Adiponectin Reduces Plasma Triglyceride by Increasing VLDL-Triglyceride Catabolism

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Abbreviations: VLDL: very low density lipoprotein; TG: triglyceride; HDL: high density lipoprotein; LPL: lipoprotein lipase; VLDLR: VLDL receptor; ACRP30: adipocyte complement-related protein of 30 kDa, adiponectin; BSA: bovine serum albumin.

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ABSTRACT

Objective: Adiponectin is an adipocyte-derived hormone that plays an important role in glucose and lipid metabolism. The main aims of this study are to investigate the effects of adiponectin on very low density lipoprotein triglyceride (VLDL-TG) metabolism and the underlying mechanism.

Research Design and Methods: Adenoviruses were used to generate a mouse model with elevated circulating adiponectin. HepG2 and C2C12 cells were treated with recombinant human adiponectin.

Results: Three days after Ad-mACRP30 adenovirus injection, plasma adiponectin protein levels were increased 12-fold. All three main multimeric adiponectin molecules were proportionally elevated. Fasting plasma triglyceride levels were significant decreased (~40%) in the mice with elevated adiponectin in circulation, as were the plasma levels of large and medium-sized VLDL subclasses. Although apolipoprotein B mRNA levels were robustly suppressed in the livers of adiponectin overexpressing mice and in cultured HepG2 cells treated with recombinant human adiponectin, hepatic VLDL-TG secretion rates were not altered by elevated plasma adiponectin. However, Ad-mACRP30-treated mice exhibited a significant increase of post-heparin plasma lipoprotein lipase (LPL) activity compared with mice that received control viral vector. Skeletal muscle LPL activity and mRNA levels of LPL and VLDL receptor were also increased in Ad-mACRP30 treated mice. Recombinant human adiponectin treatment increased LPL and VLDL receptor mRNA levels in differentiated C1C12 myotubes.

Conclusions: These results suggest that adiponectin decreases plasma triglyceride levels by increasing skeletal muscle LPL and VLDL receptor expression and consequently VLDL-TG catabolism.
Dyslipidemia is a major component of the metabolic syndrome and a strong risk factor for the development of cardiovascular disease. Adiponectin is an adipocyte-derived hormone that enhances insulin sensitivity and plays an important role in glucose metabolism (1). Paradoxically, adiponectin gene expression is diminished with the development of obesity (1). Thus, hypoadiponectinemia and dyslipidemia are commonly observed in obesity and obesity-associated metabolic syndrome. In addition, studies have showed that plasma adiponectin levels are inversely correlated with very low density lipoprotein-triglyceride (VLDL-TG) and positively correlated with high density lipoprotein (HDL)-cholesterol (2-4), which suggest that adiponectin may influence in lipid metabolism.

Effects of adiponectin on lipid metabolism have been studied in adiponectin transgenic and gene deficient mouse models. In adiponectin transgenic mice, although plasma adiponectin is only moderately elevated, plasma triglyceride concentrations are markedly reduced (5-7). In contrast, hypertriglyceridemia has been reported in adiponectin deficient mice (8). Furthermore, administration of adiponectin normalized high fat diet-induced hypertriglyceridemia in mice (9). These studies demonstrate that adiponectin plays an important role in triglyceride metabolism. However, the mechanism by which adiponectin regulates triglyceride metabolism is largely unknown.

VLDL is the main carrier of circulating triglyceride during fasting. Plasma VLDL-TG level is determined by the balance of VLDL-TG hepatic secretion and catabolism in peripheral tissues. VLDL-TG is synthesized and secreted by hepatocytes after a series of complex intracellular events involving the synthesis of apolipoprotein B (apoB) and lipid and their assembly into lipoprotein particles. Triglyceride is the main lipid component of VLDL. At the luminal surface of capillaries in peripheral tissues, VLDL-TG is hydrolyzed by lipoprotein lipase (LPL) with the release of fatty acids, which are transported into adipocytes for storage or into heart and skeletal muscle for oxidation as fuel (10).

In order to determine the effect and underlying mechanism of adiponectin on triglyceride metabolism, a mouse model with acute elevation of plasma adiponectin was generated for this study using adenovirus-mediated gene transduction. Recombinant human adiponectin and cultured myotubes were also used. Our study showed that elevation of plasma adiponectin reduced fasting circulating triglyceride in mice. Despite decreased apoB mRNA levels in adiponectin-overexpressing mice, elevated adiponectin did not affect hepatic VLDL-TG or hepatic apoB protein secretion. Increases in plasma adiponectin did not alter mean VLDL particle size. However, the large and medium VLDL subclass particle concentrations were significantly reduced in adiponectin-overexpressed mice. Furthermore, a significant increase of post-heparin LPL activity and of LPL and VLDLr expression in skeletal muscle were observed in the mice with elevated adiponectin. Therefore, we proposed that adiponectin reduces plasma triglyceride by increasing LPL, VLDLr expression and VLDL-TG catabolism in peripheral tissues.

