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Apolipoprotein A-V associates with intrahepatic lipid droplets and influences triglyceride accumulation

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Abstract

Apolipoprotein A-V (apoA-V), secreted solely by the liver, is a low abundance protein that strongly influences plasma triglyceride (TG) levels. In vitro, in transfected hepatoma cell lines apoA-V is largely retained within the cell in association with cytosolic lipid droplets (LD). To evaluate if this is true in vivo, in the present study the amount of apoA-V in the plasma compartment versus liver tissue was determined in *APOA5* transgenic (Tg) mice. The majority of total apoA-V (~80%) was in the plasma compartment. Injection of *APOA5* Tg mice with heparin increased plasma apoA-V protein levels by ~25% indicating the existence of a heparin-releasable pool. Intrahepatic apoA-V was associated with LD isolated from livers of wild type (WT) and *APOA5* Tg mice. Furthermore, livers from *APOA5* Tg mice contained significantly higher amounts of TG than livers from WT or *apoa5* knockout mice suggesting the apoA-V influences intrahepatic TG levels.

Keywords

apolipoprotein A-V; *APOA5* transgenic mice; liver; lipid droplets; triglyceride

1. Introduction

Apolipoprotein (apo) A-V, first described in 2001 [1,2], has emerged as an important modulator of triglyceride (TG) metabolism [3]. The protein is exclusively expressed in liver and is found in plasma associated with HDL and VLDL [4,5]. The concentration of apoA-V in plasma, compared to other apolipoproteins, is extremely low. In rat it is estimated at about 1 g/mL [2] while in humans it ranges from 114 - 258 ng/mL [4]. This corresponds to a 1,000-fold lower concentration than apoB-100 on a molar basis. Since apoA-V in humans was not found in the LDL fraction [4] and ~6% of circulating apoB-100 is associated with VLDL, it is estimated that about one apoA-V molecule is present per 24 VLDL particles [6]. Transgenic (Tg) mice that overexpress the human apoA-V gene (*APOA5*) yet lack an endogenous mouse *apoa5* gene (*APOA5* Tg mice) were generated and characterized in our laboratory [5]. The average plasma concentration of apoA-V in these mice was ~12.5 μg/mL, which is ~50 to 100-fold higher than that reported for normolipidemic humans.

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Newly synthesized apoA-V consists of 366 amino acids where the first 23 residues form the cleavable signal peptide. Amino acid sequence analysis reveals a stretch of 42 residues between amino acid 186 and 227 that lacks negatively charged side chains yet contains eight positively charged amino acids [7]. This highly positively charged region has been shown to be involved in binding to heparin as well as members of the LDL receptor family [8]. It has also been demonstrated that apoA-V binding to cell surface heparan sulfate proteoglycans (HSPG) enhances lipoprotein lipase (LPL) mediated hydrolysis of TG-rich particles [9].

In hepatocytes, cytosolic lipid droplets (LD) are a storage form of neutral lipids, particularly TG, which may be used for production of VLDL. We recently reported that apoA-V associates with LD in transfected hepatoma cell lines, despite being synthesized with a signal peptide [10,11]. Furthermore, in transfected hepatoma cells newly synthesized apoA-V is poorly secreted. The portion of apoA-V that is secreted associates with lipoproteins in conditioned medium. This observation suggests there are two pools of apoA-V, one intracellular and the other extracellular. Although in vitro cell culture studies provide valuable insight into intracellular trafficking of apoA-V, it has yet to be demonstrated in vivo, that apoA-V has a similar trafficking itinerary. To address this, genetically engineered mouse models were utilized to determine whether apoA-V associates with LD in vivo and define the extent to which this association influences intracellular TG content. Results obtained reveal that, unlike the situation in transfected hepatoma cells, the extracellular pool of apoA-V in mouse plasma is larger than the intracellular pool in mouse liver. ApoA-V is found associated with cytosolic LD and appears to facilitate accumulation of intracellular TG.

