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Genetic deficiency of apolipoprotein D in the mouse is associated with nonfasting hypertriglyceridemia and hyperinsulinemia

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ABSTRACT

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. 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, and plasma metabolites were studied. Phenotypic characterization of glucose metabolism was performed using glucose tolerance test. β-Cell mass, islet volume, and islet number were analyzed by histomorphometry. Apolipoprotein D deficiency results in nonfasting hypertriglyceridemia in young (P = .01) and aged mice (P = .002). In young ApoD-KO mice, hypertriglyceridemia was associated with 30% to 50% increased food intake in nonfasting and fasting conditions, respectively, without changes in body weight. In addition, lipoprotein lipase levels were reduced by 35% in adipose tissue (P = .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 = .02). Furthermore, nonfasting plasma insulin levels were elevated by 2-fold in young (P = .016) and aged (P = .004) ApoD-KO mice, without changes in blood glucose levels, glucose tolerance, β-cell mass, or islet number. 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.

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1. 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 (HDLs) and to a lesser extent in very low-density lipoproteins (VLDLs) [7,8]. Apolipoprotein D is an atypical apolipoprotein, unrelated to other apolipoproteins in both structure and evolutionary origins [9]. Because ApoD is mainly located in HDLs, it was soon proposed to have a role in lipid homeostasis [10].

The *Drosophila* genome contains 2 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 (TG) content and resistance to starvation in young flies [11,12], whereas aging is accompanied by neutral fats accumulation in NLaz-deficient flies [13]. In contrast, overexpression of NLaz increases total TG 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 TG clearance, whereas VLDL-TG 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 TG levels [16,17]; and the *Taq*I polymorphism of the APOD gene is associated with the development of obesity, insulin resistance, hyperinsulinemia, and type 2 diabetes mellitus [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 TG 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 TG levels, contributing to the pathogenesis of dyslipidemia. To address this hypothesis, we evaluated the impact of losing ApoD on TG metabolism in young and aged ApoD-deficient mice (ApoD knockout [KO]). Here we show that genetic ablation of ApoD results in hypertriglyceridemia and hyperinsulinemia in nonfasting conditions. These findings underscore the importance of ApoD in the regulation of TG metabolism and in insulin-dependent processes.

2. Methods

2.1. *Ethical approval*

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

2.2. *Experimental animals*

Wild-type (WT) and ApoD-KO mice were bred at the animal facility of the University of Valladolid, Spain. Apolipoprotein D–KO mice were generated and genotyped as previously described [5]. Mice were fed standard rodent chow and water ad libitum in ventilation-controlled cages in a 12-hour light/dark cycle. The experimental cohorts used in this study were males of the F1 generation of homozygous crosses. The parental generation was composed of ApoD–/– and ApoD+/+ littermates from heterozygous crosses of the ApoD-KO line in C57BL/6 background. This strategy avoids the potential maternal effects of ApoD and generates WT and ApoD-KO cohorts with a homogeneous genetic background. Two independent cohorts were used for the collection of tissues at 2 ages: 3 months (n = 10/genotype) and 21 months old (n = 11/genotype).

2.3. *Plasma biochemistry*

Blood samples were obtained from mice under fasting conditions (16 hours) or under nonfasting conditions (animals had free access to food pellets ad libitum for 48 hours after the fasting period). This paradigm compares fasting vs nonfasting conditions because the exact timing of food intake with respect to sample collection is not determined. Blood was collected from the tail vein into capillary tubes precoated with potassium-EDTA (Sarstedt, Nümbrecht, Germany) for the preparation of plasma. Blood glucose levels were determined using a Glucometer Xceed (Abbott Diabetes Care, Oxon, United Kingdom). Plasma TG and cholesterol levels were determined using the Wako TG and cholesterol reagents (Wako Chemicals, Neuss, Germany). Plasma insulin levels were measured using ultrasensitive mouse enzyme-linked immunosorbent assay (ALPCO Diagnostics, Salem, NH).

2.4. *Food intake*

For food intake determination, ApoD-KO and WT mice were separated in individual cages (n = 7-11 mice per genotype).

