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Structure and function of the apoA-IV T347S and Q360H common variants

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ABSTRACT

Human apolipoprotein A-IV (apoA-IV) is involved in chylomicron assembly and secretion, and in reverse cholesterol transport. Several apoA-IV isoforms exist, the most common in Caucasian populations being apoA-IV-1a (T347S) and apoA-IV-2 (Q360H). The objective of the present study was to investigate the impact of these common aminoacid substitutions on the ability of apoA-IV to bind lipids, to promote cell cholesterol efflux via ABCA1, and to maintain endothelial homeostasis. Recombinant forms of wild-type apoA-IV, apoA-IV Q360H, and apoA-IV T347S were produced in *Escherichia coli*. ApoA-IV Q360H and apoA-IV T347S showed a slightly higher α-helical content compared to wild-type apoA-IV, and associated with phospholipids faster than wild-type apoA-IV. The capacity to promote ABCA1-mediated cholesterol efflux was significantly greater for the apoA-IV T347S than the other apoA-IV isoforms. No differences were observed in the ability of apoA-IV isoforms to inhibit the production of VCAM-1 and IL-6 in TNFα-stimulated endothelial cells. In conclusion, the apoA-IV T347S common variant has increased lipid binding properties and cholesterol efflux capacity, while the apoA-IV Q360H variant has only slightly increased lipid binding properties. The two common aminoacid substitutions have no effect on the ability of apoA-IV to maintain endothelial homeostasis.

Introduction

Apolipoprotein A-IV (apoA-IV) is a 46 kDa glycoprotein that circulates as lipid-free protein or in association with chylomicrons and high density lipoproteins (HDL) [1]. In humans, apoA-IV is synthesized predominantly by the enterocytes of the small intestine and is secreted into the lymph bound to chylomicrons [1]. The 376-amino acid sequence is characterized by 22-mer amino acid repeats predicted to form the amphipathic α-helices that are a hallmark of the exchangeable apolipoproteins. The α-helices are considered to be operational for lipid binding and, therefore, for the functionality of these apolipoproteins. In the closely related protein apoA-I, disruption of helix 10 markedly reduces its ability to bind lipids and therefore promote cell cholesterol efflux via the ATP binding cassette transporter A1 (ABCA1) [2,3]. The opposite is observed in the case of apoA-IV, where the removal of the C-terminus results in increased lipid binding efficiency [4], and indeed intact apoA-IV displays a more labile lipid binding affinity than other apolipoproteins and a significant fraction of apoA-IV circulates lipid-free in plasma [5,6]. Besides facilitating chylomicron assembly and secretion [1], apoA-IV has been postulated to play many roles in reverse cholesterol transport [7]. ApoA-IV can accept, like all the other exchangeable apolipoproteins, cellular cholesterol via ABCA1 [8], it can activate the enzyme lecithin: cholesterol acyltransferase [9], and it can facilitate the action of cholesteryl ester transfer protein [10]. ApoA-IV has also been shown to possess anti-inflammatory and antioxidant properties [11,12].

APOA4, encoding apoA-IV is polymorphic with several cSNPs [13]. The most common polymorphism worldwide is rs675, an ACT > TCT substitution at codon 347, with an allele frequency of 0.20–0.25, that encodes a serine for threonine substitution (T347S) previously identified as isoform apoA-IV-1a. In Caucasian populations, the next most common polymorphism is rs5110, a CAG > CAT substitution at codon 360, with an allele frequency of 0.03–0.12 [14–16], that encodes a histidine for glutamine substitu-

Abbreviations: ABCA1, ATP binding cassette transporter A1; apoA-IV, apolipoprotein A-IV; CD, circular dichroism; DMS, dimethylsuberimidate; DMPC, dimiristoyl-phosphatidylcholine; HDL, high density lipoproteins; IL-6, interleukin-6; POPC, palmitoyl-oleoyl-phosphatidylcholine; sHDL, synthetic HDL; HUVEC, umbilical vein endothelial cells; VCAM-1, vascular cell adhesion molecule 1.

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tion (Q360H), isoform apoA-IV-2. Here we investigate the impact of the two common isoforms on the ability of apoA-IV to bind lipids, to promote cell cholesterol efflux via ABCA1, and to protect the endothelium by inhibiting the expression and release of adhesion molecules and inflammatory cytokines.