**RESEARCH DESIGN AND METHODS**

**Materials.** Recombinant human adiponectin and anti-mouse adiponectin antibody were purchased from R&D system (Minneapolis, MN). Poloxamer-407 was a generously provided by BASF Corporation (Mount Olive, NJ). Fatty acid free bovine serum albumin (BSA) and actinomycin D were purchased from Sigma (St. Louis, MO). The L-type TG-H and HDL-cholesterol kits were purchased from Wako Diagnostics (Richmond, VA). Penicillin, streptomycin,
Dulbecco’s modified Eagles’ medium (DMEM) and horse serum were purchased from Invitrogen.

**Experimental animals.** Male C57BL/6J mice were purchased from Jackson Laboratory (Bar Harbor, ME). All mice were maintained under standardized conditions with 12h/12h light/dark cycle and used between 8-10 weeks. The mice were randomly divided into two groups (n=6). The experiments using mouse models were carried out under the Association for Assessment and Accreditation of Laboratory Animal Care guidelines with approval of the University of Kentucky Animal Care and Use Committee. Purified adenoviruses were diluted in phosphate-buffered saline (PBS) immediately prior to infusion. The adenovirus was injected through tail vein with a dosage of 1 x 10^9 pfu/mouse in 100 µl volume (11). Most experiments were conducted 3 days after injection unless otherwise stated. Adenovirus encoding GFP was used as control.

**Cell Culture.** HepG2 and C2C12 myoblasts were purchased from ATCC. The cells were maintained in DMEM supplemented with 10% fetal bovine serum (FBS, Gemini Bio-Products, Woodland, CA), 200 mM L-glutamine, 200 units/ml penicillin and 50 µg/ml streptomycin in an atmosphere of 95% air and 5% CO2. When C2C12 cells reached 90% confluency, myotube differentiation was induced by switching the medium to DMEM supplemented with 2% horse serum. The culture medium was changed daily. Multinucleated myotubes were obtained within 4 days. For adiponectin treatment studies, both HepG2 and C2C12 myotubes were washed twice with PBS. Recombinant human adiponectin (10 µg/ml) was added to serum-free DMEM and cultured overnight or for the indicated times.

**mRNA stability measurement.** After treating HepG2 cells with adiponectin overnight, actinomycin D (10 µg/ml) was added into medium. The cells were further cultured for the indicated times. Total mRNA was extracted and mRNA levels were quantified by real-time PCR using primers listed in Table 1. 18S ribosomal RNA was used as an internal control.

**Hepatic VLDL-TG secretion and apoB secretion.** Hepatic VLDL-TG secretion and apoB secretion rates were measured using poloxamer-407, which inhibits endogenous LPL with less adverse effects on lipoprotein metabolism than Triton WR-1339 (12). Mice were fasted for 4 hours prior to detergent injection. Poloxamer-407 was dissolved in PBS, and injected into mouse (1,000 mg/kg) through tail vein (12). Blood samples were drawn in heparin capillary tubes at the indicated times and serum triglyceride concentrations were measured using a Wako kit. ApoB protein levels were quantified by Western blotting. Serum samples were prepared in non-reducing protein loading buffer (10 mM Tris-HCl, 5% glycerol, and 1% SDS, pH: 6.8) or 2-fold concentrated Laemmli buffer (4% SDS, 20% glycerol, 10% 2-mercaptoethanol and 0.125 M Tris-HCl, pH: 6.8) without boiling. The anti-mouse apoB-48/100 antibody was purchased from Meridian Life Science (Saco, ME). The antibody was specifically designed to recognize both apoB-100 and apoB-48.

The total lipoprotein fraction of plasma (100 µl) was prepared by gel filtration chromatography using a Superose 6 10/300GL column (GE), run at a flow rate of 0.5 ml/min. Fractions were collected at 0.5 min intervals. Total cholesterol concentrations of fractions were measured and plotted to identify the positions of the major lipoprotein classes. 25 µl fractions (12-35) were mixed with non-reducing protein loading buffer and separated by SDS-PAGE. ApoB protein was probed by western blotting.

**LPL activity measurement.** Post-heparin and tissue LPL activity was measured using a
kit and protocol provided by the manufacturer (Research Diagnostics, INC). Fluorescent triglyceride, which is intramolecularly quenched by a trinitrophenyl group, was used as substrate. Hydrolysis by lipase results in pyrene fluorescence. Hepatic lipase activity can be distinguished in the presence of 1 Mol/l NaCl, which totally abolishes LPL activity. Plasma samples were collected from mice 5 min after a tail vein injection of heparin (300 units/kg) (13). Tissue samples were collected after overnight fasting, and homogenized for LPL activity measurement.

