High-Density Lipoprotein Maintains Skeletal Muscle Function by Modulating Cellular Respiration in Mice

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Background—Abnormal glucose metabolism is a central feature of disorders with increased rates of cardiovascular disease. Low levels of high-density lipoprotein (HDL) are a strong predictor for cardiovascular disease. We used genetic mouse models with increased HDL levels (apolipoprotein A-I transgenic [apoA-I tg]) and reduced HDL levels (apoA-I–deficient [apoA-I ko]) to investigate whether HDL modulates mitochondrial bioenergetics in skeletal muscle.

Methods and Results—ApoA-I ko mice exhibited fasting hyperglycemia and impaired glucose tolerance test compared with wild-type mice. Mitochondria isolated from gastrocnemius muscle of apoA-I ko mice displayed markedly blunted ATP synthesis. Endurance capacity during exercise exhaustion test was impaired in apoA-I ko mice. HDL directly enhanced glucose oxidation by increasing glycolysis and mitochondrial respiration rate in C2C12 muscle cells. ApoA-I tg mice exhibited lower fasting glucose levels, improved glucose tolerance test, increased lactate levels, reduced fat mass, associated with protection against age-induced decline of endurance capacity compared with wild-type mice. Circulating levels of fibroblast growth factor 21, a novel biomarker for mitochondrial respiratory chain deficiencies and inhibitor of white adipose lipolysis, were significantly reduced in apoA-I tg mice. Consistent with an increase in glucose utilization of skeletal muscle, genetically increased HDL and apoA-I levels in mice prevented high-fat diet–induced impairment of glucose homeostasis.

Conclusions—In view of impaired mitochondrial function and decreased HDL levels in type 2 diabetes mellitus, our findings indicate that HDL-raising therapies may preserve muscle mitochondrial function and address key aspects of type 2 diabetes mellitus beyond cardiovascular disease. (Circulation. 2013;128:2364-2371.)

Key Words: cellular respiration ■ cholesterol, HDL ■ exercise ■ obesity

Recent years have seen an alarming rise in the incidence of cardiovascular disease linked to obesity-related metabolic factors. Epidemiological studies have confirmed a strong association between fat intake, plasma cholesterol levels, and cardiovascular disease mortality rates. Of particular concern is the incidence of diabetes mellitus in obese patients, because diabetes mellitus itself carries a substantially elevated cardiovascular disease risk. One of the strongest independent predictors of cardiovascular disease is a low level of high-density lipoprotein (HDL) particles and their major protein constituent apolipoprotein A-I (apoA-I). Besides its critical role in reverse cholesterol transport and cellular cholesterol efflux, apoA-I also has anti-inflammatory, antithrombotic, and antioxidant functions that contribute to its well-known antiatherogenic function. Low circulating HDL and apoA-I levels are also a hallmark of insulin-resistance, a pathological precondition leading to type 2 diabetes mellitus (T2D). It remains unclear, however, whether circulating HDL levels exert an effect on insulin resistance and the development of diabetes mellitus.

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Several lines of evidence suggest that HDL and apoA-I modulate glucose homeostasis: Infusions of reconstituted HDL particles have been shown to reduce circulating glucose levels and increase insulin levels in patients with T2D through insulin-dependent and -independent mechanisms.\textsuperscript{18} Cell-based assays have confirmed that both HDL components apoA-I and apoA-II increase β-cell insulin secretion.\textsuperscript{13} HDL and apoA-I have been also demonstrated to directly enhance glucose uptake in cultured mouse and human skeletal muscle cells, thus confirming an insulin-independent effect of HDL.\textsuperscript{14,16} Thus, these results show that HDL and apoA-I are enhancing muscular glucose uptake.