Materials and methods

Apolipoproteins. ApoA-IV was purified from the plasma fraction with density >1.25 g/ml following the method of Weinberg and Scanu [17] and separated from crude protein extracts by size-exclusion chromatography. ApoA-IV was purified from human plasma, as previously described [18]. ApoA-IV and apoA-I purity was checked by SDS–PAGE stained with Coomassie Blue and by immunoblotting using specific antibodies (anti-apoA-I from Calbiochem and anti-apoA-IV kindly provided by Professor F. Kronenberg).

Recombinant wild-type apoA-IV and apoA-IV Q360H and apoA-IV T347S variants were produced as previously described [19] and were purified to be endotoxin free using Detoxi-Gel Endotoxin removing gel (Pierce, Rockford Il, USA).

Preparation and characterization of synthetic HDL. Synthetic HDL (sHDL) containing palmitoyl-oleoyl-phosphatidylcholine (POPC, Sigma–Aldrich Chemie, Steinheim, Germany) and either plasma-derived apoA-IV, recombinant wild-type apoA-IV, apoA-IV Q360H, or apoA-IV T347S, or apoA-I were prepared by the cholate dialysis technique [20], with a starting POPC:protein weight ratio of 2.5:1. Protein and phospholipid concentrations were measured as previously described [20]. sHDL preparations were dialyzed against sterilized saline immediately before use. sHDL particle size was evaluated by non-denaturing polyacrylamide gradient gel electrophoresis (GGE). The number of protein molecules per sHDL particle was determined by cross-linking experiments with dimethylsuberimidate (DMS) [21].

Spectroscopic analyses. Circular dichroism (CD) spectra were recorded with a Jasco J-810 spectropolarimeter (Jasco, Tokyo, Japan) at the constant temperature of 25 °C. All the spectra were baseline-corrected and normalized for the protein concentration. The α-helical content was calculated by the method of Chang et al. [22], using a Jasco software. Solubilization of dimiristoyl-phosphatidylcholine (DMPC) by the various apolipoproteins was monitored by turbidity change over time at 24 °C. Multilamellar DMPC liposomes (0.5 mg/mL) were mixed with apoA-I or the various apoA-IV at a DMPC:apolipoprotein ratio of 2.5:1 (w/w), and the clearance of liposomal turbidity was monitored continuously at 325 nm at 24 °C in a Perkin-Elmer Lambda2 spectrophotometer. Time courses were analyzed by means of the non-linear statistics package in Sigma Plot 2001 (Jandel Scientific Co.).

Cholesterol efflux measurement. J774 mouse macrophages were cultured in RPMI with 10% FCS. Cells were incubated at 37 °C, 5% CO_2 seeded in 12-well plates and utilized at 80–90% of confluence. J774 monolayers were washed with PBS and incubated for 24 h in RPMI containing [^{3}H]cholesterol (4 μCi/ml), as previously described [23]. The labeling medium contained 1% FCS and 2 μg/ml of an ACAT inhibitor (Sandoz 58–035) to ensure that all labeled cholesterol was present as free cholesterol. Following 24 h labeling period, cells were washed and incubated with 0.2% BSA in RPMI, with or without 0.3 mM cpt-cAMP for 18 h. After this incubation, some wells were washed with PBS, dried, and extracted with 2-propanol. These cells provide baseline (time 0) values for total [^{3}H]cholesterol content. Stimulated and unstimulated monolayers containing [^{3}H]cholesterol were washed with PBS and incubated for efflux time (4 h) in the presence of the apolipoproteins (protein concentrations 0–12.0 μg/ml). Cell media were centrifuged to remove floating cells, and radioactivity in the supernatant was determined by liquid scintillation counting. Cholesterol efflux was calculated as: ((cpm in medium at 4 h/cpm at time 0) × 100).

Anti-inflammatory activities of sHDL. Primary cultures of human umbilical vein endothelial cells (HUVEC) were purchased from PromoCell (Heidelberg, Germany) and subcultured as previously described [24]. HUVEC were incubated overnight with increasing concentrations of sHDL (0.125–1.0 mg of protein/ml), washed twice with PBS, and then stimulated with tumor necrosis factor alpha (TNFα, R&D Systems, Minneapolis, MN, USA) at the concentration of 10 ng/ml for 8 h. At the end of the experiment, cells and conditioned media were immediately frozen at −80 °C. The concentrations of soluble vascular cell adhesion molecule 1 (sVCAM-1) and interleukin-6 (IL-6) in the conditioned media, which correlate with endothelial expression and mRNA levels [25], were evaluated by ELISA on MaxiSorp plates (Nunc GmbH & Co., Wiesbaden, Germany) using the CytoSets™ kit (BioSource International, Camarillo, CA) and the Endogen matched antibody pairs [24], respectively. For each sample, the concentrations of sVCAM-1 and IL-6 in the conditioned medium were normalized by the protein concentration of the total cell lysate, assessed by the micro BCA method (Pierce, Rockford Il, USA).