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After the 16-hour fasting period, food pellets (50 g) were added to each cage; and food intake of each mouse was estimated from the difference in remaining food weight at 24 and 48 hours. These weights were averaged to provide an estimate of the mean food intake of each genotype in the 48-hour period following fasting.

2.5. *Glucose tolerance test and insulin sensitivity index*

Mice were fasted for 16 hours and injected intraperitoneally with glucose at 2 g/kg of body weight. Blood glucose levels were determined and plotted as a function of time. Insulin sensitivity index (ISI) was calculated using the formula ISI = 2/[(INS × GLU) + 1], where INS is fasting plasma insulin levels and GLU is fasting blood glucose levels with values converted to picomoles per liter and millimoles per liter, respectively [20].

2.6. *Hepatic TG determination and liver histology*

Hepatic TG 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].

2.7. *Determination of islet mass and islet histomorphometry*

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

2.8. *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 per lane) were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis and electrotransferred onto polyvinylidene difluoride membranes for conventional immunoblotting. After probing with LPL-specific antibody (1:1000; Santa Cruz Biotechnology, 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) were detected in the linear range for quantification purposes.

2.9. *Statistical analysis*

Statistical analyses of data were performed by Student *t* test and by analysis of variance. Data were expressed as mean ± SD. *P* values < .05 were considered significant.

3. Results

3.1. *Effect of ApoD deficiency on TG 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 TG metabolism in the ApoD-KO mice. When compared with control, nonfasting 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

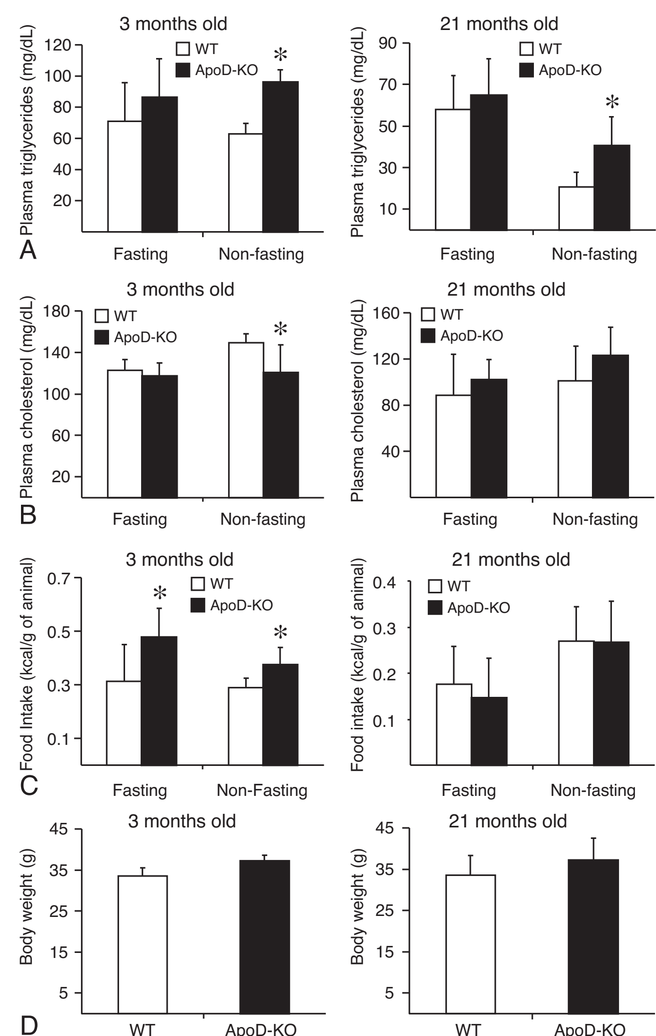

Fig. 1 – Effect of ApoD knockout on lipid metabolism in mice. Blood samples were collected from male ApoD-KO and WT mice at 3 and 21 months of age in nonfasting or fasting state for the determination of plasma TGs (A) and cholesterol (B). The effect of ApoD depletion on food intake (C) and body weight (D) were determined at 3 and 21 months of age. *P < .05 vs control.