In addition to glucose uptake, intracellular glucose metabolism is known to play a pivotal role in the pathogenesis for insulin resistance and diabetes mellitus. After entry into the muscle cell, glucose is oxidized through mitochondrial phosphorylation to ATP as an energy source for cellular metabolism. Glucose is also used to generate glycogen and converted into fat (lipogenesis) for storage and later use as energy.\textsuperscript{17} All 3 metabolic routes have been shown to contribute to the development of diabetes mellitus. First, oxidative phosphorylation and ATP synthesis are impaired in skeletal muscle from relatives of patients with T2D, strongly suggesting that defects in mitochondrial oxidative metabolism are a primary cause of insulin resistance.\textsuperscript{18} Second, dysfunctional muscle glycogen synthesis has been reported to play a dominant role in insulin resistance of diabetic patients.\textsuperscript{19} Consistently, defects in insulin-mediated glucose oxidation, glycogen synthesis, and storage have been already revealed in skeletal muscle from individuals with normal glucose tolerance but with peripheral insulin resistance,\textsuperscript{20} underscoring the importance of intramyocellular glucose metabolism as crucial player in the development of insulin resistance. Finally, an excessive conversion of glucose into lipid resulting from defects in mitochondrial function leads to accumulation of intramyocellular triglycerides, which also has been implicated in insulin resistance in several population studies.\textsuperscript{21} Although it has been shown that HDL and apoA-I do increase glucose uptake in muscle cells and thus decrease circulating glucose levels, it has not been determined whether HDL and apoA-I also affect intracellular glucose oxidation, glycogen synthesis, and lipogenesis.

In this study, we investigated the requirement for circulating HDL and apoA-I in normal glucose oxidation of skeletal muscle using a genetic loss- and gain-of-function mouse model. We show that, in the absence of apoA-I, mitochondrial oxidative phosphorylation is reduced in skeletal muscle, resulting in increased circulating glucose levels and impaired muscular function. We provide in vitro and in vivo evidence that HDL and apoA-I enhance glycolysis and mitochondrial oxidative phosphorylation rates of glucose. Overexpression of apoA-I in mice resulted in protection against age-induced decline of endurance capacity, against age-induced fat mass gain, and against diet-induced hyperglycemia. Improved mitochondrial function in apoA-I transgenic (apoA-I tg) mice was further confirmed indirectly by the marked reduction of circulating fibroblast growth factor 21 (FGF-21), a novel biomarker for mitochondrial dysfunction. Our findings point to a key role for circulating HDL and apoA-I in regulating skeletal muscle metabolism and highlight a possible target for the treatment of metabolic diseases such as insulin resistance and T2D.

**Methods**

An expanded online-only Methods and Results section is available in the online-only Data Supplement.

**Mice**

Age-matched male apoA-I-deficient (apoA-I ko), human apoA-I tg, and control (wild-type [wt]) C57/B16J mice (The Jackson Laboratories, Bar Harbor, ME) were housed in specific pathogen-free facilities with a 12-hour light/12-hour dark cycle and were fed basal rodent chow 5058 PicoLab Mouse Diet 20 (LabDiet, Richmond, IN). Mice that underwent the diet-induced obesity study were fed a low-fat diet containing 4.8% fat by weight (D12328; Research Diets, New Brunswick, NJ) or a high-fat diet containing 35.8% fat by weight (D12330; Research Diets) for 12 weeks. All experimental procedures conformed to institutional guidelines for animal experiments and were approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Cincinnati.

**Respiration Studies in Isolated Skeletal Muscle Mitochondria and Cultured Muscle Cells**

After euthanization, gastrocnemius muscles were harvested and mitochondria were isolated immediately as described.\textsuperscript{22} Muscle mitochondria respiration measurements were made in triplicate by the Seahorse 24 XF analyzer (Seahorse Biosciences Inc., North Billerica, MA). For the determination of extracellular acidification rate (ECAR) and mitochondrial oxygen consumption rate in the murine skeletal muscle C2C12 cell line (ATCC, Manassas, Virginia), cells were incubated for 4 hours with increasing amounts of human HDL and 4.5 mg/ml glucose using the Seahorse XF24 analyzer as published.\textsuperscript{23}