Statistical analysis. Results are reported as means ± SEM. Statistical significance was determined by One-way Anova, followed by the Student–Newman–Keuls method.

Results

Lipid binding properties

The secondary structure of plasma-derived and recombinant wild-type apoA-IV and of two apoA-IV common variants in the lipid-free and lipid-associated states was examined by CD in the far-ultraviolet at the protein concentration of 0.1 mg/ml. Lipid-free plasma-derived and recombinant wild-type apoA-IV show superimposable spectra with 53–56% α-helical content, only slightly higher than the value recently derived from a homology model [26]. Of the two common variants, apoA-IV Q360H showed a much higher α-helical content than either the wild-type apoA-IV or the apoA-IV T347S mutant (Table 1), in agreement with previous reports on a plasma-derived variant [27]. When the various apoA-IV isoforms were associated with phospholipids, a significant increase in α-helical content was observed; again, the spectra

Table 1
Alpha-helical content and kinetic parameters for binding to DMPC for apoA-IV and its variants.

Protein	α-Helical content (%)	Fast		Slow	
		Rate constant (min^{-1})	Amplitude (molar fraction)	Rate constant (h^{-1})	Amplitude (molar fraction)
Plasma apoA-IV	53	0.31 ± 0.03	0.60 ± 0.03	2.52 ± 0.11	0.4 ± 0.02
rApoA-IV	56	0.43 ± 0.06	0.49 ± 0.05	2.52 ± 0.14	0.51 ± 0.06
rApoA-IV Q360H	64	0.30 ± 0.02	0.69 ± 0.01	1.98 ± 0.05	0.32 ± 0.01
rApoA-IV T347S	51	0.34 ± 0.01	0.72 ± 0.02	1.80 ± 0.09	0.27 ± 0.02
Plasma apoA-I	45	0.28 ± 0.01	0.86 ± 0.02	1.21 ± 0.08	0.14 ± 0.01

Data are expressed as means ± SD ($n = 3$).

Table 2
Properties of synthetic HDL containing apoA-IV and its variants.

sHDL	Diameter (nm)	POPC:protein (w:w)	Apolipoproteins/particle	α-Helical content
Plasma apoA-IV	12.1	2.6	2	75
rApoA-IV	12.5	2.3	2	75
rApoA-IV Q360H	12.4	2.6	2	68
rApoA-IV T347S	12.4	2.3	2	64
Plasma apoA-I	9.8	2.4	2	72

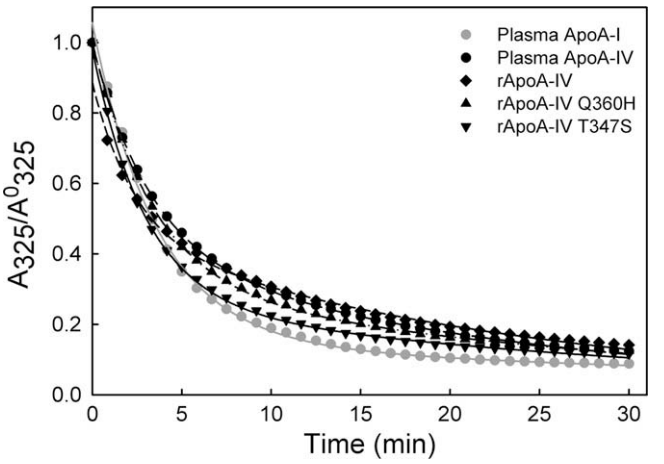

Fig. 1. Solubilization of DMPC by the various apoA-IV and by apoA-I, as monitored by turbidity change over time at 24 °C. Multilamellar DMPC liposomes (0.5 mg/mL) were mixed with the various proteins at a DMPC:apolipoprotein ratio of 2.5:1 (w/w) and the clearance of liposomal turbidity was continuously monitored at 325 nm. Thin lines on each experimental curve represent a computer fitting to simultaneously occurring reactions, as described in the text.

obtained for the plasma-derived and recombinant apoA-IV were superimposable, whereas the two variants showed a reduced α-helical content (Table 2).