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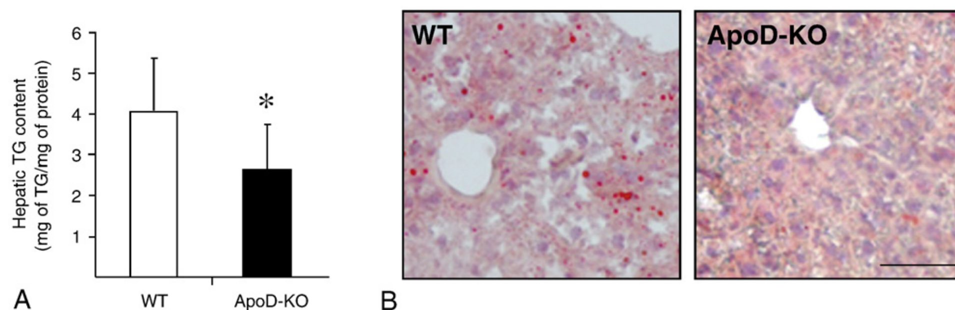

Fig. 2 – Hepatic TG content. Mice were killed at 21 months of age. A, Frozen liver tissues (20 mg) were used to quantify hepatic TG content in ApoD-KO and WT mice. B, Cryostat sections of livers stained with oil red O and counterstained with hematoxylin. Calibration bar: 50 μm. *P < .05 vs control.

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 TG 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 with 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 TG 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 nonfasting hypertriglyceridemia, we analyzed the expression level of 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% to 40% in ApoD-KO mice compared with WT control animals. These results shed light on the mechanism by which ApoD deficiency is associated with nonfasting hypertriglyceridemia and spur the hypothesis that ApoD deficiency reduces TG clearance through decreased LPL activity.

3.2. *Effect of ApoD deficiency on glucose metabolism in mice*

Elevated serum TGs 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 with control, ApoD-KO mice exhibited similar fasting and nonfasting blood glucose levels (Fig. 4A). However, nonfasting 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 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 nonfasting insulin to glucose ratio (Fig. 4E), suggesting that ApoD deficiency is associated with inappropriate hyperinsulinemia to maintain normoglycemia in nonfasting conditions.

Insulin resistance usually precedes the development of glucose intolerance and type 2 diabetes mellitus. Before this happens, the pancreas compensates for insulin resistance by increasing insulin secretion sustaining normoglycemia. β-Cell compensation can be accomplishing by increasing β-cell mass or enhancing cellular secretory capacity. To investigate why an ApoD deficiency leads to hyperinsulinemia, we performed a histomorphometric analysis of β-cell mass in ApoD-KO and control mice. As shown in Fig. 5, pancreatic β-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

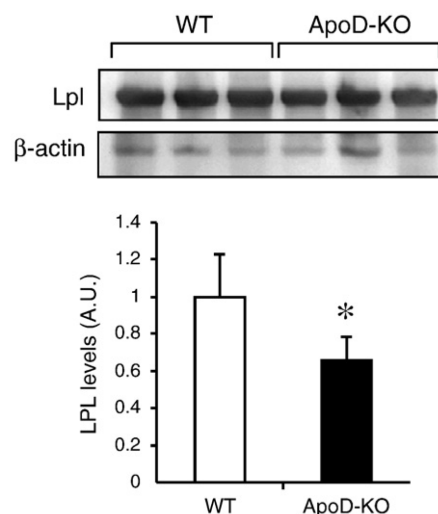

Fig. 3 – Effect of ApoD deficiency on LPL levels in adipose tissue. Cell lysates (20 μg protein) of epididymal fat isolated from WT and ApoD-KO mice were subjected to immunoblot analysis using anti-LPL antibody. After normalizing to β-actin, the relative amounts of LPL were compared between WT and ApoD-KO in mice at 3 months of age. *P < .05 vs WT.