**Results**

**Circulating HDL Is Required for Normal Glucose Homeostasis**

To understand the role of circulating HDL levels in glucose homeostasis, we used a genetic loss- and gain-of-function mouse model for apoA-I, the main protein component of HDL. Fast protein liquid chromatography (FPLC) analysis of apolipoprotein profiles revealed a severely reduced HDL particle concentration in apoA-I ko and a markedly increased HDL particle concentration in human apoA-I tg mice compared with wt controls (Figure 1A and 1B). ApoA-I ko mice exhibited increased hepatic triglyceride content compared with wt and apoA-I tg mice (Figure 1C). We detected markedly higher fasting glucose and HbA1c levels in chow-fed apoA-I ko mice compared with wt littermates (Figure 1E and F). In contrast, apoA-I tg mice had significantly lower fasting glucose levels compared with wt and apoA-I tg mice compared with wt littermates (Figure 1D). These results indicate that circulating HDL levels are important for the efficient clearance of glucose from the circulation.

To find out which tissue is responsible for HDL-mediated improvement of glucose tolerance and whether HDL modulates basal or insulin-mediated uptake of glucose, we determined deoxyglucose uptake under euglycemic-hyperinsulinemic clamp conditions (Figure 1G and Figure 1 in the online-only Data Supplement). This analysis revealed a
significant decrease of glucose transport in muscles with a high content of glycolytic (white) fibers IIB like the tibialis lateralis anterior and the extensor digitorum longus muscle of apoA-I ko mice compared with wt and apoA-I tg mice. However, no difference was observed in muscles with a high content of oxidative (red) fibers I and IIA, like the soleus, or in muscles with a mixed content of IIA and IIB fibers, like the gastrocnemius muscle. Because white fibers are far less responsive to insulin than red fibers as a result of markedly lower protein levels of glucose transporter 4 (GLUT4), the rate-limiting transporter for insulin-mediated glucose uptake,24–26 we conclude that apoA-I is modulating glucose transport in muscle directly and independently of insulin. Similar glucose transport rates in fat and liver of wt, apoA-I tg and apoA-I ko mice rule out these tissues as potential mediators for the HDL-mediated effect on glucose metabolism and point to a muscle-specific effect of HDL (Figure 1G).

Figure 1. High-density lipoprotein (HDL) modulates whole body glucose homeostasis through a direct effect on glycolytic muscle fibers: Fasting cholesterol (A) levels, lipoprotein profiles (B, HDL=fraction 40–53), hepatic triglyceride levels (C), glucose tolerance tests (D), fasting glucose levels (E), and Hba1c levels (F, n=6–15 per group); deoxyglucose uptake (G) in tibialis lateralis anterior (TLA) muscle, extensor digitorum longus (EDL) muscle, soleus (SOL) muscle, gastrocnemius (GAST) muscle, subcutaneous adipose tissue (ScAT), and liver under hyperinsulinemic-euglycemic clamp conditions (n=6–8 per group); basal and insulin-induced AKT phosphorylation in quadriceps muscle (H, n=2–3 per group), glycogen content in quadriceps (I) and liver (J) of chow fed and age-matched wt (open bars, filled circles), apolipoprotein A-I transgenic (apoA-I tg; filled bars, open squares), and apolipoprotein A-I–deficient mice (apoA-I ko; hatched bars, open triangles; n=6–14 per group). AUC indicates area under the curve; exe, exercised; and sed, sedentary. Data are expressed as means±SEM. *P<0.05; **P<0.005; ***P<0.0005 vs wt mice; #P<0.05 vs sedentary wt mice, †P<0.05 vs sedentary apoA-I tg mice, ‡P<0.005 vs sedentary apoA-I ko mice.

After insulin injection (Figure 1H). These results also point to an insulin-independent effect of HDL on skeletal muscle.