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2. Materials and methods

2.1 Materials

Protease inhibitor cocktail, Pefabloc SC, gentamycin sulfate, chloramphenicol, Trolox, Tween 20, and bovine serum albumin Fraction V were from Sigma-Aldrich. Heparin was from Baxter. Primary antibodies included polyclonal goat anti-human apoA-V [12], polyclonal rabbit anti-human apoA-V [4], polyclonal guinea pig anti-adipocyte differentiation-related protein (ADRP) (PROGEN Biotechnik), polyclonal goat anti-calreticulin (CRT) (kindly provided by Dr. Marek Michalak, University of Alberta) and polyclonal rabbit anti-calnexin (CNX) (Sigma). Secondary antibodies included horseradish peroxidase (HRP) -conjugated affinity-purified donkey anti-goat immunoglobulin (IgG), and HRP-conjugated affinity-purified goat anti-rabbit IgG (Jackson ImmunoResearch Laboratories). Novex precast 4-20% polyacrylamide gradient gels in Tris-glycine buffer were from Invitrogen. Enzymatic assay kit for determination of TG was purchased from Wako Chemicals, USA.

2.2 Mice

Mice used in these studies, including *apoa5* knockout (KO), *APOA5* Tg and wild type (WT), have been described [5]. Studies were carried out on male mice approximately 4 months of age after a 4 h fast. Research on the mice was conducted in conformity with the Public Health Service Policy on the Humane Care and Use of Laboratory Animals and was approved by the Children's Hospital Oakland Research Institute Animal Care and Use Committee.

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2.3 Immunoblotting

Samples were electrophoresed on 4-20% polyacrylamide gradient gels, transferred to PVDF membranes and processed as previously described [12]. The polyclonal goat anti-human apoA-V antibody [12] used in this study cross reacts with mouse apoA-V in highly concentrated samples, such as isolated LD.

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2.4 ApoA-V liver versus plasma distribution

APOA5 Tg mice were used for these studies since apoA-V in liver lysate and plasma from WT mice was too low for detection and reliable quantification. Plasma was prepared from mouse blood collected by submandibular venipuncture. Protease inhibitors and stabilizers were added as previously described [5]. Liver tissue was obtained from anaesthetized mice after perfusion with Hanks balanced salt solution to remove blood. Tissue was rapidly frozen in cryovials and stored at −80°C until utilized. Separate liver extracts were prepared using weighed portions of individual frozen livers, in 5 volumes of ice-cold Tissue Protein Extraction Reagent (T-PER, Pierce) to which protease inhibitor cocktail and Pefabloc SC had been added. Supernatants were pooled, aliquoted and subjected to immunoblotting with anti-apoA-V IgG. Immunoblot band intensity of plasma and liver homogenate samples was measured by densiometry using the NIH ImageJ software and compared to the corresponding intensity of known amounts of recombinant apoA-V standard on the same blot. Estimation of total apoA-V in the plasma compartment was made from known weights of animals, assuming plasma volume is 3% of body weight. Calculation of apoA-V in the liver compartment was made from the known weight of the excised liver.

2.5 Isolation of LD

Lipid droplets were isolated from liver tissue as described by Wang et al [13] with modification. Briefly, liver tissue from *APOA5* Tg, WT and *apoa5* KO mice were homogenized in ice cold homogenization buffer (1:5 w/v) consisting of 250 mM sucrose in 20 mM Tris, pH 7.4 containing 1 mM EDTA, and protease inhibitors. Cellular debris and the mitochondria were separated by centrifugation at 1,000 × g for 10 min and 15,000 × g for 10 min, respectively. The supernatant was centrifuged at 100,000 × g for 1 h to pellet microsomes. Cytosolic LD were carefully collected from the top of the tube and mixed with glycerol 1:1 (v/v). The LD fraction was overlaid in the centrifuge tube with equal volumes of homogenization buffer, and subsequently, Tris buffered saline (TBS). The sample was centrifuged in a SW41 rotor at 40,000 rpm for 2 h. LD were removed from the top of the tube and mixed with protease inhibitors.

2.6 Measurement of liver TG content

Lipid extracts of liver tissue were assayed for triglyceride according to the manufacturer's protocol using the Triglyceride Quantification Kit (BioVision, Mountain View, CA). Briefly, liver tissue was homogenized in 5% Triton-X100 in water (1:10 w/v). Samples were slowly heated to 80°C for 5 min. Insoluble material s were removed by centrifugation. TG concentration in the supernatant was determined by an enzyme based colorimetric assay.

