**Genetic deficiency of Apolipoprotein D in the mouse is associated with
non-fasting hypertriglyceridemia and hyperinsulinemia**

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Abstract

Objective: Apolipoprotein D (ApoD) is an atypical apolipoprotein with an incompletely understood function in the regulation of triglyceride and glucose metabolism. We have demonstrated that elevated ApoD production in mice results in improved postprandial triglyceride clearance. This work studies the role of ApoD deficiency in the regulation of triglyceride and glucose metabolism and its dependence on aging.

Methods/materials: We used ApoD knockout (ApoD-KO) mice of 3 and 21 months of age. Body weight and food intake were measured. Hepatic histology, triglyceride content, lipoprotein lipase levels (LPL) and plasma metabolites were studied. Phenotypic characterization of glucose metabolism was performed using glucose tolerance test. Beta-cell mass, islet volume and islet number were analyzed by histomorphometry.

Results: ApoD deficiency results in non-fasting hypertriglyceridemia in young ($p=0,01$) and aged mice ($p=0,002$). In young ApoD-KO mice, hypertriglyceridemia was associated with 30-50% increased food intake in non-fasting and fasting conditions respectively, without changes in body weight. In addition, LPL levels were reduced by 35% in adipose tissue ($p=0,006$). In aged ApoD-KO mice, hypertriglyceridemia was not associated with changes in food intake or body weight, whereas hepatic triglyceride levels were reduced by 35% ($p=0,02$). Furthermore, non-fasting plasma insulin levels were elevated by 2-fold in young ($p=0,016$) and aged ($p=0,004$) ApoD-KO mice, without changes in blood glucose levels, glucose tolerance, beta cell mass or islet number.

Conclusions: These findings underscore the importance of ApoD in the regulation of plasma insulin levels and triglyceride metabolism, suggesting that ApoD plays an important role in the pathogenesis of dyslipidemia.

Key words: Dyslipidemia, lipoprotein lipase, insulin resistance, beta-cell function, lipocalins.

Abbreviations: ApoD, Apolipoprotein D; HDL, high density lipoprotein; LDL, low density lipoprotein; VLDL, very low density lipoprotein; WT, wild type mice; ApoD-KO, Apolipoprotein D knockout mouse; TG, triglycerides; CHL, total cholesterol; LCN2, Lipocalin-2; LPL, Lipoprotein lipase; RBP4, Retinol-Binding Protein 4.

Introduction

Apolipoprotein D (ApoD) is a Lipocalin widely expressed in mammalian tissues and known to bind a series of hydrophobic ligands *in vitro* with high affinity (pregnenolone, progesterone and arachidonic acid) [1-3] as well as cholesterol with very low affinity [4]. The expression of ApoD is prominent in the nervous system, particularly upon aging or induced damage. We have shown that it exerts protective roles in both situations: by controlling the levels of brain peroxidated lipids in a model of accelerated aging by oxidative insult [5], or by controlling the extent and duration of inflammatory processes after peripheral nerve injury [6], influencing this way the rate of nerve regeneration.

Curiously, ApoD was simultaneously discovered in the human breast cyst fluid and as an apolipoprotein present in high-density lipoproteins (HDL) and to a lesser extent in very low-density lipoproteins (VLDL) [7,8]. ApoD is an atypical apolipoprotein, unrelated to other apolipoproteins in both structure and evolutionary origins [9]. Because ApoD is mainly located in HDL, it was soon proposed to have a role in lipid homeostasis [10].

The *Drosophila* genome contains two Lipocalin homologues of vertebrate ApoD, Glial Lazarillo (GLaz) and Neural Lazarillo (NLaz), mainly expressed in glia and neurons respectively [11,12]. Genetic ablation of GLaz or NLaz reduces total triglyceride content and resistance to starvation in young flies [11,12], while aging is accompanied by neutral fats accumulation in NLaz deficient flies [13]. In contrast, overexpression of NLaz increases total triglyceride content and resistance to starvation [11]. In addition to its role in the regulation of lipid metabolism, NLaz mutants exhibit low glucose levels, whereas flies overexpressing NLaz show

elevated glucose levels [11]. Taken together, these studies illustrate that the *Drosophila* ApoD homologues GLaz and NLaz play a role in the regulation of lipid and glucose metabolism, besides their roles in nervous system physiology.