Glycogen levels are an important determinant of exercise capacity,27 and defects in glycogen synthesis have been shown to play a dominant role in the development of insulin resistance.19 To understand whether circulating HDL levels are also modulating intracellular glycogen storage, we assessed glycogen levels in skeletal muscle of sedentary and exercised wt, apoA-I tg, and apoA-I ko mice. Although muscle glycogen levels were similar between sedentary groups, exercised apoA-I ko mice failed to increase glycogen levels to the same extent as wt and apoA-I tg mice (Figure 1I). These results suggest that in absence of apoA-I, metabolic adaptations to exercise are hampered in skeletal muscle. Because liver glycogen is quantitatively more important than muscle glycogen for endurance capacity in rodents,27–30 we also determined hepatic glycogen content in our mouse groups: Sedentary apoA-I ko mice exhibited markedly increased glycogen levels compared with wt mice. However, upon training glycogen was severely reduced in apoA-I ko mice whereas it increased in exercised wt and apoA-I tg mice (Figure 1J). Our results indicate that glycogen metabolism is dysfunctional in the absence of apoA-I.
HDL Modulates Muscle Function Through an Effect on Mitochondrial Bioenergetics

We characterized body composition and muscle function in our gain- and loss-of-function models. Lean mass was significantly decreased in apoA-I ko mice (Figure 2A and 2B) compared with wt littermates and, conversely, slightly increased in apoA-I tg mice. Because loss of lean mass typically reflects altered muscle biology, we next tested for differences in muscular function by determining oxidative capacity of skeletal muscle in endurance tests using mouse treadmills. We found that reduced circulating HDL in apoA-I ko mice correlated with considerably decreased endurance capacity (Figure 2D). Our results suggest that circulating HDL and apoA-I play an important role in normal skeletal muscle metabolism and function.

To understand whether HDL and apoA-I can alter mitochondrial function in skeletal muscle in vivo, we analyzed mitochondrial bioenergetics using Seahorse analyzer. In the absence of apoA-I, oxygen consumption rate was markedly reduced and ATP synthesis was clearly blunted in mitochondria isolated from gastrocnemius muscle (Figure 2E). Elevated HDL was associated with a moderate increase in ATP synthesis (Figure 2E). This small enhancement of mitochondrial ATP synthesis was associated with increases in expression of the ATP synthase α and β subunits in muscle homogenate of apoA-I tg mice compared with wt mice (Figure 2F and 2G). In contrast, no differences in oxygen consumption rate were detected in mitochondria isolated from liver between the different genotypes (Figure II in the online-only Data Supplement). These results provide evidence that circulating HDL is required for normal mitochondrial function in skeletal muscle.

HDL and ApoA-I Directly Enhance Cellular Respiration of Glucose in Skeletal Muscle and Prevent Age-Induced Decline of Endurance Capacity

To address whether the observed effect of HDL on mitochondrial bioenergetics occurs cell autonomously in skeletal muscle, we applied cell-based in vitro assays. Using Seahorse XF24 analyzer we detected that HDL isolated from human subjects increases glycolysis and oxygen consumption rate in murine C2C12 myoblasts in a dose dependent manner (Figure 3A and 3B). Subsequently we determined that HDL and apo-A-I, but not LDL or phospholipid vesicles, enhanced glycolysis considerably (Figure 3C). Our results indicate that HDL and apoA-I directly affect the breakdown of glucose by increasing both components of cellular respiration, glycolysis and mitochondrial oxidative phosphorylation. Thus, the observed effects on cellular respiration are direct and cell autonomous, and are specific to HDL and apo-A-I.

Our observations that normal circulating HDL levels are required for proper skeletal muscle function raised the question whether increasing HDL levels above normal circulating HDL concentrations further improve skeletal muscle glucose utilization and muscle function in vivo. We therefore analyzed fasting glucose and lactate levels in mice with normal (wt) and genetically raised HDL levels (apoA-Itg). Consistent with an increase in glucose utilization, we found that physiologically relevant increases in HDL levels correlated strongly with reduced fasting glucose levels compared with wt mice (Figure 3D). HDL-induced increases in glycolysis were reflected by higher circulating lactate levels (Figure 3E). To address the question whether raising HDL levels may further improve muscular function we investigated whether age-induced decline in muscle performance is prevented in apoA-I tg mice. Endurance capacity was better maintained in aging apoA-I tg mice than wt mice (Figure 3F). Thus, HDL and apoA-I appear to play a role in the preservation of muscle function during aging by directly enhancing glucose utilization in skeletal muscle.

