Apolipoprotein A2 reduces the levels of circulating triglyceride-rich lipoproteins, an effect blocked by apolipoprotein E

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ABSTRACT

Apolipoprotein A2 (APOA2), the second in quantity apolipoprotein of high density lipoprotein (HDL) is synthesized by the liver and much less by the intestine. Studies in humans, failed to establish a clear role for APOA2 in coronary heart disease and overall human physiology. Even though we know that APOA2 plays a key role in the biogenesis and functionality of HDL particles and can interact physically with other apolipoproteins such as apolipoprotein E (APOE), forming dimers, our knowledge on its role in triglyceride-rich lipoprotein (TRL) metabolism remains limited. Here, we investigated how functional interactions between APOA2 and APOE may affect plasma lipoprotein metabolism in the absence of apolipoprotein A1 (APOA1). For this purpose, APOA1 deficient and APOA1xAPOE double deficient mice were fed high fat diet for two weeks and were subsequently infected with either an adenovirus expressing the human APOA2 (AdAPOA2) or a control adenovirus AdGFP. Five days post-infection blood was collected, and plasma and lipoprotein fractions were isolated. After confirmation of human APOA2 expression in vivo by western blot we measured plasma and lipoprotein total cholesterol and triglyceride levels. APOA2 expression increased total cholesterol and triglyceride levels in APOA1 deficient mice. To the contrary, when APOA2 was expressed in APOA1xAPOE double deficient mice, which lack functional APOE a significant reduction in both plasma cholesterol and triglyceride levels associated with a notable reduction in TRL was observed. Overall, our data support that a significant functional interaction between APOA2 and APOE impacts plasma TRL metabolism.

KEY WORDS: Apolipoprotein A2, apolipoprotein E, triglyceride-rich lipoproteins

INTRODUCTION

Apolipoprotein A2 (APOA2) is the second, most abundant apolipoprotein of high density lipoprotein (HDL) particles after apolipoprotein A1 (APOA1). APOA2 consists of 77 amino acids and is synthesized mainly by the liver...
and to a lesser extent by the intestine. It has been suggested that APOA2 impacts the synthesis and functionality of HDL particles.

In transgenic mice, APOA2 overexpression is associated with abnormal lipoprotein composition, elevated HDL-cholesterol (HDL-C) levels, and susceptibility to atherosclerosis. Clinical data from animal models and humans show that elevated APOA2 levels affect the size and distribution of HDL particle subpopulations as well as their APOA1 content. Similarly, Mice expressing human APOA2 have smaller size APOA2-containing HDL particles and significantly reduced levels of APOA1-containing HDL particles. Furthermore, the HDL particles of these mice show reduced levels of esterified cholesterol in relation to total cholesterol, due to reduced activity of lecithin–cholesterol acyltransferase (LCAT). Mice overexpressing endogenous APOA2 showed increased levels of triglycerides in HDL particles. In addition, transgenic mice expressing human APOA2 contain high levels of APOA2 in their very low density lipoprotein (VLDL) particles and reduced activity of lipoprotein lipase (LpL) and hepatic lipase (HL) in plasma. Some atherosclerotic lesions develop in these mice, even when they are fed a standard low-fat diet. These lesions develop further when these mice are fed an atheromatous diet. In contrast, human studies have so far failed to identify a precise role of APOA2 in coronary heart disease (CHD). In some studies, APOA2 appears to promote the development of atherosclerosis. However, in a study of 126 subjects with different degrees of atherosclerosis (calcified and non-calcified atherosclerotic plaques), APOA2 appeared to be positively correlated with reverse cholesterol transport and negatively correlated with non-calcified plaque burden.

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Furthermore, the interactions of APOA2 with APOA1 but also with other lipoproteins involved in the HDL metabolic pathway, affect the distribution of HDL particle subpopulations, as well as their functionality. Despite that 40 years have elapsed since Apoa2 gene was sequenced and the structure of APOA2 protein was identified, its functional role in human physiology and disease development have not yet been elucidated.