To gain insights into the role of ApoD in the regulation of lipid metabolism in a vertebrate model organism, we have previously used a gain-of-function approach to overexpress ApoD in mouse. Elevated ApoD production in the liver of young mice results in enhanced lipoprotein lipase (LPL) activity and improved postprandial triglyceride clearance, whereas VLDL-triglyceride production remained unchanged [14]. However, brain overexpression of human ApoD in middle-aged mice results in hepatic steatosis, despite normal lipid concentration in circulation, glucose intolerance and insulin resistance [15]. Finally, epidemiological studies in humans associate ApoD genetic variants with elevated plasma triglyceride levels [16,17], and the TaqI polymorphism of the APOD gene is associated with the development of obesity, insulin resistance, hyperinsulinemia and type 2 diabetes [18,19].

Thus, the precise role of mammalian ApoD in the regulation of lipid metabolism has only recently started to be addressed and to fully understand the role of ApoD in triglyceride metabolism, an analysis of the loss-of-function mouse model is required. In this study, we hypothesized that a loss of ApoD would increase plasma triglyceride levels, contributing to the pathogenesis of dyslipidemia. To address this hypothesis, we evaluated the impact of losing ApoD on triglyceride metabolism in young and aged ApoD-deficient mice (ApoD-KO). Here we show that genetic ablation of ApoD results in hypertriglyceridemia and hyperinsulinemia in non-fasting conditions. These findings underscore the importance of ApoD in the regulation of triglyceride metabolism and in insulin-dependent processes.

121 **Methods**

122 **Ethical approval**

123 Experimental procedures were approved by the Animal Care and Use Committee of
124 the University of Valladolid in accordance with the Guidelines for the Care and Use of
125 Mammals in Research (European Commission Directive 86/609/CEE and Spanish
126 Royal Decree 1201/2005).

127 **Experimental Animals**

128 WT and ApoD-KO mice were bred at the animal facility of the University of Valladolid,
129 Spain. ApoD-KO mice were generated and genotyped as previously described [5].
130 Mice were fed standard rodent chow and water ad libitum in ventilation-controlled
131 cages in a 12 h-light/dark cycle. The experimental cohorts used in this study were
132 males of the F1 generation of homozygous crosses. The parental generation was
133 composed of ApoD^{-/-} and ApoD^{+/+} littermates from heterozygous crosses of the
134 ApoD-KO line in C57BL/6 background. This strategy avoids the potential maternal
135 effects of ApoD and generates wild-type and ApoD-KO cohorts with a homogeneous
136 genetic background. Two independent cohorts were used for the collection of tissues
137 at two ages: 3 months (n=10/genotype) and 21 months old (n=11/genotype).

138 **Plasma Biochemistry**

139 Blood samples were obtained from mice under fasting conditions (16 hours) or under
140 non-fasting conditions (animals had free access to food pellets ad libitum for 48 h
141 after the fasting period). This paradigm compares fasting versus non-fasting
142 conditions, since the exact timing of food intake with respect to sample collection is
143 not determined. Blood was collected from the tail vein into capillary tubes precoated
144 with potassium-EDTA (Sarstedt, Nümbrecht, Germany) for the preparation of plasma.

145 Blood glucose levels were determined using a Glucometer Xceed (Abbott Diabetes
146 Care Ltd., Oxon, UK). Plasma triglycerides and cholesterol levels were determined
147 using the Wako triglyceride and cholesterol reagents (Wako Chemicals GmbH,
148 Neuss, Germany). Plasma insulin levels were measured using ultrasensitive mouse
149 ELISA (ALPCO Diagnostics, NH, USA).

150 **Food intake**

151 For food intake determination, ApoD-KO and WT mice were separated in individual
152 cages (n=7-11 mice per genotype). After the 16 h fasting period, food pellets (50 g)
153 were added to each cage, and food intake of each mouse was estimated from the
154 difference in remaining food weight at 24 and 48 h. These weights were averaged to
155 provide an estimate of the mean food intake of each genotype in the 48 h period
156 following fasting.