The association with DMPC of plasma-derived and recombinant wild-type apoA-IV and of the two apoA-IV common isoforms was followed by measuring the clearance of liposomal turbidity as a function of time at 24 °C, i.e., the transition temperature of the phospholipid, in comparison with apoA-I. Time courses similar to those presented in Fig. 1 were analyzed from a kinetic standpoint. In all cases, and for all proteins, these time courses could be deconvoluted into two simultaneous reactions, a fast one and a slow one, that accounted for a different fraction of the observed changes, as summarized in Table 1. These data made it evident that the extent to which the fast phase accounted for the observed changes decreased in the order: apoA-I >> apoA-IV T347S > apoA-IV Q360H > plasma-derived apoA-IV >> recombinant wild-type apoA-IV. The rate of changes observed in the fast component of the simultaneous reaction were comparable, if not for recombinant wild-type apoA-IV, that was about 25% faster than all other proteins. In view of the very low rates observed for the slow component of the overall reaction and of the fact that often it accounted for a marginal portion of the lipid–protein interaction, both mutants seem to be more efficient than the wild-type apoA-IV, and less efficient than apoA-I.

All the apoA-IV isoforms were completely included in discoidal sHDL when reconstituted with POPC (Fig. 2). The size of the various sHDL was very similar, with diameters ranging from 12.1 to 12.5 nm (Fig. 2A and Table 2). All the sHDL contained 2 apoA-IV molecules per particles as detected by cross-linking (Fig. 2B). When apoA-I was reconstituted with POPC at the same POPC:protein ratio, a major component of 9.6 nm containing two apoA-I molecules per particle was obtained [20].

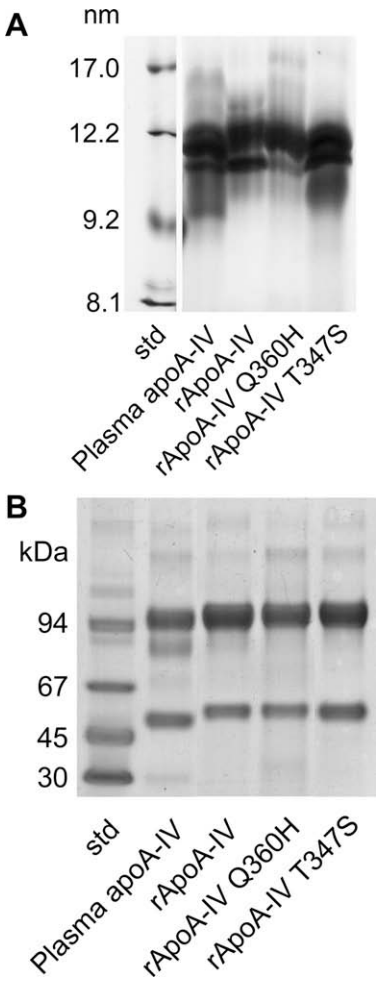

Fig. 2. Non-denaturing gradient gel electrophoresis (A) and SDS–PAGE analysis after cross-linking with DMS (B) of sHDL containing plasma-derived apoA-IV or recombinant wild-type apoA-IV, apoA-IV Q360H, and apoA-IV T347S.

Cell cholesterol efflux capacity

The ability of apoA-IV and its variants to promote cell cholesterol efflux was tested in unstimulated and cAMP-stimulated J774 murine macrophages, in comparison with apoA-I. ABCA1-mediated cell cholesterol efflux was calculated as the percentage efflux from cAMP-stimulated J774 cells minus the percentage efflux from unstimulated cells. As shown in Fig. 3, apoA-I was significantly more efficient in promoting ABCA1-mediated cholesterol efflux than all the apoA-IV forms. Among the apoA-IV forms, the apoA-IV T347S variant was the most efficient, and accepted significantly more cholesterol than all the others apoA-IV isoforms at all tested concentrations (Fig. 3).