Raising HDL Levels Decreases Fat Mass in Association With Reduced Circulating FGF21 Levels and Enhanced Free Fatty Acid Release From White Adipose Tissue

To address whether HDL-enhanced muscular metabolism also results in a leaner phenotype, we monitored body composition of apoA-I tg and wt mice for 10 months: Age-induced increases in fat mass did not occur in apoA-I tg mice. Sedentary apoA-I tg mice retained a remarkably stable fat mass throughout their life span (Figure 4A). To understand whether the observed difference in fat mass is similar to that induced by chronic physical activity, we compared fat mass of sedentary apoA-I tg mice to that of apoA-I tg and wt mice subjected to daily aerobic exercise training throughout the study. As shown in Figure 4A, sedentary apoA-I tg mice exhibit fat mass values close to the levels of running mice. These results suggest that raising HDL levels under sedentary conditions may lead to a...
leaner phenotype that seems metabolically more similar to a physical active condition.

FGF21, a novel biomarker for mitochondrial deficiencies, is increased in patients with the metabolic syndrome and correlates inversely with circulating HDL levels in humans and monkeys. We measured FGF21 levels in sedentary apoA-I tg and wt mice as an additional indicator of improved mitochondrial function. Both hepatic expression and circulating levels of FGF21 were significantly lower in apoA-I tg mice compared with wt mice, indicating that lipolysis in white adipose tissue is enhanced. Consequently, FGF21-mediated inhibition of lipolysis may be reduced in apoA-I tg mice.

### Raising HDL Protects Against Diet-Induced Hyperglycemia Through Increased Glucose Utilization

To investigate the therapeutic potential of HDL in metabolic disease, we fed age-matched male apoA-I tg and wt mice HFD for 12 weeks and analyzed body composition and whole body glucose homeostasis. Although raising circulating HDL levels did not protect against diet-induced obesity (Figure 5A and 5B), fasting glucose levels were significantly lower in apoA-I tg mice compared with wt mice throughout the HFD feeding study (Figure 5C). This difference was even increased compared with the difference observed when mice were fed chow diet (data not shown). HbA1c levels were slightly reduced in apoA-I tg mice compared with wt mice (3.8±0.2 vs 4.2±0.08 %, n=8). Mice of either group developed fasting hyperinsulinemia to the same extent (Figure 5D). However, glucose tolerance test revealed that raising HDL levels improves the development of diet-induced impairment of glucose homeostasis (Figure 5E) independently of body-weight gain and diet-induced hyperinsulinemia.

### Discussion

The present studies establish that circulating HDL is required for normal glucose homeostasis in skeletal muscle. We show that apoA-I directly increases glucose utilization by enhancing cellular respiration in skeletal muscle cells. Furthermore, increased apoA-I, and presumably increased HDL levels, lead to reduced body fat mass and enhanced free fatty acid release by white adipose tissue via a mechanism that involves
reduction of circulating FGF21 levels. Taken together, these findings establish a key role for circulating apoA-I in maintaining muscle metabolism and function and suggest a novel role for HDL or some of its subspecies in the prevention and treatment of metabolic diseases.

Our finding that HDL and apoA-I directly enhance glucose oxidation in the skeletal muscle cell provides additional support for the hypothesis that HDL and apoA-I exert intracellular functions independent of their lipid transport function. Previously, Drew et al. reported that the actions of HDL and apoA-I on glucose uptake occur through a receptor-mediated event involving ABCA1, an ABC class of transporter. The authors suggested that, in addition to mediating reverse cholesterol transport, HDL also initiates a calcium-sensitive signaling cascade through ABCA1. Consistent with these findings, Han et al. showed that selectively extracting cholesterol from the plasma membrane using methyl beta-cyclo-dextrin had no effect on AMPK phosphorylation in C2C12 myotubes, indicating that cholesterol efflux itself does not affect AMPK phosphorylation in skeletal muscle. However, HDL and apoA-I may also mediate their effect on glucose uptake through alternative pathways to ABCA1 signaling. Accordingly, Han et al. provided in vitro evidence that endocytosis of apoA-I through the clathrin-dependent pathway is required for apoA-I-mediated AMPK and ACC activation. HDL has been also shown to transport and deliver endogenous microRNAs to recipient cells with functional gene regulatory consequences. ApoA-I may also colocalize with lipid droplets and mitochondria within the skeletal muscle cell, further strengthening the hypothesis that apoA-I acts intracellularly besides through its known surface receptors. Thus, although there is some evidence that HDL may mediate its effects on glucose uptake through one of its known receptors, it seems obvious that alternative and not yet determined underlying mechanisms are involved.