157 **Glucose tolerance test and insulin sensitivity index**

158 Mice were fasted for 16 hours and injected intraperitoneally with glucose at 2g/Kg of
159 body weight. Blood glucose levels were determined and plotted as a function of time.
160 Insulin sensitivity index (ISI) was calculated using the formula $ISI = 2/[(INS \times GLU) + 1]$,
161 where INS is fasting plasma insulin levels and GLU is fasting blood glucose levels
162 with values converted to pmol/L and mmol/L respectively [20].

163 **Hepatic triglyceride determination and liver histology**

Hepatic triglyceride determination was performed as previously described [14]. For liver histology, standard paraffin and cryostat sections were performed after fixation in 4% paraformaldehyde as previously described [6]. Oil-red O staining was performed on 10 µm cryostat sections using isopropanol as diluent. Hematoxylin-Eosin staining was performed on 3 µm paraffin sections following standard procedures [6].

Determination of islet mass and islet histomorphometry

Pancreata were excised, fixed, sectioned, stained with insulin, and quantitative islet histomorphometry was performed as previously described [21].

Immunoblot analysis

To determine the effect of ApoD deficiency on LPL protein expression, epididymal fat tissue was collected from experimental and control mice. Cell extracts were obtained in lysis buffer (Cell Lysis Buffer, Cell Signaling, Beverly, MA) supplemented with protease inhibitors (Protease Inhibitor Cocktail Sigma, St. Louis, MO). Solubilized proteins (20 µg/lane) were separated by SDS-PAGE and electrotransferred onto polyvinylidene difluoride membranes for conventional immunoblotting. After probing with LPL-specific antibody (1:1000, Santa Cruz Biotechnology Inc., Heidelberg, Germany) the membranes were stripped and reprobed with antibody against β-actin (1:5000, Sigma). Chemiluminescence signals (ECL Plus detection system, Amersham Biosciences, Piscataway, NJ, USA) were detected in the linear range for quantification purposes.

Statistical analysis

Statistical analyses of data were performed by Student-t test and by analysis of variance (ANOVA). Data were expressed as mean ± SD. P values <0.05 were considered significant.

Results

Effect of ApoD deficiency on triglyceride metabolism in mice

We reported that elevated ApoD production resulted in significant reduction in plasma TG levels in mice [14]. Here, we determined the impact of ApoD on triglyceride metabolism in the ApoD knockout mice (ApoD-KO). When compared to control, non-fasting ApoD-KO mice exhibited significantly increased TG levels at 3 and 21 months of age (Fig. 1A). In contrast, fasting plasma TG levels remained unchanged (Fig. 1A). Whereas ApoD deficiency reduced plasma cholesterol levels in ApoD-KO mice at 3 months of age, this reduction was not observed at 21 months of age (Fig 1B). In addition, ApoD deficiency resulted in a significantly increased food intake at 3 months of age (Fig. 1C) without differences in body weight (Fig. 1D). However, food intake and body weight at 21 months remained unchanged (Fig. 1C-D).

To investigate the potential effect of ApoD deficiency on hepatic fat metabolism we determined hepatic triglyceride content. When compared with control mice, ApoD-KO mice exhibited a trend (16% reduction) in hepatic TG content at 3 months of age (however differences did not reach statistical significance, data not shown). In contrast, hepatic TG content was significantly reduced by 35% in ApoD-KO mice at 21 months of age compared to control mice (Fig. 2A). To confirm these findings, liver tissues from both ApoD-KO and control groups were stained with Oil Red O.

Histological examination of liver sections revealed significant differences in hepatic triglyceride content in ApoD-KO and control mice at 21 months of age (Fig. 2B).

To investigate the mechanism by which ApoD deficiency is associated with non-fasting hypertriglyceridemia we analyzed the expression level of lipoprotein lipase

(LPL), a key enzyme in the hydrolysis and clearance of TG-rich particles, in peripheral tissues of young mice. As shown in Fig.3, LPL levels in adipose tissue from young mice were reduced by 30-40% in ApoD-KO mice compared to WT control animals. These results shed light on the mechanism by which ApoD deficiency is associated with non-fasting hypertriglyceridemia and spur the hypothesis that ApoD deficiency reduces TG clearance through decreased LPL activity.