2.7 Heparin Injection

APOA5 Tg mice were injected via tail vein with 50 μl heparin (50 units) or phosphate buffered saline (PBS). At 0, 1, 3, 8, and 13 min after injection, blood samples were collected by submandibular venipuncture. Mice were anesthetized with Isoflurane during the entire procedure. Plasma was obtained by centrifuging blood at 2,000 × g for 10 min at 4°C and aliquots analyzed for TG and apoA-V [5].

3. Results

3.1 ApoA-V partitioning between liver and plasma compartment

In general, apolipoproteins mediate their biological effects in the plasma compartment. Their functions include stabilization of lipoprotein structure, modulation of lipid metabolic enzyme activity or serving as ligands for cell surface receptors. Given the extremely low concentration of apoA-V in plasma, its poor secretion efficiency from transfected cells and its ability to

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associate with intracellular LD, it is conceivable that apoA-V functions to modulate TG within hepatocytes. An important question related to this is the distribution of apoA-V between liver and plasma in vivo. ApoA-V levels in liver versus plasma were determined in *APOA5* Tg mice overexpressing apoA-V. Liver lysates and plasma samples were subjected to a semi-quantitative Western blot analysis using known amounts of isolated recombinant apoA-V as standard. Two independent experiments with pools from 5 mice per study revealed that 80 ± 2% (Mean ± SE) of apoA-V was found in plasma while the remainder was present in liver, the sole tissue that expresses apoA-V.

3.2 A heparin releasable pool of apoA-V

ApoA-V contains a stretch of amino acids between residues 186 and 227 that possess a strong positive charge character. Based on studies that show apoA-V binds heparin as well as cell surface HSPG [8,9], it is possible that a pool of HSPG-bound apoA-V exists in vivo. To test this, *APOA5* Tg mice were injected with heparin and plasma apoA-V concentration measured as a function of time (Fig. 1A). A rapid 25% increase in apoA-V concentration was noted after heparin injection while there was a slight decrease of apoA-V concentration in PBS injected controls. Plasma TG levels were also determined for the same time points (Fig. 1B). As expected [14], injection of heparin dramatically lowered plasma TG levels compared with PBS.

3.3 ApoA-V association with isolated hepatic LD

In hepatoma cell lines transfected with apoA-V a large proportion of the protein is associated with intracellular LD [10,11]. Unlike transfected hepatoma cell lines, < 20% of the total apoA-V pool is present in the liver in vivo. Therefore, we sought to determine whether this pool of apoA-V associates with LD. Equivalent amounts of apoA-V were recovered in the LD (together with ADRP marker protein) and microsomal fractions in both *APOA5* Tg mice and WT mice (Fig. 2). Little or no microsomal marker proteins, including calreticulin (CRT) and calnexin (CNX), were recovered in the LD fraction suggesting that apoA-V identified in this fraction does not arise from microsomal contamination. As expected, no apoA-V was detected in *apoa5* KO mouse liver. To confirm apoA-V association with cytosolic LD in hepatocytes, the experiment was repeated using primary hepatocytes isolated from *APOA5* Tg mouse liver. Again, apoA-V was found associated with LDs as well as microsomes (data not shown). Taken together, these data indicate apoA-V associates with hepatic LD.

3.4 Effect of apoA-V on liver TG accumulation

Intracellular LD are the site of TG storage in hepatocytes. Given that apoA-V associates with LD in vivo, we hypothesized that apoA-V may affect liver TG levels. To examine this, livers were harvested from *APOA5* Tg, WT and *apoa5* KO mice and TG levels determined and normalized to liver weight (Fig. 3). *Apoa5* KO and WT mouse livers contained similar amounts of TG while the amount in livers from *APOA5* Tg mice was significantly ($p<0.05$) higher. These data suggest that apoA-V facilitates liver TG accumulation / retention.