Anti-inflammatory activity of sHDL

The anti-inflammatory activity of sHDL containing apoA-IV or its variants was evaluated as the ability to inhibit the production

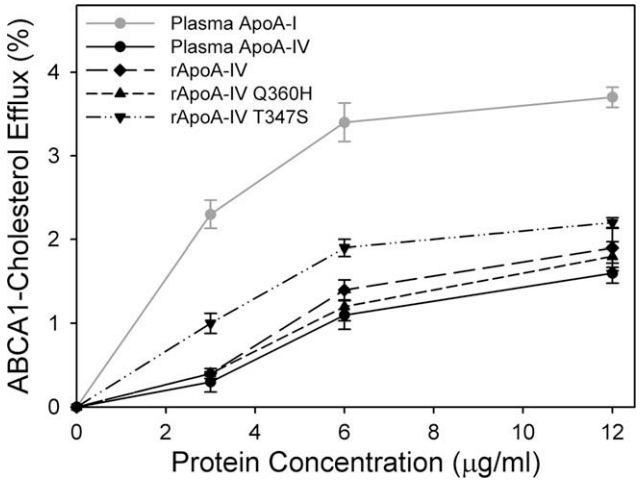

Fig. 3. Efflux of cholesterol from J774 macrophages to apoA-I and to the various apoA-IV isoforms. Monolayers were radiolabeled and incubated in the presence or absence of 0.3 mM cAMP. Following this treatment, monolayer were incubated with either apoA-I or the various apoA-IV (3.0–12.0 μg/ml) as cholesterol acceptors for 4 h. ABCA1-mediated cell cholesterol efflux was calculated as the percentage efflux from cAMP-stimulated J774 cells minus the percentage efflux from unstimulated cells. Data are expressed as mean ± SEM ($n = 3$).

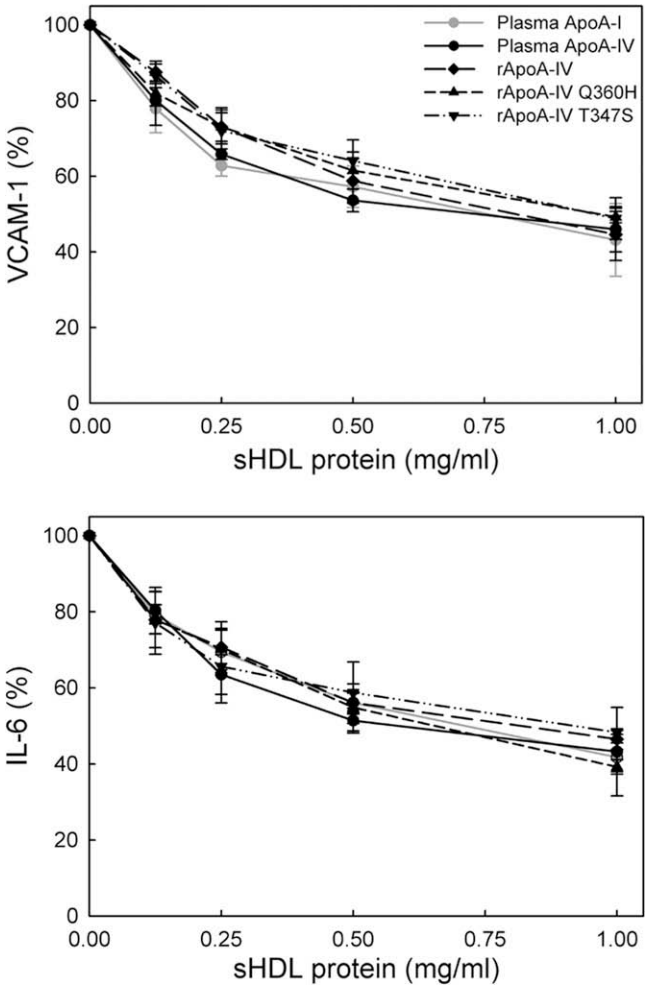

Fig. 4. IL-6 and VCAM-1 release in sHDL-treated, TNFα-stimulated HUVEC. Cells were incubated overnight with the sHDL containing apoA-I or the various apoA-IV before stimulation with TNFα for 8 h. Results are expressed as percentage of IL-6 or VCAM-1 concentration in conditioned media of untreated, TNFα-stimulated HUVEC. Data are expressed as mean ± SEM ($n = 3$).