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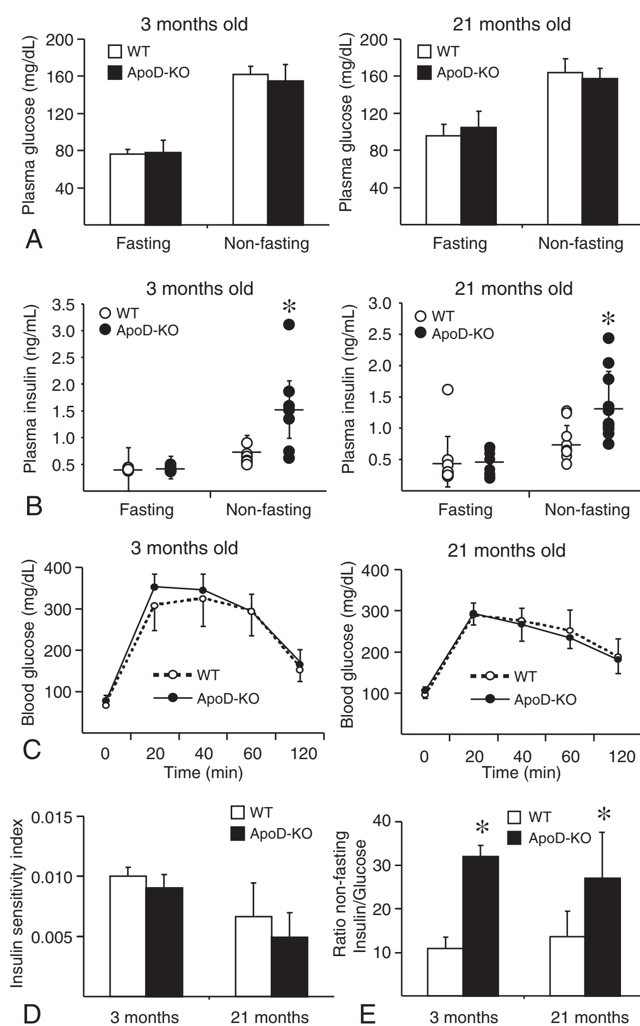

Fig. 4 – Effect of ApoD-KO on glucose metabolism in mice. Nonfasting or fasting blood samples were collected from male ApoD-KO and WT mice for the determination of plasma glucose (A) and insulin (B). C, Intraperitoneal glucose tolerance test. D, Insulin sensitivity indexes. E, Ratio of nonfasting insulin to glucose.

nonfasting ApoD-KO mice was not due to β-cell mass changes or growth, but related to β-cell function.

4. Discussion

In this study, we hypothesized that ApoD deficiency would increase plasma TG levels and could contribute to the pathogenesis of dyslipidemia. To contrast this hypothesis, we tested whether mice lacking ApoD gene have elevated plasma TG levels. We show that ApoD deficiency is associated with hypertriglyceridemia and decreased LPL protein levels in adipose tissue in nonfasting conditions. Consistently, we previously showed that elevated ApoD production was associated with increased LPL activity in mice, contributing to improved postprandial TG clearance [14]. In parallel with these results, epidemiological studies in African populations have identified 3 missense mutations (namely, Phe36Val, Tyr108Cys, and Thr158Lys) in the ApoD gene associated with significantly elevated plasma TG 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 TG metabolism and suggest that ApoD deficiency contributes to the pathogenesis of dyslipidemia.

Interestingly, hypertriglyceridemia was not accompanied by hepatic accumulation of TGs in nonfasting ApoD-KO mice. At first inspection, the effect of ApoD deficiency, promoting hypertriglyceridemia and reducing hepatic TG 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 nonfasting conditions. Second, ApoD deficiency may increase fatty acid oxidation, which would reduce hepatic TG 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 TG levels.

Apolipoprotein D 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 were 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-month-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 TG metabolism, young ApoD-KO mice have reduced nonfasting plasma cholesterol levels compared with 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 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 [15] reported that transgenic mice overexpressing human ApoD show hepatic steatosis with normal plasma TG levels. The discrepancies in the transgenic mouse phenotypes observed could be explained by the different methodological approaches used in both studies. First, 2 different gain-of-function paradigms (chronic overexpression in transgenic mice vs acute overexpression using adenoviral vectors) were used. Second, mouse and human ApoD complementary DNA was used to overexpress ApoD in mice. Although human ApoD 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

