Apolipoproteins A-I and A-V as gene therapeutic targets to intervene in lipid metabolism
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Plasma apolipoprotein A-V levels in mice are positively associated with plasma triglyceride levels

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Abstract

Apolipoprotein A-V (apoA-V) overexpression causes a decrease in plasma triglyceride (TG) levels, while deficiency of apoA-V causes hypertriglyceridemia in both men and mice. However, contrary to what would be expected, plasma apoA-V and TG levels in humans are positively correlated. To address this apparent paradox, we determined plasma apoA-V levels in various mouse models with median TG levels ranging from 30 mg/dL in wild-type mice to 2089 mg/dL in glycosylphosphatidylinositol-anchored HDL binding protein 1-deficient mice. The data show that apoA-V and TG levels are positively correlated in mice ($r = +0.798$, $P < 0.001$). In addition, we show that lipoprotein lipase (LPL) gene transfer caused a simultaneous decrease in TG and apoA-V in LPL deficient mice. The combined data suggest that apoA-V levels follow TG levels due to an intimate link between the apoA-V molecule and TG-rich lipoproteins, comprising both secretion and removal of these lipoproteins. Taken together, the data suggest that higher plasma apoA-V levels reflect an increased demand for plasma TG hydrolysis under normal physiological conditions.

Introduction

Since its discovery in 2001, apolipoprotein A-V (apoA-V) has been postulated as a key regulator of plasma triglyceride (TG) levels.\(^1\) Relevance for TG homeostasis was initially demonstrated using genetically engineered mice: Overexpression of human apoA-V decreased plasma TG levels by 64% while apoA-V deficient mice had 4-fold increased plasma TG levels.\(^1\) In humans, it was furthermore shown that variation at the $APOA5$ gene locus was associated with TG levels\(^2-4\), while complete apoA-V deficiency was associated with severe hypertriglyceridemia.\(^5,6\) Combined, these data suggested that plasma levels of apoA-V are in general inversely correlated with TG levels. However, we and others subsequently showed that plasma apoA-V and TG levels are, unexpectedly, positively correlated in patients with type 2 diabetes and in the general population.\(^7-11\) Considering the suggested negative correlation between plasma apoA-V and TG in mice, based on the overexpression models, these subsequent observations in humans have led several investigators to suggest an inherent difference between humans and mice.\(^10-12\) This despite the fact that the phenotypic presentation of complete apoA-V deficiency in mice and
humans is similar. Moreover, in mice with endotoxemia or in rats fed poly-unsaturated fatty acids, semi-quantification of apoA-V levels by immuno-blotting also suggested a positive correlation with TG levels. In addition, Nelbach and co-workers recently showed a positive correlation between plasma apoA-V and TG concentrations in mice overexpressing human apoA-V. In this study, we further addressed this hypothesis by determining the relationship between plasma apoA-V and TG levels in wild-type mice and various genetically engineered mouse models that are characterised by a wide range of elevated levels of plasma triglycerides.

**Methods**

**Mouse models**

To determine the association between plasma apoA-V and TG levels in mice, we collected plasma samples from different mouse models on a C57Bl/6 background with variable plasma TG levels. The etiology of increased TG levels in these mice and the collaborators who provided plasma samples are given below. Human-APOCI-transgenic-APOE3Leiden mice develop moderately elevated plasma TG levels due to an inhibition of lipoprotein lipase (LPL) activity (n=16, courtesy Dr. P. Rensen, Leiden University, Leiden, the Netherlands). Glycosylphosphatidylinositol-anchored HDL binding protein 1 deficient (Gpihbp1/-) mice are severely hypertriglyceridemic because the lack of GPIHBP1 prevents binding of LPL to the vascular endothelium, resulting in an inability to lipolyse triglycerides in apoB48- and apoB100-containing lipoproteins (n=26, courtesy of Dr. S. Young and M. Weinstein, University of California, Los Angeles, CA, USA). Both murine-apoA2 transgenic (n=60) as well as murine-apoA2 transgenic-apoE deficient mice (n=38) develop moderately elevated plasma TG levels due to both an overproduction of VLDL by the liver as well as an impaired LPL-mediated TG hydrolysis (both strains are courtesy of Dr. L. Castellani, University of California, Los Angeles, CA, USA). Lpl/- mice develop severe hypertriglyceridemia due to the inability to hydrolyse plasma TG. Plasma samples of heterozygous and homozygous LPL deficient mice (both n=5) were provided by Dr. C. Ross (University of British Columbia, Vancouver, Canada). To determine whether an induced change in TG levels in mice affects apoA-V levels, we used plasma samples of lpl/- mice that were injected intramuscularly with adeno-associated virus (AAV) encoding the natural variant LPL, as
previously described.\textsuperscript{19} Wild-type mice (n=66), housed in our institute, were obtained from Harlan (Horst, the Netherlands). Fasting (4-12 h.) plasma samples of all mice indicated above were prepared and immediately frozen at -80°C at the respective institutes. The samples were sent on dry ice to the laboratory in Amsterdam where they were stored at -80°C until use for measurements.