Effect of ApoD deficiency on glucose metabolism in mice

Elevated serum triglycerides are often associated with insulin resistance in rodents and humans [22]. To investigate the effect of ApoD deficiency on glucose metabolism we determined blood glucose and insulin levels in ApoD-KO and control mice. When compared to control, ApoD-KO mice exhibited similar fasting and non-fasting blood glucose levels (Fig. 4A). However, non-fasting plasma insulin levels were significantly elevated in ApoD-KO mice (Fig. 4B). To evaluate the impact of ApoD deficiency on whole-body glucose disposal rates, glucose tolerance tests were performed. As shown in Fig. 4C, similar glucose profiles were observed in ApoD-KO and control mice in response to intraperitoneal glucose infusion. However, ApoD-KO mice show a trend to increase insulin release during the glucose tolerance test, although this trend did not achieve statistical significance (data not shown). Based on fasting blood glucose and plasma insulin levels, we calculated the insulin sensitivity index (ISI). As shown in Fig. 4D, ApoD-KO mice exhibited similar ISI regardless of age. However, ApoD-KO mice at 3 and 21 months of age were associated with increased non-fasting insulin:glucose ratio (Fig. 4E), suggesting that ApoD deficiency is associated with inappropriate hyperinsulinemia to maintain normoglycemia in non-fasting conditions.

Insulin resistance usually precedes the development of glucose intolerance and type 2 diabetes. Before this happens, the pancreas compensates for insulin resistance by increasing insulin secretion sustaining normoglycemia. Beta-cell compensation can be accomplished by increasing beta-cell mass or enhancing cellular secretory capacity. To investigate why an ApoD deficiency leads to hyperinsulinemia, we performed a histomorphometric analysis of beta-cell mass in ApoD-KO and control mice. As shown in Fig 5, pancreatic beta-cell mass (A-D and C), islet volume (B) and islet number (D) were not significantly different between ApoD-KO and control mice. These findings suggest that the hyperinsulinemia observed in non-fasting ApoD-KO mice was not due to beta-cell mass changes or growth, but related to beta-cell function.

Discussion

In this study, we hypothesized that ApoD deficiency would increase plasma triglyceride levels and could contribute to the pathogenesis of dyslipidemia. To contrast this hypothesis, we tested whether mice lacking ApoD gene have elevated plasma triglycerides levels. We show that ApoD deficiency is associated with hypertriglyceridemia and decreased LPL protein levels in adipose tissue in non-fasting conditions. Consistently, we previously showed that elevated ApoD production was associated with increased LPL activity in mice, contributing to improved postprandial triglyceride clearance [14]. In parallel with these results, epidemiological studies in African populations have identified three missense mutations (namely Phe36Val, Tyr108Cys and Thr158Lys) in the ApoD gene associated with significantly elevated plasma triglyceride levels [16,17]. In addition, plasma ApoD levels are significantly lower in patients with hyperchylomicronemia

[23]. Taken together, these findings demonstrate a role for ApoD in the regulation of triglyceride metabolism and suggest that ApoD deficiency contributes to the pathogenesis of dyslipidemia.

Interestingly, hypertriglyceridemia was not accompanied by hepatic accumulation of triglycerides in non-fasting ApoD-KO mice. At first inspection, the effect of ApoD deficiency, promoting hypertriglyceridemia and reducing hepatic triglyceride levels, might seem be contradictory. There are several possible explanations. First, ApoD deficiency may enhance hepatic VLDL-TG secretion, which would explain the reduced hepatic TG levels and hypertriglyceridemia in non-fasting conditions.

Second, ApoD deficiency may increase fatty acid oxidation, which would reduce hepatic triglyceride levels. Third, ApoD deficiency may decrease hepatic “de novo” fatty acid biosynthesis and/or esterification of exogenous fatty acids. Although the precise effect of ApoD deficiency on hepatic lipid metabolism needs further investigation, these possibilities may explain, at least in part, the observed reduced hepatic triglyceride levels.

ApoD regulation in response to fat load has not been extensively studied. In support of this notion, we showed that elevated plasma ApoD levels in diet-induced obese mice was associated with reduced body weight and fat pad mass [14]. Here, we show an age-dependent effect of ApoD deficiency on food intake and body weight.