In a recent study, we showed that overexpression of human APOA2 in full-genome expressing mice resulted in the generation of APOA2-containing HDL particles with distinctly different apolipoprotein composition and geometry from control HDL particles, with features which were associated with increased functionality. It is possible that these changes in HDL functionality are not due to a direct effect of APOA2, but rather arise from the functional interaction of APOA2 with other apolipoproteins, such as apolipoprotein E (APOE). In vitro studies have shown that APOA2 forms dimers with APOE and that this interaction was proposed to affect the ability of APOE to bind to lipoprotein particles. However, to this date there is limited knowledge on the effect of APOA2-APOE interaction on TRL metabolism.

To fill this void, we investigated how functional interactions between APOA2 and APOE may affect plasma lipoprotein metabolism. To this end we used APOA1 deficient mice (apoai−/−) and mice with a combined deficiency in APOA1 and APOE (apoai−/−×apoe−/−), fed a lipid-rich diet for 2 weeks and subsequently infected them with a recombinant adenovirus expressing either human APOA2 (AdAPOA2) or a control adenovirus expressing the green fluorescent protein (AdGFP).

Our results show that in apoai−/− mice hepatic production of APOA2 and subsequent generation of APOA2-containing HDL, is associated with an increase in both plasma cholesterol and triglycerides due to increased deposition of TRL particles in blood. In contrast, in apoai−/−×apoe−/− mice that do not express APOE, a significant decrease in TRL particles was observed and was associated with a measurable decrease in plasma cholesterol and triglyceride levels.

**MATERIALS AND METHODS**

**Animals**

APOA1 deficient (apoai−/−) mice and mice deficient in both APOA1 and APOE (apoai−/−×apoe−/−) on the C57BL/6 genetic background were purchased from Jackson Labs (Bar Harbor, Maine, USA). Mice aged 16-20 weeks were individually caged under a 12-hour light/dark cycle and had unrestricted access to food and water. Their age was approximately 16-20 weeks. For a period of 2 weeks mice were fed a standard Western-type diet (WTD, Mucedola SRL, Milano, Italy, 4.5 kcal/g) composed of 17.3% protein, 48.5% carbohydrate, 21.2% fat and 0.2% cholesterol (0.15% added, 0.05% from fat source). Immediately after the 2 weeks mice were infected with either an adenovirus expressing human APOA2 or a control adenovirus. Sample size was determined based on the desired power of statistical analysis, using an online statistical tool (http://www.stat.ubc.ca/~rollin/stats/ssize/n2.html). All animal experiments were conducted according to the EU guidelines of the Protocol for the Protection and Welfare of Animals. The work was authorized by the Laboratory Animal Centre com-
mittee of The University of Patras Medical School and the Veterinary Authority of the Prefecture of Western Greece.

Development and purification of recombinant adenovirus expressing human APOA2

The development and purification of the recombinant adenovirus expressing human APOA2 (AdAPOA2) was performed as described previously. Briefly, both AdAPOA2 and AdGFP (control adenovirus expressing the green fluorescent protein) were expanded in HEK293 cells and then purified by double CsCl ultracentrifugation, followed by titration. The adenovirus title was approximately 5×10¹⁵ pfu/L.

Expression of human APOA2 in mice

Mice were infected with 2x10⁹ pfu of AdGFP-APOA2, by tail vein injection, after WTD feeding. To assess potential non-specific effects of viral infection in subsequent analyses, an additional mouse group will also be infected with 2x10⁹ pfu of the control AdGFP virus. Five days post-infection and four hours after fasting, mice were euthanized, and blood and tissue samples were collected for further analyses. To assess the expression of APOA2 in the infected mice plasma and lipoprotein fractions were analyzed by western blot as described previously.

Lipid levels of plasma and lipoproteins

Total cholesterol and triglycerides levels were measured spectrophotometrically in plasma samples and lipoprotein fractions, using the DiaSys Cholesterol FS kit (ref# 11300, Diagnostic Systems, GmbH, Holzheim, Germany) and the DiaSys Triglycerides FS kit (ref# 15710, Diagnostic Systems, GmbH, Holzheim, Germany), respectively, according to manufacturers’ instructions and as described previously.

Plasma density gradient ultracentrifugation and isolation of lipoprotein fractions

Pooled plasma (0.4 ml) from each mouse group was fractionated by KBr density gradient ultracentrifugation, over a 4 ml KBr (Sigma-Aldrich, St. Louis, MO, USA) gradient (1.23 g/ml over 1.21 g/ml over 1.063 g/ml over 1.019 g/ml over saline), as described previously.