Our present study provides in vivo and in vitro evidence that HDL and apoA-I increase glucose oxidation, thus giving insight into how the cell handles HDL-enhanced glucose entry downstream of AMPK activation. Our finding that skeletal muscle ATP synthesis is severely blunted in the absence of apoA-I ex vivo in association with an impairment of endurance capacity highlights for the first time the importance of circulating HDL in normal muscle cell metabolism. Drew and colleagues previously showed that palmitate oxidation is increased in human skeletal muscle cells incubated with HDL or apoA-I. Their results complement ours by determining the effect of HDL-mediated AMPK activation on fatty acid utilization and, taken together, give a more detailed view of HDL-induced intracellular metabolic pathways. There is recent evidence that HDL particles also increase glycogen synthesis in the rat skeletal muscle cell line L6 after an overnight starvation period. In contrast, we did not detect differences in glycogen content in muscle of apoA-I ko or apoA-I tg mice compared with wt mice. We think that this discrepancy may arise as a result of different experimental conditions. Our animal experiments did not include a fasting period and may therefore better portray the state of normal intracellular glycogen content.

Our in vitro results determining that glycolysis rates in skeletal muscle are markedly increased by HDL and apoA-I were confirmed by our in vivo findings that fasting lactate levels were significantly higher in mice with genetically raised HDL and apoA-I levels. One explanation for this observation is that at the point when glycogen stores in skeletal muscle have been replenished, the glucose taken up is converted into lactate to maintain enhanced glucose utilization. The lactate released by skeletal muscle is taken up by the liver and converted into glycogen by the so-called “indirect pathway of glycogen synthesis” to preserve this energy for the future. Our findings that lactate levels are increased in apoA-I tg mice suggest that this may enhance the Cori cycle and allow lactate released by skeletal muscle to be used for oxidation but also for anabolic purposes by all tissues. According to this hypothesis, the lactate taken up would then be converted to pyruvate, a precursor for acetyl-CoA, which has many important anabolic functions. In light of our observation that apoA-I tg mice exhibit a lower age-induced decline in muscle performance compared with age-matched wt mice, we conclude that the endurance capacity may be better preserved partially through an increased flux through the Cori cycle in addition to the enhancement in glucose oxidation. Simultaneously, HDL particles also increase glucose uptake in cultured adipocytes via a mechanism involving GLUT 4 translocation and AMPK activation. Because adipose tissue releases significant amounts of lactate, our observations that fasting lactate levels were increased and fat mass was reduced in apoA-I tg mice may be in part attributable to adipocytes not completely using the glucose taken up, but instead releasing it as lactate which then serves as a substrate in the Cori cycle for anabolic processes in other tissues. This conclusion is supported by a leaner body composition of apoA-I tg mice. In contrast to data provided by Ruan et al., we show that apoA-I tg mice are not protected against diet-induced obesity. Although the initial difference in fat mass disappeared after 12 weeks on HFD, apoA-I tg mice exhibited markedly lower glucose levels and better glucose tolerance than wt mice,
indicating that raising HDL levels may offer a potential therapeutic option for obese and insulin-resistant patients who do not respond to weight-reducing measures.