**Plasma TG and apoA-V analyses**

Plasma samples were analysed for TG using a standard enzymatic assay (Roche, Basel, Switzerland). Murine apoA-V levels were determined using a newly developed ELISA. A rabbit-anti-rat apoA-V polyclonal antibody\textsuperscript{20}, with strong cross-reactivity to murine apoA-V, was used both as capture and as biotinylated secondary antibody. Purified recombinant murine apoA-V was used to generate standard curves. Plates (Maxisorb, Nunc, Thermo Fisher Scientific, Roskilde, Denmark) were coated overnight with the capture antibody (100μl, 5 μg/mL in 0.1M carbonate buffer, pH 9.6) at 4°C. After washing with PBX (PBS containing 0.1% Triton X-100), wells were blocked for 1 h at room temperature with PBXC (PBX containing 1% casein; Hammerstan grade, Merck, Darmstadt, Germany). After extensive washing, wells were incubated for 2 h with 100μL of standard or sample (1:10) all diluted in PBXC. Next, wells were washed and incubated with 100μL of the biotinylated secondary antibody (2 μg/mL in PBXC) for 1 h. Following washing, wells were incubated with streptavidin-horseradish peroxidase (1:5000 in PBXC, DAKO, Glostrup, Denmark) for 1 h. After extensive washing, the plate was incubated with o-phenylenediamine dihydrochloride (OPD) substrate (Sigma, St. Louis, MO, USA). The color reaction was stopped after exactly 10 minutes with 2M sulphuric acid and absorbance was read at 490nm (Easia reader, Medgenix Diagnostics, Springfield, MO, USA). The inter-assay variation coefficient was 3.7 ± 1.6%.

**Statistics**

Statistics were performed using SPSS (version 16.0; Chicago, IL, USA). Concentrations are expressed as mean ± standard deviation (SD). Plasma TG levels show a skewed distribution; therefore, logarithmically transformed values were used. Correlations between plasma apoA-V levels and TG levels were calculated using Pearson correlation. Comparisons between pre- and post-treatment TG values of \textit{lpl}\textsuperscript{-/-} mice were performed using the Student’s \textit{t}-test. \textit{P}-values < 0.05 were considered significant.
Results

To determine the association between plasma apoA-V and TG levels in mice, we have tested plasma samples from mouse models with median TG levels ranging from 30 mg/dL (range 13-88) in wild-type mice to 2089 mg/dL (range 200-6677) in *gpihbp1−/−* mice (Table 1). Figure 1 shows that plasma apoA-V and TG are positively correlated in mice (*r* = +0.798, *P* < 0.001) over the entire TG range. Table 1 shows that this positive correlation was observed in each of the following subgroups: wild-type mice (*r* = +0.368; *P* < 0.01), *gpihbp1−/−* mice (*r* = +0.455; *P* < 0.001), murine-*apoA2* transgenic mice (*r* = +0.596; *P* < 0.001) and murine-*apoA2* transgenic-*apoE−/−* mice (*r* = +0.882; *P* < 0.001).