**Lipoprotein particle size and subclass profile.** Three days after virus injection and overnight fasting, blood samples were collected by heart puncture. Plasma was prepared and stored at 4 °C. Assays were carried out within three days after collecting plasma samples. Lipoprotein particle size and subclass profile were analyzed using whole plasma sample and nuclear magnetic resonance (NMR) lipoProfile-II at LipoScience (Raleigh, NC). All lipoprotein particle concentrations are provided in units of moles of particle per liter (14). 1 mole of particles equals to $6.02 \times 10^{23}$ particles per liter. The measured NMR signal amplitude from each lipoprotein subclass is directly proportional to the particle concentration of that subclass and not the amount of cholesterol or triglyceride in that particle (14).

**Plasmid constructs and generation of adenovirus.** Mouse adiponectin cDNA was cloned by RT-PCR and inserted into pENTR vector (Invitrogen). The sequences of the primers are the following: forward primer, 5’-caccaggATGgTACTGTTGCAAGC-3’; reverse primer, 5’-TCAGTTGATCATGGTAGA-3’. Adiponectin encoding DNA was then transferred to pAd/CMV/V5-Dest vector using Gateway technique following protocol provided by manufacturer (Invitrogen). Adenovirus was produced in HEK293 cells, and purified by cesium chloride as described previously (15).

**Serum adiponectin immunoblotting.** Serum adiponectin protein levels and adiponectin multimers were analyzed by Western blotting. Blood samples were collected through the tail vein after overnight fasting. For total adiponectin measurements, serum (0.4 μl) was separated by SDS-PAGE using Laemmli sample buffer. For multimeric adiponectin measurements, serum samples were prepared by non-reducing loading buffer (1% SDS, 5% glycerol, and 10 mM Tris-HCl, pH 6.8) at room temperature for 10 min and separated by 4–20% gradient SDS-PAGE. Proteins were transferred to PVDF membrane, and adiponectin was probed with antibody raised against mouse adiponectin. Protein bands were quantified by using Bio-Rad Chemidoc system with Quantity one software.

**Quantitative RT-PCR Analysis.** Total RNA was prepared from liver tissues, HepG2 cells and C2C12 myotubes with Trizol following the manufacturer’s protocol (Invitrogen, Carlsbad, PA). cDNA was synthesized using SuperScript III Reverse Transcriptase and oligo(dT)$_{12-18}$ primer (Invitrogen). Real-time PCR was performed using a Mx3000P Real-Time PCR system (Stratagene) and SYBR Green dye (Molecular Probes, Eugene, OR). The sequences for the primers are listed in Table 1. The levels of PCR product were calculated from standard curves established for each primer pair. Expression data were normalized to the amount of 18S rRNA.

**Statistical Analysis.** Data are expressed as mean ± standard error (S.E.). Statistical analysis was performed using the Student t test or ANOVA, followed by contrast test with Tukey or Dunnett error protection. Differences were considered significant at $p<0.05$.

**RESULTS**
**Adenovirus-mediated hepatic adiponectin transduction increases circulating adiponectin in mice.** Most previous animal studies regarding the modulatory effects of adiponectin on lipid metabolism were conducted in adiponectin transgenic or gene deficient mouse models, which illustrate a long term impact of adiponectin on lipid metabolism. A life-time increase or absence of adiponectin may induce changes in multiple systems that may obscure the direct modulatory effects of adiponectin on lipid metabolism in vivo. In order to investigate the effects of adiponectin on lipid metabolism, we generated a mouse model with an acute elevation of plasma adiponectin using adenovirus-mediated in vivo gene transduction (15). Similar mouse or rat models have been reported in several studies for other purposes (16; 17).

Consistent with our previous study (15), viral vector-mediated adiponectin gene expression was observed almost exclusively in liver tissues (data not shown). Plasma adiponectin protein levels were measured by Western blot. As shown in Fig. 1A, a significant increase of serum adiponectin was detected 24h after viral vector injection. Serum adiponectin concentration reached maximal levels around day 4-5. Ad-mACRP30-treated mice exhibited a 12-fold increase of circulating adiponectin 72 hours after injection (Fig.1B), without any significant changes of body weight compared with Ad-GFP-treated mice (24.45 ± 1.64g vs. 24.10 ± 1.45 g). In order to minimize any possible effects of virus-induced inflammation, subsequent experiments were performed 48-72 hours following injection. These results demonstrate that adenovirus vector-mediated adiponectin gene expression in liver increases adiponectin levels in plasma.

Circulating adiponectin proteins can exist as multimers. It has been suggested that the different multimeric forms of adiponectin may have different biological functions or potency (1; 18). Our results showed that adenovirus-mediated adiponectin gene transduction proportionally increased all three main molecular weight adiponectin multimers in circulation (Fig. 1C).