Our studies furthermore may provide some intriguing insights into the inverse relationship of circulating HDL and FGF21 levels. A recent multi-center study showed that measuring circulating FGF21 concentrations reliably identified primary respiratory chain deficiencies in skeletal muscle of humans. The authors report that circulating FGF21 levels are about 10-fold increased in patients with mitochondrial disorders compared with healthy individuals, and their data suggest that FGF21 induction is triggered by primary respiratory chain deficiencies. Based on our findings presented herein we propose that FGF21 expression has been downregulated by the improved mitochondrial function in apoA-I tg mice. FGF21 is also known to function as an inhibitor of lipolysis in mice, monkeys, and human subjects. FGF21 is mainly expressed in the liver and thymus, and once secreted into circulation, it exerts its effects on adipose tissue like an endocrine hormone by binding its receptor β-klotho. Thus, our finding that FGF21 levels are reduced in apoA-I tg mice may point to an additional novel mechanism by which genetically raised HDL modulates lipolysis.

A number of intervention studies have shown that acute and long-term physical exercise have a clear beneficial effect on circulating HDL levels and on glucose tolerance in healthy subjects and in elderly, overweight, and dyslipidemic patients. Putting our findings into perspective, we propose that, besides its direct effects on glucose tolerance, physical activity may also enhance glucose oxidation indirectly by increasing HDL levels. Our rationale is supported by the finding that physical activity–induced increases in HDL levels correlate strongly with the upregulation of gene expression sets for glycolysis and oxidative phosphorylation in physically active versus inactive co-twins in a recently published Gene Set Enrichment Analysis. Although the underlying mechanisms for the association between upregulated skeletal muscle metabolic pathways and high circulating HDL levels in physically active subjects are largely unknown, it seems plausible that current therapeutic approaches to raise HDL levels may partly mimic exercise-mediated effects on glucose homeostasis.

In conclusion, our studies show strong evidence that HDL and apoA-I are required for normal glucose homeostasis and muscle mitochondrial function. We provide evidence that the key HDL component apoA-I directly increases glucose utilization by enhancing cellular respiration in skeletal muscle cells. Furthermore, we demonstrate that genetically raising HDL levels leads to a reduction in fat mass in association with a decrease in circulating FGF21 levels. Because low and dysfunctional HDL might contribute to the exacerbation of insulin resistance, therapeutic approaches to raise HDL levels and improve HDL function may not only benefit patients with cardiovascular disease but also improve metabolic diseases such as insulin resistance and T2D.

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Disclosures
None.

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Abnormal glucose metabolism, ranging from insulin resistance to type 2 diabetes mellitus, is a central feature of disorders associated with increased rates of cardiovascular disease. Successfully preventing and treating these disorders resides among the great public health challenges of our times. One of the strongest predictors of cardiovascular disease in the metabolic syndrome is a low level of high-density lipoprotein (HDL) cholesterol and its major protein constituent apolipoprotein A-I (apoA-I). Infusions of recombinant HDL raising circulating apoA-I levels reduce plasma glucose and promote glucose uptake in skeletal muscle of type 2 diabetes mellitus patients. Our series of discoveries showing that raising HDL levels by extending mitochondrial respiratory chain deficiencies: a diagnostic study. *Lancet Neurol.* 2011;10:806–818.


CLINICAL PERSPECTIVE

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Supplemental Material

Supplemental Methods:

Mice

Age-matched male apoA-I deficient (apoA-I ko), human apoA-I transgenic (apoA-I tg), and control (wt) C57/Bl6J mice (The Jackson Laboratories, Bar Harbor, ME) were housed in specific pathogen–free facilities with a 12-hour light/12-hour dark cycle and were fed 1 basal rodent chow 5058 PicoLab Mouse Diet 20 (LabDiet, Richmond, IN). Mice that underwent the diet-induced obesity study were fed a low fat diet containing 4.8% fat by weight (D12328; Research Diets, New Brunswick, NJ) or a high-fat diet containing 35.8% fat by weight (D12330; Research Diets) for 12 weeks. All experimental procedures conformed to institutional guidelines for animal experiments and were approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Cincinnati. Body fat mass was measured in conscious mice using 1H magnetic resonance spectroscopy (EchoMRI-100; Echo- medical Systems). Body weight and body composition measurements were performed monthly.