<table>
<thead>
<tr>
<th>Mouse model</th>
<th>TG (mg/dL)*</th>
<th>ApoA-V (ng/mL)**</th>
<th>Pearson Corr.</th>
<th>Sign. (P)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type (n = 66)</td>
<td>30 (13; 88)</td>
<td>24 ± 14</td>
<td>+ 0.368</td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td>Human-<em>APOC1</em> transgenic-<em>APOE3Leiden</em> (n = 16)</td>
<td>297 (94; 464)</td>
<td>30 ± 9</td>
<td>+ 0.200</td>
<td>= 0.45</td>
</tr>
<tr>
<td><em>Gpihbp1−/−</em> (n = 26)</td>
<td>2089 (200; 6677)</td>
<td>147 ± 30</td>
<td>+ 0.455</td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td>Murine-<em>apoA2</em> transgenic (n = 60)</td>
<td>258 (54; 1360)</td>
<td>87 ± 43</td>
<td>+ 0.596</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Murine-<em>apoA2</em> transgenic-<em>apoE−/−</em> (n = 38)</td>
<td>182 (53; 1739)</td>
<td>111 ± 47</td>
<td>+ 0.882</td>
<td>&lt; 0.001</td>
</tr>
</tbody>
</table>

* Data are presented as median (minimum; maximum). ** Data are represented as mean ± SD.

This correlation did not reach statistical significance for the human *APOC1* transgenic-*APOE3Leiden* mice (*r* = +0.2; *P* = 0.45), most likely due to the relative low number of animals in this group. Thus, irrespective of the molecular cause of hypertriglyceridemia, apoA-V plasma levels are positively correlated with plasma TG in mice.
ApoA-V and triglycerides in mice

Figure 1. Plasma apoA-V and TG levels are positively correlated in mice. Correlation between log-transformed plasma TG and apoA-V levels in wild-type (n=66), human-APOC1-APOE3Leiden transgenic (n=16), GPI-anchored HDL-binding protein 1-deficient (Gpihbp1-/-, n=26), murine-apoA2 transgenic (n=60), and murine-apoA2 transgenic-apoE-/- mice (n=38) (all on a C57Bl/6 background). Correlations within each group are presented in table 1.

Figure 2. LPL gene transfer simultaneously reduces TG and apoA-V levels in LPL deficient mice. Plasma TG (A) and apoA-V (B) levels in lpl+/+, lpl+/-, lpl-/- and AAV-LPL-treated lpl-/- mice four weeks post-treatment. * Significantly different (P < 0.05) compared to lpl-/- mice that were not treated with LPL gene transfer.
To study apoA-V in a context of changing TG levels, we assessed plasma apoA-V levels in lpl⁻/⁻ mice before and after LPL gene transfer. We observed that apoA-V was elevated in lpl⁺/+ mice (TG of 110 ± 52 mg/dL) and even more so in lpl⁻/⁻ mice (TG levels of 5033 ± 2141 mg/dL) confirming our finding in the other mouse models (Figure 2). LPL gene transfer in lpl⁻/⁻ mice led to simultaneous significant reductions of both TG and apoA-V concentrations (Figure 2), showing that induction of TG lowering is closely followed by a decrease of apoA-V levels.