**Adiponectin reduces fasting plasma triglyceride and FFA in mice.** Three days after virus injection, mice were fasted for 6 hours and plasma total triglyceride and FFA concentrations were determined. As showed in Fig. 2A, plasma triglyceride concentrations were reduced approximately 40% (p<0.01) in Ad-mACRP30 treated mice. A similar reduction in plasma FFA was observed (Fig. 2B). Fasting plasma HDL-cholesterol concentrations were significantly increased in Ad-mACRP30 treated mice compared with control group (48.08 ± 6.86 vs. 25.59 ± 5.65 mg/dL, p=0.021). The finding that adiponectin reduces fasting total TG is consistent with the observed reduction in plasma TG in adiponectin transgenic mice (5). However, 4 hours after re-feeding the difference of TG between Ad-mACRP30 and Ad-GFP-treated mice vanished (Supplemental Fig. 1), presumably due to high levels of chylomicrons.

An insulin challenge test, conducted 72 hours after adenovirus administration, showed that there was no significant difference in insulin-stimulated glucose disposal between Ad-GFP and Ad-mACRP30-treated mice (Fig. 2C). In addition, there was no difference in fasting plasma glucose concentrations between these two groups of mice (Fig. 2C). Fasting plasma insulin levels in Ad-mACRP30-treated and Ad-GFP-treated mice were similar (0.3346 ± 0.04 vs. 0.3105 ± 0.01 ng/ml). However, consistent with a previous study (19), significant decreases of PEPCK and G6Pase mRNA were observed in the livers of mice with elevated plasma adiponectin (data not shown). More precise measurements, e.g. hyperinsulinemic-euglycemic clamps, are required to determine
the effect of acute increased adiponectin on insulin sensitivity. Our results imply that adiponectin may modulate lipid metabolism through a mechanism independent of adiponectin-enhanced insulin sensitivity.

**Adiponectin reduces apoB mRNA but does not alter hepatic apoB protein secretion.** We measured mRNA levels of apoB and several other apolipoproteins in the livers of Ad-mACRP30-treated mice to test if adiponectin regulates apolipoprotein expression. As showed in Fig. 3A, hepatic apoB mRNA was reduced nearly 50% in Ad-mACRP30-treated mice compared with control mice. The expression of apoE, apoA-I, apoA-V and apoC-III was also measured. Studies have reported that adiponectin can regulate PPARα (18) and that PPARα regulates both apoA-V and apoC-III transcription (20; 21). However, our results showed no differences in the mRNA levels of either apoA-V or apoC-III between Ad-mACRP30 and Ad-GFP treated mice (Fig. 3A). However, apoA-I mRNA levels were decreased in Ad-mACRP30 treated mice (Fig. 3A).

In order to further verify these observations, an *in vitro* study was carried out using HepG2 hepatocytes and recombinant human adiponectin protein. Consistent with the *in vivo* results, the apoB mRNA level was also markedly suppressed in HepG2 cells after 24 hours of adiponectin treatment (10 μg/ml) (p<0.01, Fig.3B). Adiponectin treatment did not alter apoA-V mRNA, however, it reduced apoC-III mRNA (Fig. 3B). The increase in apoA-I mRNA level following adiponectin treatment was opposite to the results of the *in vivo* study (Fig. 3A). These differences in the effects of adiponectin on apoA-I and apoC-III expression in the *in vivo* and *in vitro* studies may reflect differences in these two models.

We also measured the effects of adiponectin treatment on apoB mRNA stability in HepG2 cells. The results showed that there was no significant difference of apoB mRNA half-life between adiponectin treated and control cells (data not shown). Together, these studies indicate that adiponectin suppresses hepatic apoB gene expression without altering apoB mRNA stability, implying that adiponectin inhibits apoB expression at the transcription level.

While this manuscript was in preparation, Neumeier et al reported that adiponectin reduced apoB expression in human hepatocytes by reducing HNF4α (22). However, our study did not show any changes of HNF4α at both protein (Fig. 4) and mRNA levels (data not shown). Microsomal triglyceride transfer protein plays a key role in VLDL assembly in liver. Our studies showed that the MTP mRNA levels were not significantly altered in the livers of Ad-mACRP30-treated mice or adiponectin-incubated HepG2 cells (data not shown).

To further study the effect of adiponectin on apoB expression in liver, basal serum apoB protein levels and hepatic apoB protein secretion rates were studied using fasting serum and serum samples collected at different time points following LPL inhibition by Poloxamer-407 (see details in the Methods section for measurement of hepatic VLDL secretion rate). Mouse hepatocytes edit apoB mRNA and both apoB-100 and apoB-48 protein can be produced from mouse liver (23). As shown in Fig. 3C, despite slightly reduced fasting apoB-100 and apoB-48 levels in Ad-mACRP30-treated mice, there was no statistical difference of basal total apoB protein levels between Ad-mACRP30- and Ad-GFP-treated mice (p>0.05).