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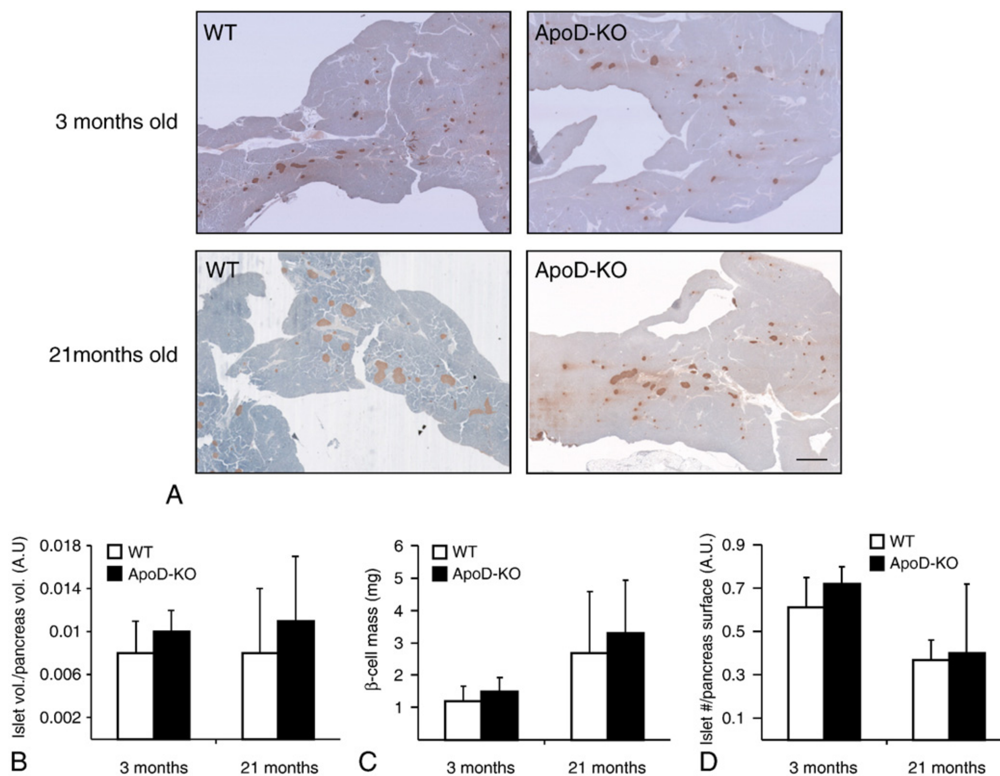

Fig. 5 – Quantitative islet histomorphometry of WT and ApoD-KO pancreas. A, Insulin staining sections of whole pancreas from WT mice and ApoD-KO mice at 3 and 21 months of age. Islet volume (B), histomorphometry of islet mass (C), and islet number (D). Pancreas weight in the 2 groups was not significantly different (data not shown). Calibration bar: 1 mm.

covalent cross-link with Cys6 of apolipoprotein A-II within HDL particles [24]. Finally, ApoD was overexpressed in different tissues. Do Carmo et al [15] expressed human ApoD under the control of neuron specific promoters, whereas we overexpressed mouse ApoD under the control of cytomegalovirus (CMV) promoter in liver [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 TG metabolism.

Finally, the 2 ApoD homologues in *Drosophila*, NLaz and GLaz, also regulate total TG content and neutral fat storage. Total TG content is decreased in the absence of NLaz and GLaz, whereas overexpression of NLaz increases total TG content in young flies [11,12]. Curiously, whereas 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 are elevated in obese humans, correlating with lower insulin sensitivity [25,26]. Likewise, circulating concentrations of RBP4 and lipocalin-2 are elevated in obese mice [26,27]. 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 *Taq*I polymorphism of the ApoD gene and insulin resistance, hyperinsulinemia, obesity, and type 2 diabetes mellitus [18,19,29] 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 nonfasting blood glucose levels, hyperinsulinemia, and glucose intolerance [15]. In this study, we show that nonfasting ApoD-KO mice exhibit elevated TG levels associated with hyperinsulinemia and normoglycemia. The higher insulin levels and insulin to 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 nonfasting 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 β-cell compensatory mechanism to overcome insulin resistance. However, the observed hyperinsulinemia was not accompanied by changes in islet morphology, total β-cell mass, β-cell volume, or islet number, suggesting that ApoD deficiency does not alter β-cell growth. Taken together, these

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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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Conflict of Interest

The authors reported no potential conflict of interest.

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