Three months old ApoD-KO mice show augmented food intake without an increase in body weight. Thus, our results are consistent with the hypothesis that ApoD regulates body weight and energy homeostasis by a potential mechanism that would implicate enhanced energy expenditure.

In addition to its effect on triglyceride metabolism, young ApoD-KO mice have reduced non-fasting plasma cholesterol levels compared to WT mice, sustaining the

concept that ApoD regulates cholesterol metabolism in mice. Supporting such a role, we have previously shown that hepatic overexpression of ApoD decreased plasma cholesterol levels in mice [14]. Interestingly, the effect of ApoD deficiency on cholesterol metabolism was lost with aging. It is plausible that other apolipoproteins involved in cholesterol regulation such as Apolipoprotein A-I (ApoA-I) compensate for a deficit in ApoD during aging. Nonetheless, the role of ApoD in the regulation of cholesterol metabolism remains to be deciphered and further work is warranted. Also in the context of lipid metabolism, Do Carmo et al. reported that transgenic mice overexpressing human ApoD show hepatic steatosis with normal plasma triglyceride levels [15]. The discrepancies in the transgenic mouse phenotypes observed could be explained by the different methodological approaches used in both studies. First, two different gain-of-function paradigms (chronic overexpression in transgenic mice vs. acute overexpression using adenoviral vectors) were used. Second, mouse and human ApoD cDNA were used to overexpress ApoD in mice. Although human and mouse ApoD present a high degree of similarity in their sequences, there are some structural differences that may be of importance to explain the phenotypes. Mouse ApoD lacks Cys116, a residue involved in the intermolecular covalent cross-link with Cys6 of Apolipoprotein A-II (ApoA-II) within HDL particles [24]. Finally, ApoD was overexpressed in different tissues. Do Carmo et al. expressed human ApoD under the control of neuron specific promoters, while we overexpressed mouse ApoD under the control of cytomegalovirus (CMV) promoter in liver [15,14]. In summary, further work using transgenic and knockout tissue-specific mouse models is warranted to decipher the tissue-specific contribution of ApoD on the regulation of triglyceride metabolism.

Finally, the two ApoD homologues in *Drosophila*, NLaz and GLaz, also regulate total triglyceride content and neutral fat storage. Total triglyceride content is decreased in the absence of NLaz and GLaz, whereas overexpression of NLaz increases total triglyceride content in young flies [11,12]. Curiously, while reduction of neutral fats is maintained through aging in GLaz mutants, aged flies lacking NLaz in fact accumulate fat [13].

Lipocalins are emerging also as significant players in the regulation of systemic insulin action and glucose metabolism. The Lipocalin retinol-binding protein 4 (RBP4) and lipocalin-2 (LCN2) are elevated in obese humans correlating with lower insulin sensitivity [25,26]. Likewise, circulating concentrations of RBP4 and LCN2 are elevated in obese mice [27,26]. Transgenic overexpression of RBP4 in normal mice decreases insulin sensitivity, whereas genetic ablation of RBP4 improved insulin sensitivity [28]. Our findings suggest that the Lipocalin ApoD may play a role in the regulation of systemic insulin action and glucose metabolism. This hypothesis is strengthened by epidemiological studies that demonstrate a linkage between TaqI polymorphism of the ApoD gene and insulin resistance, hyperinsulinemia, obesity and type 2 diabetes [18,29,19], and that circulating concentrations of ApoD are reduced in obese mice [14]. In *Drosophila*, the genetic ablation of NLaz decreased glycogen and glucose levels, whereas transgenic overexpression increased glucose levels. Furthermore, NLaz function antagonizes the insulin/IGF signaling pathway and is critical for the regulation of metabolic adaptations to stress [11]. In rodents, transgenic overexpression of ApoD in mice is associated with normal non-fasting blood glucose levels, hyperinsulinemia and glucose intolerance [15]. In this study, we show that non-fasting ApoD-KO mice exhibit elevated triglyceride levels associated with hyperinsulinemia and normoglycemia. The higher insulin levels and