Assessment of hepatic VLDL-triglyceride secretion

Mice were infected with 2x10⁹ pfu of AdGFP-APOA2, by tail vein injection, after WTD feeding. To assess potential non-specific effects of viral infection in subsequent analyses, an additional mouse group was also infected with 2x10⁹ pfu of the control AdGFP virus. On the 4th day post-infection mice were fasted for 16h and subsequently injected with Tyloxapol (Triton-WR1339) at a dose of 500 mg/kg body weight using a 15% solution (w/v) in 0.9% NaCl. Blood samples were isolated at 5, 10, 20, 30, 40, 50, and 60 min after injection with Triton-WR 1339. As a control, blood samples were isolated 1 min immediately after the injection with the detergent. Triglyceride levels were assessed spectrophotometrically in plasma samples using the DiaSys Triglycerides FS kit (ref# 15710, Diagnostic Systems, GmbH, Holzheim, Germany). The rate of VLDL-triglyceride secretion (expressed in mg/dl/min) was calculated from the slope of the linear regression for each individual mouse. Then, slopes were grouped together and reported as means ± SEM in the form of a bar graph.

Statistical analyses

All data sets were tested for normality using the Kolmogorov-Smirnov and the Shapiro-Wilk tests. Data are reported as Mean ± SEM. Parametric (p>0.1) or non-parametric tests (p<0.1) were performed using the GraphPad Prism 6 software.

RESULTS

Confirmation of human APOA2 expression in vivo

To confirm human APOA2 expression in vivo, mice were infected with 2 x 10⁹ pfu AdGFP or AdAPOA2 by tail-vein injection. Five days post-infection plasma samples were collected and analyzed by western blot for APOA2 protein, confirming the efficient expression of APOA2 in vivo. In apoa1−/− mice, infection with the AdAPOA2 resulted in the presence of significant amounts of APOA2 on both HDL and LDL, but not VLDL, particles. No lipid-free APOA2 was detected in the plasma of these mice. In apoa1−/−×apoe−/− mice APOA2 was present mainly in HDL particles and to a lower extent in LDL particles with a significant amount of APOA2 present in its lipid-free form.

Effects of human APOA2 expression on plasma cholesterol and triglyceride levels

Mice were infected with 2x10⁶ pfu of AdAPOA2 or AdGFP to assess potential non-specific effects of viral infection in subsequent analyses. Analysis of plasma lipid levels five days post-infection showed that the expression of human APOA2 resulted in a significant increase of plasma total cholesterol and triglyceride levels of apoa1−/− mice, compared to the same mice
infected with the control AdGFP adenovirus (Figure 2A, 2B). This increase was associated with an increase in chylomicrons/VLDL, IDL, LDL and HDL cholesterol and an increase in chylomicrons/VLDL and IDL triglycerides (Figure 3A, 3B). On the other hand, the expression of human APOA2 in apoa1−/−×apoe−/− mice five days post-infection, resulted in a significant decrease of both cholesterol and triglyceride levels (Figure 2A, 2B). This decrease was also evident in the analysis of lipoprotein fractions (Figure 3C, 3D). Specifically, both cholesterol and triglyceride levels were significantly lower in chylomicrons/VLDL, IDL and LDL fractions of apoa1−/−×apoe−/− mice infected with AdAPOA2 compared with control group (Figure 3C, 3D). Moreover, our data show that APOA2 expression in the absence of functional APOE, results in elevated triglycerides levels in the HDL fractions (Figure 3C, 3D).

**Effects on hepatic VLDL-triglyceride secretion**

The lower VLDL-triglyceride content detected in the apoa1−/−×apoe−/− mice infected with AdAPOA2 could be a result of reduced VLDL-triglyceride secretion rate. Therefore, we performed a VLDL-triglyceride secretion assay in both apoa1−/− and apoa1−/−×apoe−/− mice following infection with AdGFP or AdAPOA2. Our results show that APOA2 expression led to a significant reduction of the hepatic VLDL-triglyceride secretion rate apoa1−/−×apoe−/− mice infected with AdAPOA2 compared to those infected with the control AdGFP adenovirus (1.396 ± 0.13 mg/dL/min for AdAPOA2 group vs 3.314 ± 0.83 mg/dL/min for AdGFP group, p<0.05) (Figure 4B, 4C). Interestingly, in apoa1−/− mice which express APOE, infection with AdAPOA2 did not alter the rate of hepatic VLDL-triglyceride secretion (7.460 ± 0.59 mg/dL/min for AdAPOA2 group vs 7.779 ± 0.06 mg/dL/min for AdGFP group, p>0.05) (Fig. 4A, C).
APOA2 in TRL metabolism