Unexpectedly, the fasting plasma apoB-100 band was markedly shifted in Ad-mACRP30-treated mice under non-reducing conditions, while the size difference disappeared under reducing conditions (Fig. 3C). These observations suggest that elevated adiponectin may induce a conformational change or modification of plasma apoB-100
in the treated mice. Further studies are being undertaken to identify the mechanism through which adiponectin alters plasma apoB-100 protein mobility and its biological consequences.

Results in supplemental Fig. 2A show that plasma apoB-100 protein levels are higher than apoB-48 in mice in the fasting state. Plasma lipoproteins were fractionated by FPLC and cholesterol concentrations in the fractions were determined. Interestingly, the large bulk of apoB-100 in Ad-mACRP30-treated mice and also control mice (Ad-GFP) was detected in fractions corresponding to the position of LDL (Supplemental Fig. 2B). The study also showed that at 4h after inhibition of LPL by Poloxamer-407 (12; 24) there was an expected large accumulation of apoB-48 and also of apoB-100, although to a lesser extent (Fig. 3D and Supplemental Fig. 2A). However, the increased rate of apoB-100 protein was significantly less than of apoB-48 (Supplemental Fig. 2A), which may be caused by P-407-induced blockage of formation of new LDL and continued catabolism of preexisting LDL (Supplemental Fig. 2B). Therefore, we used apoB-48 protein accumulation to calculate hepatic apoB secretion rate (Fig. 3D). Our study showed no significant difference of hepatic apoB-48 secretion between adiponectin overexpressing and control mice (Fig. 3E). These results suggest that adiponectin has no effect on hepatic apoB protein secretion despite reducing apoB mRNA in hepatocytes.

**Elevated plasma adiponectin has no effect on hepatic VLDL-TG secretion.** The rate of hepatic VLDL-TG secretion was directly measured by the technique in which Poloxamer-407 is used to inhibit endogenous LPL and VLDL processing (12; 24). Serum TG levels increased markedly following Poloxamer-407 addition and at the same rate in the Ad-mACRP30-treated and control mice. Calculated hepatic VLDL-TG secretion rates in the two groups of mice were similar (Fig. 5B). This study indicates that although adiponectin suppresses apoB transcription, hepatic VLDL-TG secretion is not inhibited. This conclusion is supported by previous studies, which suggest that apoB gene expression in liver is constitutive, and that apoB synthesis is not usually rate-limiting for VLDL assembly and VLDL-TG secretion (25; 26).

**Decrease of large and medium VLDL particle levels in adiponectin-overexpressed mice.** Studies have demonstrated that VLDL particle size plays a significant role in VLDL catabolism and that multimeric adiponectin is related to lipoprotein subclass profiles (27). Our studies showed that blood triglyceride concentrations were significantly reduced in adiponectin overexpressing mice without an alteration in the rate of hepatic apoB and VLDL-TG secretion rate. This raises the question whether VLDL particle size is altered in adiponectin overexpressing mice. We measured lipoprotein particle size and the subclass profile using NMR. As shown in Fig. 5C, mean particle sizes of LDL or HDL were comparable between Ad-mACRP30-treated and control mice. VLDL mean particle size was slightly decreased in adiponectin-overexpressed mice but without statistical significance. However, the levels of large and medium VLDL subclasses in Ad-mACRP30-treated mice were significantly lower than in control mice (Fig. 5D). Interestingly, small VLDL levels tended to increase in Ad-mACRP30-treated mice (Fig. 5D). These results are consistent to the human study which reported that circulating total and HMW adiponectin are negatively correlated with large VLDL particle levels (27).

**Post-heparin plasma LPL activity is increased in the mice with elevated plasma adiponectin.** The level of fasting plasma VLDL-TG is determined by the balance of hepatic VLDL-TG secretion and peripheral VLDL-TG catabolism. Our results showed that elevated adiponectin reduces serum
triglyceride concentrations, and has no effect on hepatic VLDL-TG secretion. Therefore, adiponectin-induced low serum triglyceride is most likely caused by increasing VLDL-TG catabolism.

Our results showed that post-heparin plasma LPL activities were significantly increased in mice that received Ad-mACRP30 treatment (Fig. 6A). Tissue LPL activity was also measured using homogenized epididymal fat, gastrocnemius muscle or heart. No difference was detected in LPL activities in epididymal fat or heart between Ad-mACRP30 and Ad-GFP virus treated mice (Fig. 6B). However, skeletal muscle LPL activities were increased by 41% in Ad-mACRP30-treated mice (p<0.05, Fig. 6B).