of VCAM-1 and IL-6 in TNFα-stimulated HUVEC, in comparison with apoA-I containing sHDL. Synthetic HDL were used in a range of protein concentrations which includes the physiological plasma levels of both apoA-IV and apoA-I (0.125–1.0 mg/ml). Pre-treatment with sHDL caused a concentration-dependent decrease of VCAM-1 and IL-6 levels in the conditioned media of TNFα-stimulated cells (Fig. 4). The inhibitory effect of sHDL containing plasma-derived apoA-I or apoA-IV on VCAM-1 levels was comparable within the whole concentration range. When compared to untreated TNFα-stimulated cells, VCAM-1 level varied between 77.9 ± 6.4% and 43.1 ± 9.6% in cells pre-treated with 0.125–1.0 mg/ml of apoA-I sHDL and between 80.1 ± 6.6% and 46.0 ± 6.0% in cells pre-treated with the same concentrations of apoA-IV sHDL. sHDL containing the recombinant forms of apoA-IV were as efficient as plasma-derived apoA-IV at all tested concentrations. The inhibitory effect of the different sHDL particles on IL-6 production was superimposable along the whole tested concentration range; IL-6 levels varied between 83.3 ± 6.4% and 77.1 ± 8.3% at 0.125 mg/ml, and between 45.0 ± 5.8% and 41.7 ± 3.8% at 1.0 mg/ml.

Discussion

The present study demonstrates that (i) the common variants of apoA-IV, T347S and Q360H, have increased ability to bind lipids; (ii) the variant T347S has enhanced capacity to promote macrophage cholesterol efflux; (iii) the two aminoacid substitutions have no impact on apoA-IV ability to inhibit the expression and release of adhesion molecules and cytokines on endothelial cells.

An important function of the exchangeable apolipoproteins is to bind to lipids to form lipoproteins. The results described here demonstrate that apoA-IV is significantly less efficient than apoA-I at clearing a phospholipid suspension, as already shown by others [28]. The substitutions of the aminoacid Ser for Thr at position 347 and His for Gln at position 360 both increase the ability of apoA-IV to bind lipids. These two residues are located in helix 10 of the molecule [26], in a region very critical in determining apoA-IV lipid association [4]. Residue Thr347 is part of a region which deletion strongly increases the lipid association capacity of apoA-IV [29], and indeed the apoA-IV T347S variant is the one showing the fastest rate of lipid association among the different apoA-IV isoforms. Residue Gln360 is farther from the crucial residues involved in lipid association [29], and the effect of its substitution on the lipid binding capacity of apoA-IV is less evident.

Besides the ability to bind lipids and form lipoproteins, apoA-IV shares with apoA-I and other exchangeable apolipoproteins the ability to accept cholesterol via ABCA1. ApoA-IV is significantly less efficient than apoA-I in promoting ABCA1-mediated cholesterol efflux from macrophages [28]. This has been explained by the presence of a C-terminal domain enriched in Gln/Glu which can attenuate the apoA-I-like functions of apoA-IV [28]; indeed, the removal of the C-terminal 44 aminoacids increases the ability of apoA-IV to promote cholesterol efflux [28]. In agreement with this observation, and similar to what observed for phospholipid binding, the substitution of the aminoacid Ser for Thr at position 347 significantly increased the ability of this variant to promote cholesterol efflux compared to the other isoforms, although this value was not comparable to that of apoA-I.

When bound to lipids, either in plasma-derived or synthetic HDL, apoA-I can also modulate some endothelial functions, such as the expression of adhesion molecules and the release of cytokines and vasoactive substances [30]. Again, this property is not peculiar to apoA-I but it is shared by other HDL apolipoproteins, such as apoA-II [20]. Here we show for the first time that sHDL containing apoA-IV are as efficient as sHDL containing apoA-I in inhib-

iting the cytokine-induced expression of adhesion molecules and release of cytokines in endothelial cells. Moreover, no differences were observed between wild-type apoA-IV and the two common variants. This observation, in agreement with our previous results obtained with different apolipoproteins [20], support the hypothesis that the HDL apolipoproteins are not the active component but are likely needed to correctly pack the active lipid component. Indeed, sHDL containing apoA-I mimetic peptides, with primary sequence totally different from that of apoA-I but able to form amphipathic α-helices and to bind lipids, have anti-inflammatory activity comparable to apoA-I containing sHDL [25].

In conclusion, the substitution of the aminoacid Ser for Thr at position 347 of the apoA-IV sequence significantly increases the ability of the protein to bind lipids and to promote macrophage cholesterol efflux. The substitution of the aminoacid His for Gln at position 360 has little effects on apoA-IV lipid binding ability and no effects on its cholesterol efflux capacity. None of the substitutions affects the endothelial protection ability of apoA-IV.

Acknowledgments

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