insulin:glucose ratio in ApoD-KO mice suggest that they are insulin resistant. Noteworthy, ApoD-KO and WT mice exhibited similar glucose tolerance. Thus, in our model system a more sophisticated and sensitive technique, such as the hyperinsulinemic euglycemic glucose clamp, should be used to quantify insulin sensitivity. Interestingly, hypertriglyceridemia under non-fasting conditions is usually a characteristic associated with the development of insulin resistance [30]. The fact that hyperinsulinemia in ApoD-KO mice is not accompanied by hypoglycemia may indicate a pancreatic beta-cell compensatory mechanism to overcome insulin resistance. However, the observed hyperinsulinemia was not accompanied by changes in islet morphology, total beta-cell mass, beta-cell volume or islet number, suggesting that ApoD deficiency does not alter beta-cell growth. Taken together, these results suggest a role of ApoD in the pathogenesis of insulin resistance and further research is needed to decipher the potential role of ApoD deficiency in insulin resistance. In conclusion, our results suggest that altered plasma ApoD levels link abnormalities in the regulation of plasma insulin levels and lipoprotein metabolism with the pathogenesis of dyslipidemia.

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Disclosure statement

The authors reported no potential conflict of interest.

Author contributions

The experiments were performed at the laboratories of DS, MDG, GP and IC. GP, DS and IC participated in the conception, design, analysis and interpretation of the data. GP wrote the manuscript and DS, IC and MDG revised it critically for important intellectual content. MJP and MDG participated in the collection, analysis and interpretation of the data. All authors approved the final version of the manuscript.

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Figures and legends

Figure 1: Effect of ApoD knockout on lipid metabolism in mice. Blood samples
were collected from male ApoD-KO and WT mice at 3 and 21 months of age in non-
fasting or fasting state for the determination of plasma triglycerides (A) and
cholesterol (B). The effect of ApoD depletion on food intake (C) and body weight (D)
were determined at 3 and 21 months of age. * $p < 0.05$ versus control.

Figure 2: Hepatic triglyceride content. Mice were sacrificed at 21 months of age.
Frozen liver tissues (20 mg) were used to quantify hepatic triglyceride content in
ApoD-KO and WT mice (A) Cryostat sections of livers stained with Oil red O and
counterstained with hematoxylin (B). Calibration bar: 50 μ m. * $p < 0.05$ versus control.

Figure 3: Effect of ApoD deficiency on LPL levels in adipose tissue. Cell lysates
(20 μ g protein) of epididymal fat isolated from WT and ApoD-KO mice were

476 subjected to immunoblot analysis using anti-LPL antibody. After normalizing to β
477 actin, the relative amounts of LPL were compared between WT and ApoD-KO in
478 mice at 3 months of age. * $p < 0.05$ versus WT.

479 **Figure 4: Effect of ApoD-KO on glucose metabolism in mice.** Non-fasting or
480 fasting blood samples were collected from male ApoD-KO and WT mice for the
481 determination of plasma glucose (A) and insulin (B). Intraperitoneal glucose tolerance
482 test (C). Insulin sensitivity indexes (D). Ratio non-fasting insulin:glucose (E).

483 **Figure 5: Quantitative islet histomorphometry of WT and ApoD-KO pancreas.**

484 Insulin staining sections of whole pancreas from WT mice and ApoD-KO mice at 3
485 and 21 months of age (A). Islet volume (B), histomorphometry of islet mass (C) and
486 islet number (D). Pancreas weight in the two groups was not significantly different
487 (data not shown). Calibration bar: 1 mm.

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

Dr. Christos Mantzoros, MD, DSc
Editor-in-Chief
Metabolism

April 25th, 2011

Manuscript METABOLISM-D-11-00018R1. Genetic deficiency of Apolipoprotein D in the mouse is associated with non-fasting hypertriglyceridemia and hyperinsulinemia.

Dear Dr. Mantzoros:

Thank you for your e-mail of April 21st, 2011 regarding our manuscript cited above.

We are very pleased that you and the Reviewers find the work acceptable for publication in Metabolism. Following the Reviewers' comments, we have rewritten the abstract section, including principal results and levels of statistical significance. The changes made are highlighted in red font.

Thank you again for your consideration of our manuscript.

Sincerely,

German Perdomo and Diego Sanchez

ANSWERS TO REVIEWERS' COMMENTS

Q1: The abstract should be revised to include principal results, more hard data to attract readers into the paper and levels of statistical significance for major variables.

A1: We have rewritten the abstract section. Specifically, we have included principal results and statistical significance for major variables.