**Adiponectin increases LPL and VLDLR expression in skeletal muscle.** Altered LPL gene expression and post-translational modification are two mechanisms that regulate LPL activity. The mRNA levels of LPL were measured by real-time PCR. Results showed that LPL mRNA was significantly increased in both skeletal muscle and kidney of Ad-mACRP30 treated mice (p<0.01, Fig.7A). No difference was observed in heart, epididymal fat or liver between these two groups of mice.

In order to further verify the effects of adiponectin on LPL expression, cultured C2C12 myotubes were treated with recombinant human adiponectin (10 μg/ml). A 24-hour incubation of cells with adiponectin significantly increased LPL mRNA levels, consistent with the results from the in vivo studies (p<0.05, Fig.7B). These data indicate that adiponectin directly increases LPL gene expression in skeletal muscle.

VLDLR plays an important role in VLDL-TG catabolism. It not only interacts with and enhances LPL activity, but also binds and internalizes VLDL remnants into cells (28). We measured VLDLR mRNA levels and found they were robustly increased (3.6-fold) in skeletal muscle of Ad-mACRP30 treated mice (p<0.001, Fig.7C). In contrast, the VLDLR mRNA levels in epididymal fat, heart and kidney were similar in adiponectin overexpressing and control mice (data not shown). A robust increase of VLDLR mRNA levels was also observed in adiponectin treated C2C12 myotubes (p<0.05, Fig. 7D).

**DISCUSSION**

Although compelling evidence indicates that adiponectin modulates lipid metabolism, the underlying mechanisms are largely unknown. Using adenovirus-mediated adiponectin overexpression in vivo and a cultured cellular model, our current study specifically investigated the modulatory effect of adiponectin on VLDL-TG metabolism. Consistent with previous studies, our study showed that elevated adiponectin reduces plasma TG in mice. Despite the decrease of apoB mRNA levels in the livers of Ad-mACRP30-treated mice and in adiponectin-treated HepG2 hepatocytes, elevated adiponectin did not alter hepatic VLDL-TG or apoB protein secretion in mice. On the other hand, hepatic LPL transcription and post-heparin plasma LPL activities, together with VLDLR transcription, were robustly increased in adiponectin overexpressing mice. Consistent with these findings, recombinant adiponectin increased LPL and VLDLR gene expression in cultured skeletal muscle cells. Therefore, we propose that adiponectin reduces plasma TG by increasing VLDL catabolism in skeletal muscle.

Since the discovery of the insulin sensitizing property of adiponectin, numerous
studies have observed an inverse relationship between plasma TG and adiponectin levels. Studies using adiponectin transgenic or gene deficient mice further indicated that adiponectin modulates lipid metabolism (5-9). However, transgenic or gene deficient mouse models represent long-term effects of increased or absent adiponectin on lipid metabolism. By using adenovirus-mediated gene transduction technique, we generated a mouse model with acute elevations of plasma adiponectin. Our study shows that an acute increase of circulating adiponectin reduces serum triglyceride and also increases HDL-cholesterol in mice. Together, our study further confirms that adiponectin improves the “anti-atherogenic” lipoprotein profile in mice. A recent study suggested that the positive link between HDL and adiponectin may be caused by reduced apoA1 degradation (29). Our study found a decrease of apoA1 mRNA in adiponectin overexpressing mice livers, but an opposite effect was observed in HepG2 cells (Fig. 3A&B). Further studies are required to elucidate the underlying mechanism by which adiponectin increases HDL cholesterol.

ApoB is the major apolipoprotein in VLDL and both apoB-100 and apoB-48 are produced in rodent hepatocytes (23). The results from our in vivo and in vitro studies show that adiponectin suppresses apoB transcription in hepatocyte, which is an agreement with a most recent report (22). Importantly, our in vivo study demonstrated that adiponectin did not alter basal serum apoB protein levels or the rate of hepatic apoB secretion, despite decreasing apoB transcription. These results are in line with previous reports that hepatic apoB mRNA is abundant and that the rate of apoB translation exceeds apoB secretion (25; 26). Thus, the majority of newly synthesized apoB protein is degraded within hepatocytes before lipoprotein assembly and secretion (26; 30). In addition, our study found that adiponectin overexpression did not alter liver VLDL-TG secretion in mice. These results lead us to conclude that although adiponectin reduces hepatic apoB gene transcription, adiponectin has no significant effect on hepatic apoB and VLDL-TG secretion. Nevertheless, our study clearly showed that fasting plasma triglyceride was decreased in adiponectin overexpressing mice. Therefore, we postulate that increased VLDL-TG catabolism, rather than decreased synthesis accounts for the reduced plasma TG levels in response to adiponectin.