Discussion

This study provides unequivocal evidence for a positive correlation between plasma apoA-V and TG in mice, underlining that humans and mice are not different in this regard as has been suggested by other investigators. The current data also show that measuring apoA-V plasma levels does not provide direct biological insight into the function of apoA-V in vivo. It has been shown that apoA-V functions as a regulator for LPL to improve TG hydrolysis, at least in vitro, and that apoA-V may be involved in remnant uptake, both suggestive of a role for apoA-V in regulating TG levels. However, our current data suggest that apoA-V plasma levels change according to changes in plasma TG levels. The current data show that apoA-V levels are positively correlated with TG levels irrespective of the different genetic causes of hypertriglyceridemia that were studied. This correlation was present in mouse models with primary defects in LPL-mediated TG hydrolysis: human APOC1 transgenic –APOE3Leiden mice in which overexpression of human apoC-I inhibits LPL-mediated TG hydrolysis, gpihbp1⁻/⁻ mice that lack the ability to bind LPL in the capillaries of the heart, skeletal muscle and adipose tissue resulting in the accumulation of chylomicrons and VLDL, and, finally, lpl⁻/⁻ mice that can not lipolyse plasma triglycerides in the absence of LPL. In addition, the positive correlation was also observed in animals with increased hepatic VLDL-TG secretion in addition to a decreased LPL-mediated TG hydrolysis. Specifically, the two murine-apoA2 transgenic strains have increased hepatic VLDL-TG secretion due to increased hepatic lipogenesis and a reduced LPL-mediated TG hydrolysis because the presence of apoA-II makes VLDL a poorer substrate for hydrolysis. A closer look into our data furthermore revealed that apoA-V plasma levels vary strongly, especially relative to the observed TG levels in the different
strains of mice. Specifically and relative to TG levels, apoA-V levels are only moderately elevated in the gpihbp1⁻/⁻ and lpl⁻/⁻ models, which present with the highest TG levels. On the other hand, apoA-V levels are more strongly increased in the murine-\textit{apoA2} transgenic lines that present with less severe hypertriglyceridemia. It thus appears that plasma apoA-V levels are most-intimately linked to VLDL secretion and that the moderately elevated plasma apoA-V levels in models with primary defects in LPL hydrolysis reflect a delayed catabolism of chylomicrons and VLDL particles. In line with this hypothesis, targeted reduction of plasma TG through LPL gene therapy, i.e. increasing TG hydrolysis in lpl⁻/⁻ mice, caused a concomitant decrease in plasma apoA-V levels. This finding indicates that plasma apoA-V levels are thus also related to TG-removal, since this effect may be seen in the context of an apparently unchanged VLDL-TG secretion in these animals.\textsuperscript{25} It is difficult to speculate on the cause for this phenomenon; however, we wish to refer to the idea that apoA-V-containing lipoproteins are rapidly removed from the circulation after LPL-mediated lipolysis that is induced upon binding to GPIHBP1.\textsuperscript{17} Nilsson and co-workers recently showed that apoA-V binds to and is internalized via peripheral receptors SorLA/LR11 and sortilin.\textsuperscript{26} It is thus conceivable that upon increased TG hydrolysis after LPL gene therapy, apoA-V internalization is also increased, offering a possible explanation for the decrease of plasma apoA-V in our experiment.

Taken together, it appears that the positive correlation of plasma apoA-V with plasma TG levels is the result of an intimate link between the apoA-V molecule and TG-rich lipoproteins which comprises both secretion and removal of these lipoproteins. Current lines of evidence suggest that apoA-V is only synthesized in the liver from which it is secreted into the circulation, a process that seems to be directly associated with the secretion of TG-rich VLDL. Upon TG-hydrolysis in VLDL into the plasma compartment, apoA-V is redistributed over all TG-rich lipoproteins, \textit{i.e.} VLDL and chylomicrons, as well as HDL\textsuperscript{27} and, in mice, possibly also LDL.\textsuperscript{15} HDL is thought to serve as a plasma reservoir for apoA-V as previously described for apoC's\textsuperscript{28,29} and apoA-II.\textsuperscript{18} In the postprandial state, this apoA-V can transfer to TG-rich lipoproteins to again facilitate TG hydrolysis. Upon adequate TG hydrolysis and effective removal of remnant lipoproteins from the circulation, apoA-V is also likely to be internalized and routed for degradation\textsuperscript{26}, putatively after being re-used as previously suggested.\textsuperscript{30} In this light, we propose that increased plasma apoA-V levels may reflect an increased demand for plasma TG hydrolysis. It does, however, not
explain the observation that mice overexpressing human apoA-V have very low plasma TG levels. In the latter situation, however, the physiological regulation of plasma apoA-V levels is overruled and under these conditions apoA-V emerges as a primary regulator of plasma TG levels due to its intrinsic TG-lowering capacity (in the presence of active LPL). On the other hand, a recent study also showed a positive relationship between plasma apoA-V and TG in mice that overexpress the human APOA5 gene, yet lack the murine apoa5 gene. However, this correlation was observed with plasma human apoA-V levels 50-100-fold higher as compared to normal human plasma apoA-V concentrations. It will be interesting to see if a similar correlation will be found in a context of lower apoA-V expression levels. On the other hand, previous murine studies suggesting a negative correlation between plasma apoA-V and TG were all performed under conditions of human apoA-V overexpression in the presence of the murine apoA-V protein. However, whether this difference contributes to the observed discrepancies between the murine overexpression studies is unclear.

In conclusion, we show that plasma TG and apoA-V levels in mice are positively correlated, as previously observed in humans, due to an intimate link between apoA-V and TG-rich lipoproteins which comprises both secretion and removal pathways of these lipoproteins. The current data suggest that apoA-V levels follow TG levels, leading to the hypothesis that increased plasma apoA-V levels reflect an increased demand for plasma TG hydrolysis under physiological conditions.

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References


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