4. Discussion

It is currently accepted that apoA-V plays a role in regulating plasma TG levels. A substantial body of evidence suggests that, extracellularly, apoA-V increases LPL mediated hydrolysis of TG-rich lipoprotein particles thereby contributing to VLDL remnant clearance [15]. Notwithstanding its extracellular role, other data suggest that apoA-V plays an intracellular role in regulating plasma TG levels. In rats, following partial hepatectomy, apoA-V mRNA is upregulated over 3-fold [2]. In this physiological setting, regenerating liver tissue may be expected to retain its lipid pool for membrane biosynthesis and energy production such that TG secretion would be inhibited. Furthermore, the findings that apoA-V associates with LD in transfected hepatoma cells and that C-terminal truncation of apoA-V results in alterations

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in LD morphology [10,11], support the premise that apoA-V may regulate intracellular TG metabolism. The present studies were carried out to assess apoA-V association with LD in vivo and examine the ability of apoA-V to influence intrahepatic TG content.

Although hepatoma cell lines transfected with apoA-V retained greater than 50% of the expressed protein intracellularly [10], the present in vivo studies with *APOA5* Tg mice demonstrate that most of the total apoA-V pool is in the plasma compartment. It is conceivable that overexpression of apoA-V in transiently transfected hepatoma cells impairs its secretion efficiency, favoring LD association. The greater distribution of apoA-V in the plasma compartment in vivo is consistent with its physiological role in mediating hydrolysis and clearance of TG-rich particles [9]. This latter observation led us to ask whether some apoA-V is bound to cell surfaces and thus, may not be accounted for in plasma samples. Treatment of mice with heparin induced a rapid increase in plasma apoA-V levels suggesting a pool of apoA-V exists that is bound to cell surface HSPG or conceivably, glycosylphosphatidylinositol-anchored HDL binding protein 1 [16]. Although the site of the bound fraction of apoA-V is likely to be endothelial cell surfaces (the site of LPL activity), the possibility that some portion of the released protein is from liver cell surfaces cannot be ruled out.

Using immunofluorescence microscopy, previous studies with transfected hepatoma cells showed that most of the apoA-V localizes to cytosolic LD [10]. This observation was unexpected and, conceivably, could result from apoA-V over-expression in these cultured cells. In the present study we unequivocally show that apoA-V is present in LD harvested from livers of both WT and *APOA5* Tg mice indicating apoA-V trafficking to LD is likely to be physiologically relevant. It is intriguing that apoA-V which is synthesized with a signal peptide that targets it for secretion is found on cytosolic LD. The signal peptide may be expected to direct newly synthesized apoA-V to the ER compartment which is also the site for lipid droplet genesis [17]. LD are assembled within the ER membrane and, when it accrues sufficient lipid, buds into the cytosol. It is conceivable that apoA-V, which is very lipophilic, associates with the budding LD during its maturation resulting in its escape from the secretory pathway. In liver, ADRP is the major LD associated protein [18] and is a marker for lipid accumulation. It has been shown that ADRP levels correlate with neutral lipid mass in the cell [18,19]. Overexpression of ADRP promotes accumulation of neutral lipid and/or LD formation in cells while, on the other hand, knockdown/knockout of ADRP reduces lipid accumulation, LD size and number. In the current study we showed that in *APOA5* Tg mouse liver, where apoA-V is overexpressed, there was a significant increase of TG content compared with WT and *apoa5* KO mice. These data support the premise that apoA-V may facilitate TG accumulation in a manner similar to overexpression of ADRP. We cannot, however, rule out the possibility that the increased TG levels in *APOA5* Tg mice may be due to increased liver uptake of TG-rich remnants. Although speculative, the reason why there is no significant difference between liver TG levels in WT and *apoa5* KO mice may be that endogenous levels of apoA-V are extremely low and ADRP is sufficient for normal TG accumulation in *apoa5* KO mouse.

The association between apoA-V and LD may also indicate that these organelles act as an intracellular storage pool of apoA-V. It was reported previously that apoA-V is very hydrophobic with high lipid binding affinity [12,20]. Thus, it is reasonable to hypothesize that association with LD stabilizes the intracellular pool of apoA-V. Since ADRP is degraded during regression of lipid storage in the cell [21], it is possible that, like ADRP, LD association protects apoA-V from degradation.