DISCUSSION

Forty years have passed since the human APOA2 nucleotide sequence was identified. Yet, our knowledge of the specific origin of APOA2-containing lipoproteins and their role in human physiology remain unclear. In vitro studies have shown that APOA2 forms dimers with APOE. It is possible that this kind of interaction affects the ability of APOE to bind to HDL particles or interact with its receptors including the low density lipoprotein receptor (LDLR). Led by previous observations from our laboratory, such as the effects of APOC3 in triglycerides levels, here we investigated the effect of APOA2 on TRL metabolism.

FIGURE 3. Lipoprotein total cholesterol (A) and triglyceride levels (B) of apoa1−/− mice and lipoprotein total cholesterol (C) and triglyceride levels (D) of apoa1−/x apoe−/− mice five days post-infection with AdAPOA2 or AdGFP (n=5).

FIGURE 4. Rate of hepatic VLDL triglyceride secretion in apoa1−/− (A) and apoa1−/x apoe−/− (B) mice five days post-infection with AdAPOA2 or AdGFP. The bar graph (C) represents the mean ± SEM of the individual secretion rates (n=6).
in the presence and absence of functional APOE, in mice lacking classical APOA1-containing HDL particles.

Western blot analysis of plasma lipoprotein fractions isolated from apoa1−/− and apoa1−/−× apoe−/− mice five days post-infection with either AdAPOA2 or AdGF, confirmed the in vivo expression of human APOA2. Specifically, human APOA2 was present in the IDL, LDL and HDL fractions of both apoa1−/− and apoa1−/−× apoe−/− mice. Our results from plasma and lipoprotein fractions lipid analysis, show that in apoa1−/− mice expression of human APOA2 results in a significant increase in both plasma cholesterol and triglycerides due to increased deposition of all lipoprotein fractions. In contrast, in apoa1−/−× apoe−/− mice that do not express APOE, a significant decrease in plasma cholesterol and triglycerides was observed, with the most significant decrease was identified in the triglyceride levels of TRLs.

Our data thus far suggest that in the absence of APOE, APOA2 suppresses hepatic VLDL-triglycerides secretion, an effect that disappears when APOE is expressed (Figure 4). However, other mechanisms could also mediate the observed phenotypes and need to be further investigated. For example, it is possible that the positive effect of APOA2 on plasma TRLs in the absence of APOE is also associated with reduced post-prandial intestinal lipid absorption. Another possibility is that the formation of APOE-APOA2 heterodimers that has been previously reported in the literature17, may lead to a potent inhibition of LpL, an effect that disappears in the absence of APOE. Another possibility is that APOA2 may also promote the direct clearance of TRLs via a receptor mediated process which is inhibited in the presence of functional APOE.

Overall, our data support that a significant functional interaction between APOA2 and APOE impacts plasma TRL metabolism.

Acknowledgements

The present study was supported by a research grant from the Hellenic Atherosclerosis Society.

Conflict of interest

The authors declare no conflict of interests/financial disclosure statement.
APOE, paratηρήθηκε σημαντική μείωση τόσο της χοληστερόλης όσο και των τριγλυκεριδίων στο πλάσμα, που χαράστηκε με αξιοσημείωτη μείωση στις TRL. Συνολικά, τα δεδομένα μας υποστηρίζουν ότι μια σημαντική λειτουργική αλληλεπίδραση μεταξύ της APOA2 και της APOE επηρεάζεται το μεταβολισμό των TRL στο πλάσμα.

ΛΕΞΕΙΣ ΚΛΕΙΔΙΑ: Απολιποπρωτεΐνη A2, απολιποπρωτεΐνη E, πλούσιες σε τριγλυκερίδια λιποπρωτεΐνες

REFERENCES


Journal of Atherosclerosis Prevention and Treatment – JAPT

55