LPL has been considered a gate-keeper for VLDL-TG catabolism (10). LPL hydrolyzes VLDL-TG and releases fatty acids for oxidation as fuel for skeletal or cardiac muscle, or storage in adipose tissue (10; 31). Recent studies have reported a positive relationship between plasma adiponectin levels and post-heparin LPL activities (32; 33). Consistent with these correlative human studies, our study demonstrated that there was significant increase of post-heparin LPL activity in the mice with elevated adiponectin. These data indicate that adiponectin increases VLDL-TG hydrolysis in peripheral tissues, thus leading to a decrease in circulating TG. Furthermore, our study showed that LPL expression and activity were increased in skeletal muscle from the mice with elevated adiponectin. In fasted animals, adipose tissue LPL expression and activity are suppressed and plasma LPL is mainly contributed by skeletal muscle and heart (34). Skeletal muscle plays an important role in TG clearance taking into account its large mass in the body. Therefore, increased post-heparin LPL activities in adiponectin overexpressing mice were likely due to increased skeletal muscle LPL expression. A previous study has reported that adiponectin increased TG clearance by increasing LPL expression in white adipose tissues in female adiponectin transgenic mice (5). However, our study did not show a significant increase of LPL
expression and activity in epididymal fat of adiponectin overexpressing mice. Differences between these animal models may cause the discrepancy. The mouse model we used is acute adiponectin overexpression. In addition, adiponectin was ectopically overexpressed and secreted from liver following adenovirus transduction. Under normal physiological conditions, adiponectin is predominantly synthesized in adipose tissues. Furthermore, our mice were male unlike the female mice used in the previous study (5). However, in both our current study and previous report (5), serum FFA were found to be reduced in adiponectin overexpressing mice.

VLDLr is a member of LDL receptor (LDLr) family (35). Similar to LPL, VLDLr is highly abundant in heart, skeletal muscle and adipose tissue whereas it is barely detectable in liver (35-37). Recent studies demonstrate that VLDLr is involved in VLDL-TG catabolism by interacting with LPL (13; 35-38). Our findings show that elevation of plasma adiponectin induced a robust increase of VLDLr mRNA in skeletal muscle and cultured myotubes. Therefore, we postulate that the combined effects of adiponectin on VLDLr and LPL in skeletal muscle contribute to increased VLDL-TG catabolism. Our recent findings have revealed that adiponectin enhances LPL gene transcription via AMP kinase activation in skeletal muscle (Qiao, L. and Shao, J. unpublished data). The detail mechanisms are under investigation.

In summary, our study indicates adiponectin reduces blood TG but has no effect on hepatic VLDL-TG secretion. Increased LPL and VLDLr expression in skeletal muscle and plasma LPL activity were observed in mice with elevated plasma adiponectin. Therefore, we propose that adiponectin reduces plasma TG by increasing VLDL-TG catabolism in skeletal muscle, at least in part by increasing LPL and VLDLr expression.

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Fig. 1. Adenovirus-mediated adiponectin overexpression in mice. Purified adenovirus vectors were injected into mice through the tail vein. Plasma adiponectin protein levels were measured by Western blot. A, time course of total serum adiponectin protein in Ad-mACRP30 viral vector treated mice. Top panel is a representative autoradiograph of four mice; B, Serum total adiponectin protein levels were increased (~12-fold) 3 days after viral vector injection; C, Proportional increase of circulating multimeric forms of adiponectin in Ad-mACRP30 vector treated mice. The multimeric forms of adiponectin were analyzed by non-reducing PAGE. HMW: high molecular weight; MMW: medium molecular weight; LMW: low molecular weight.

Fig. 2. Reduction of serum triglyceride and FFA in adiponectin-overexpressing mice. Three days after viral vector injection, the mice were fasted for 6 hours. Blood samples were collected and serum TG (A) and FFA (B) concentrations were measured using Wako kit, n=6; B, Insulin challenge tests were carried out after overnight fasting, n=5. *p<0.05 vs. Ad-GFP vector treated control mice.

Fig. 3. Adiponectin reduces apoB mRNA but has no effect on hepatic apoB secretion. A, Total mRNA was extracted from livers of mice 3d after virus injection. mRNA levels were measured by real-time PCR using 18S as internal control, n=6. B, Confluent HepG2 cells were treated with recombinant human adiponectin (10 μg/ml) or BSA control for 24 hours, n=4. Mouse serum samples were collected after 4h fasting (C) and at indicated time points after P-407 injection (D). Plasma was separated in SDS-PAGE with non-reducing loading buffer and apoB protein was probed using anti-mouse apoB specific antibody. The standards were made by stepwise dilution (1:2 dilution in PBS) using a plasma sample from a wild type mouse 2h following P-407 injection. Hepatic apoB-48 secretion rates were calculated based on the increase of plasma apoB-48 protein levels after P-407 injection (E). *p<0.05 vs. Ad-GFP or BSA treated control respectively for liver and cultured cells.

