particles and two-dimensional gel electrophoresis showed α-migrating HDL particles and preβ-like HDL particles. To exclude the possibility that the spherical HDL particles observed in these experiments did not originate from apoE, we performed gene transfer experiments in apoA-I and apoE double-deficient mice. These studies also showed the formation of spherical HDL particles and preβ-like and α-migrating HDL particles. These findings are consistent with in vivo interactions of lipid-free apoA-IV with ABCA1. As shown in Fig. 1A and documented in previous studies (20), lipid free apoA-IV promotes ABCA1 mediated cholesterol efflux to the same extend as lipid free apoA-I and apoE. The functional interactions of lipid-free apoA-IV with ABCA1 in vivo are expected to lipidate apoA-IV and lead to the generation of nascent HDL-A-IV particles. These particles may subsequently mature to spherical HDL-A-IV that can interact functionally with SR-BI. As shown in Fig. 1B, rHDL-A-IV promotes SR-BI mediated cholesterol efflux to similar extend as rHDL-A-I or rHDL-E (41, 57).

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of lipid-free apoA-IV with ABCA1 are susceptible to fast catabolism. This interpretation is supported by coexpression of apoA-IV and LCAT in LCAT−/− mice. This treatment increased the HDL cholesterol peak and the plasma apoA-IV levels, promoted the formation of spherical HDL-A-IV particles and resulted in the distribution of apoA-IV in the HDL2 and HDL3 regions. Fast catabolism of preβ-apoA-I-containing HDL particles by the kidney has been described previously (58).

**Effect of apoA-IV on lipid and lipoprotein profiles in different mouse models**

The experiments described above also showed that following gene transfer in apoA-I−/− mice, apoA-IV was distributed in the HDL2 and HDL3 regions and the mice had normal triglycerides. In contrast following gene transfer of apoA-IV in the apoA-I−/− × apoE−/− mice, 80% of apoA-IV was distributed in the VLDL/IDL region where apoB is also found and the mice developed hypertriglyceridemia. This implies that deficiency for both apoA-I and apoE increased the affinity of apoA-IV for apoB-containing lipoprotein particles and this might have triggered the hypertriglyceridemia.

**Is there a role for HDL-A-IV in atheroprotection?**

Numerous previous studies have indicated that the conventional apoA-I-containing HDL particles promote cholesterol efflux (42, 59), prevent oxidation of LDL (60), and inhibit expression of proinflammatory cytokines by macrophages (61), as well as expression of adhesion molecules by endothelial cells (62). HDL inhibits cell apoptosis (63) and promotes endothelial cell proliferation and migration (64). HDL stimulates release of NO from endothelial cells, thus promoting vasodilatation (65). Other studies have also indicated that several beneficial effects of HDL on the arterial wall cells are mediated through signaling mechanisms mediated by SR-BI or other cell surface proteins (63–67). Owing to these properties, the conventional apoA-I-containing HDL particles are thought to protect the endothelium and inhibit several steps in the cascade of events that lead to the pathogenesis of atherosclerosis and various other human diseases.

The ability of apoA-IV to form discrete populations of HDL-A-IV particles reported in this study provides the basis for exploring further the previously reported atheroprotective functions of apoA-IV. Such functions were demonstrated in mouse models expressing apoA-IV in the intestine or the liver (15, 17, 19) as well as of apoA-IV knock-out mice (14). Overall, the present study establishes that apoA-IV has the capacity to promote the de novo biogenesis of discrete HDL-A-IV particles. The formation of these particles requires the functions of ABCA1 and LCAT. Further work is required to establish whether the generation of HDL-A-IV by the liver is responsible, at least partially, for the previously reported anti-inflammatory and atheroprotective functions of apoA-IV (16–19, 29).

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