In summary, results demonstrating association of apoA-V with liver LD in vivo indicate this interaction has physiological relevance. Since *APOA5* Tg mouse liver had a higher TG level than WT or *apoa5* KO mice, it is conceivable apoA-V plays a role in facilitating hepatic TG

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accumulation and/or LD formation. Future studies on the effect of apoA-V on intracellular TG metabolism may provide insights on how apoA-V regulates TG metabolism.

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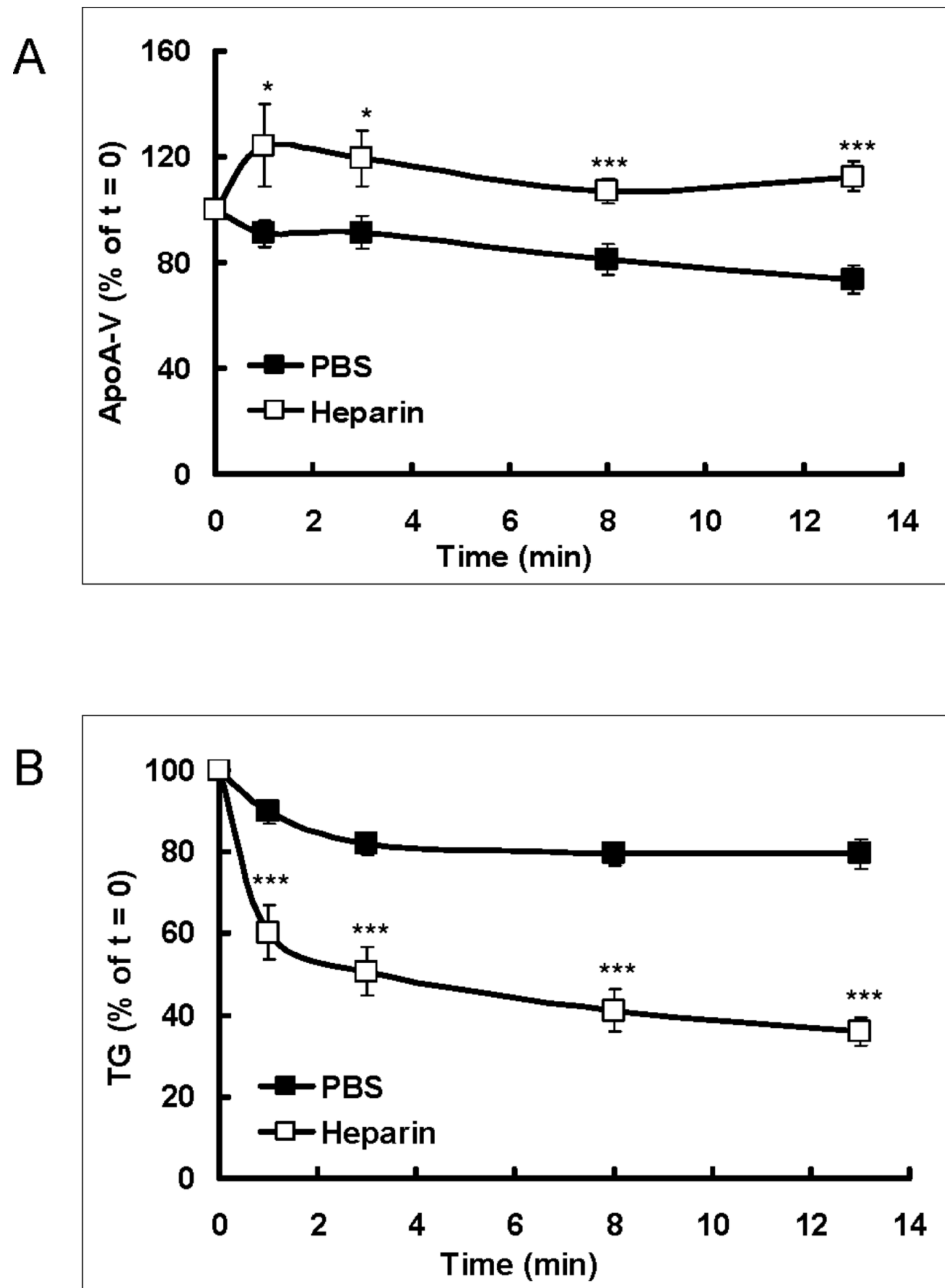

Fig. 1.