Fig. 4. Elevated adiponectin has no effect on HNF4α protein levels in mouse liver. 3 days after viral vector injection, liver tissues were collected in the fasting state following perfusion. Total liver protein was prepared and HNF4α and adiponectin were analyzed by Western blotting.

Fig. 5. Elevated plasma adiponectin has no effect on hepatic VLDL-TG secretion and lipoprotein particle size. A&B, Hepatic VLDL-TG secretion rates were studied by measuring serum TG accumulation using Poloxamer-407 to inhibit endogenous LPL. The mice were fasted for 4h, 3d after viral vector injection. Blood samples were collected at indicated time points and TG was measured; C&D, 4h fasting blood samples were collected 3 days after virus inject. Mean lipoprotein particle size and subclass profile were measured using a proton NMR analyzer, n=6. Diameter range for VLDL subclass: large >60 nm, medium 35-60 nm, small 27-35 nm. *p<0.05 vs. Ad-GFP vector treated control mice.

Fig. 6. Adiponectin increases post-heparin LPL activity in mice. A, Post-heparin plasma were collected from mice after 3 days of viral vector injection and LPL activity was measured, n=6; B, Tissue LPL activity was measured using homogenized protein samples, n=5. *p<0.05 vs. Ad-GFP treated mice.

Fig. 7. Adiponectin enhances LPL and VLDLr gene expression in skeletal muscle. A, LPL mRNA levels were measured by real-time PCR, using mRNA samples extracted from
gastrocnemius muscle, heart, kidney and epididymal fat from Ad-mACRP30 or Ad-GFP treated mice, n=6; B, differentiated C2C12 myotubes were treated with recombinant human adiponectin (10 μg/ml) or BSA control for 24 hours, n=4. The mRNA levels of CD36 and VLDLr in gastrocnemius muscle (C) and recombinant human adiponectin treated C2C12 myotubes (D) were also analyzed by real-time PCR. *p<0.05 vs. Ad-GFP or BSA treated control. SkM: skeletal muscle, WAT, epididymal fat.
FIGURE 1

A

Non specific

ACRP30

30 kDa

Serum adiponectin (arbitrary units)

Days after Ad-mACRP30 injection

B

Ad-GFP  Ad-mACRP30

ACRP30

~12-fold

Serum ACRP30 protein (arbitrary units)

Ad-GFP  Ad-mACRP30

C

Ad-GFP  Ad-mACRP30

HMW  MMW  LMW
FIGURE 2

A

TG (mg/dl)

Ad-GFP  Ad-mACRP30

B

FFA (mmol/l)

Ad-GFP  Ad-mACRP30

C

Glucose (mg/dl)

Ad-GFP  Ad-mACRP30

0 30 60 90 120

0 20 40 60 80 100 120

0 30 60 90 120 min

19
FIGURE 3C

C

<table>
<thead>
<tr>
<th></th>
<th>Ad-GFP</th>
<th>Ad-mACRP30</th>
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<tbody>
<tr>
<td>apoB-100</td>
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<tr>
<td>apoB-48</td>
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</tbody>
</table>

250 kDa

 apoB-48&100 protein (arbitrary units)

<table>
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<tr>
<th></th>
<th>Ad-GFP</th>
<th>Ad-mACRP30</th>
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</thead>
<tbody>
<tr>
<td>apoB-48&amp;100 protein</td>
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</table>
FIGURE 3D-E

D

<table>
<thead>
<tr>
<th>Time (h)</th>
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<th>Ad-GFP</th>
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<tr>
<td></td>
<td></td>
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</tbody>
</table>

250 kDa

apoB-48

E

apoB-48 secretion rates (unit/h/kg)

Ad-GFP | Ad-ACRP30

apoB-48 protein levels (arbitrary units)

Ad-GFP

Ad-ACRP30
FIGURE 5A-B

A

Plasma TG (mmol/L)

- Ad-GFP
- Ad-mACRP30

Times

B

TG secretion Rate (µM/h/kg)

Ad-GFP Ad-mACRP30
FIGURE 5C-D

C

Particle diameter (nm)

<table>
<thead>
<tr>
<th></th>
<th>Ad-GFP</th>
<th>Ad-ACRP30</th>
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<tbody>
<tr>
<td>VLDL</td>
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<tr>
<td>LDL</td>
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</tr>
<tr>
<td>HDL</td>
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D

VLDL particle concentration (nmol/L)

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<tr>
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<tbody>
<tr>
<td>Large</td>
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</tr>
<tr>
<td>Medium</td>
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<td></td>
</tr>
<tr>
<td>Small</td>
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</table>
FIGURE 7C-D

C

Relative mRNA levels (arbitrary units)

CD36  VLDLR

Ad-GFP  Ad-mACRP30

D

Relative mRNA levels (arbitrary units)

CD36  VLDLR

BSA  hAdiponectin

*