Effect of heparin injection on plasma levels of apoA-V. *APOA5* Tg mice were injected with heparin (open squares) or PBS control (filled squares) (8 mice/group) at t=0. Plasma samples collected at t=1, 3, 8, and 13 min post injection were analyzed for (A) apoA-V and (B) TG. Values are presented as percentage of the level at t=0 and expressed as mean ± SE. Student t-test versus respective controls: *, $p<0.05$; ***, $p<0.001$.

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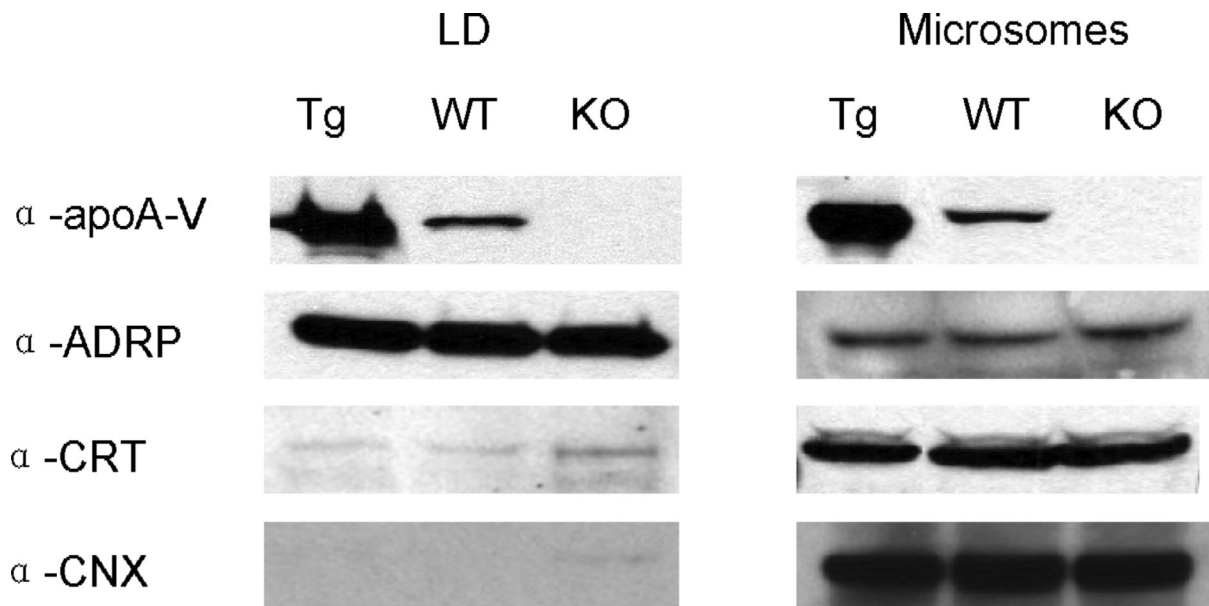

Fig. 2.

ApoA-V association with liver LD and microsomes. Livers (2 g) from *apoa5* KO, WT and *APOA5* Tg mice were harvested and LD and microsomes prepared as described in the Methods. Lipid droplets and microsomes were each taken up in 1 ml buffer and 5 l of each were subjected to electrophoresis. Western blotting was carried out using polyclonal anti-human apoA-V antibody (α-apoA-V), polyclonal anti-ADRP (α-ADRP), polyclonal anti-CRT (α-CRT) and polyclonal anti-CNX (α-CNX). Results are representative of three independent experiments.

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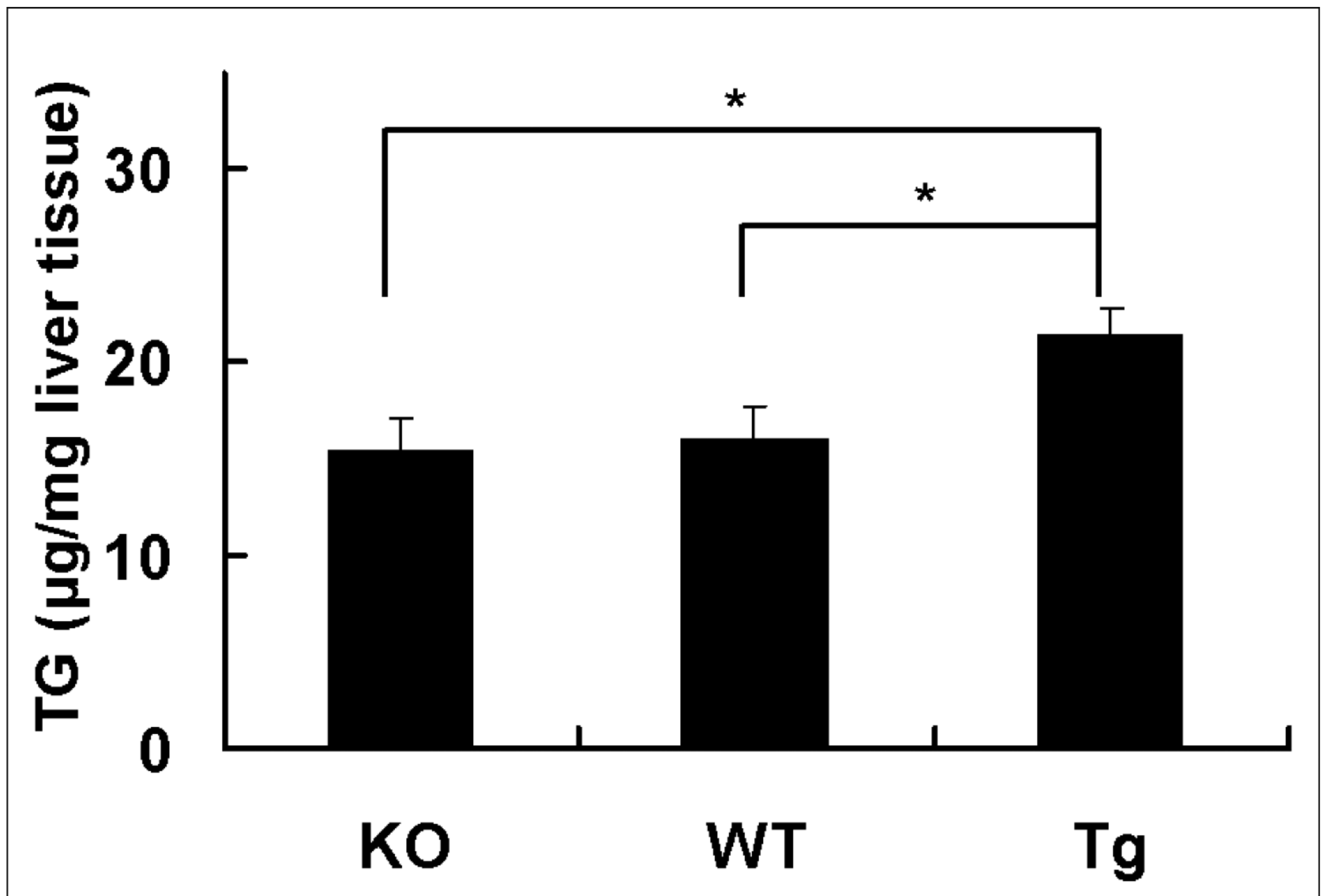

Fig. 3.

Liver TG content in different genotypes. Livers were harvested from *apoa5* KO (n=14), WT (n=15) and *APOA5* Tg (n=20) mice. Liver TG was extracted and assayed as described in the Methods. Liver TG concentration was normalized to liver tissue wet weight. All data are mean ± SE. One-way analysis of variance was used to test for significance. Post hoc analysis (Tukey-Kramer HSD) examined significance of genotype effects. All analyses were performed using JMP version 7.0 (SAS Institute Inc.). *, $p<0.05$.