For the exercise study, mice were accustomed to treadmill running for 3 days as described previously and then an exercise exhaustion test was performed in all mice. Next, mice were randomized for body weight and divided into two groups. The ‘runner’ group was subjected to treadmill running (Simplex II metabolic rodent treadmill, Columbus Instruments, Columbus, OH) during the first 4 h of the dark phase of the circadian cycle, five times a week. During the 12 months of training time (30 – 45 min), speed (5 – 7 m/min), and treadmill inclination (10 - 20°) of exercise was adjusted according to the fitness level resulting in a covered distance of 200 - 315 m/day. The ‘sedentary’ group was not forced to run except during exercise exhaustion tests. Exhaustion tests were performed during the first four hours in the dark phase of the circadian cycle at weeks 0.
and 8 of the study as described previously. Briefly, animals ran on the treadmill tilted 20° uphill starting at a warm-up speed of 5 m/min for 4 min after which speed was increased to 14 m/min for 2 min. Every subsequent 2 min, the speed was increased by 2 m/min until mice were exhausted. Exhaustion was defined as the inability of the animal to return to running within 10 s after direct contact with an electric-stimulus grid and/or terminal glucose levels below 100 mg/dl. Running time was measured and running distance calculated. Distance is the product of time and speed of the treadmill. Outside of the training schedule, all mice had unlimited access to food and water.

For the determination of downstream insulin-signaling target AKT we fasted mice for 2 hours prior to intraperitoneal injection of insulin (2 IU/kg body weight) or saline (equal volumes). 5 minutes after injection, mice were sacrificed by cervical dislocation, liver and quadiceps muscles were immediately harvested and snap frozen in liquid nitrogen for later determination of AKT phosphorylation.

**Euglycemic-hyperinsulinemic clamp studies**

Mice underwent vascular surgery under anesthesia with Isoflurane (1,5% ; Air : 0,4l/min) at least 6 days before the experiment. The right jugular vein was catheterized for infusion with a silastic catheter. The free end of the catheter was tunneled under the skin to the back of the neck. Catheters are flushed daily with ~50µl of 0,9% NaCl containing 5mg/ml ampicillin and 20U.I/ml heparin. After surgery animals were individually housed and their body weight was monitored daily. Mice that lost more than 10% of presurgery weight by postsurgery day 6 were excluded from the study. At the day of the clamp study, conscious mice were place in their home cage for the duration of the clamp experiment and food-deprivated for 5 hours. After a bolus infusion of (5 µCi) of D-[3-3H] glucose (Perkin Elmer, Courtaboeuf, France) tracer solution and 80 mU/kg insulin, the tracer was infused
continuously (0.05 μCi/min, at a constant rate of 1μl/min) for the duration of the experiment and insulin infusion was kept constant at 0.2UI/kg/h (3.33mU/kg/min). Blood glucose levels were determined from tail blood samples (1-2 μl) at t=0 and then every 20 min using the glucose analyzer Glucofix® (A. Menarini, Rungis, France). Steady state was ascertained when glucose measurements were constant for at least 20 min at a fixed glucose infusion rate and this was achieved within 50 to 80min. At steady state, 3 blood sample (10μl) were collected for determination of basal parameters, followed by a bolus injection of 2-deoxy-D-[1-14C] glucose (2DG) (3 μCi, Perkin Elmer, Courtaboeuf, France). Blood samples (10 μl) were collected from the tail at 0, 10, 20, 30, 40, 60 min until the end of the experiment where mice were sacrificed by elongation and tissues were collected. Basal and steady state plasma [3-3H] glucose radioactivity were measured as described \(^2\). Tissue Glucose turnover rate (mg/kg/min) were calculated as described \(^2\). In vivo glucose uptake (mg/mg of tissue/min) for muscle (Tibialis Anterior, Soleus, Gastrocnemius, Extensor Digitorum Longus), subcutaneous adipose tissue, and liver were calculated based on the accumulation of 2DG6P in the respective tissue and the disappearance rate of 2DG from plasma as described \(^2\). For [3-3H] glucose determination, plasma was deproteinized with Ba(OH)2 and ZnSO4. For each sample, an aliquot of the supernatant was dried to remove 3H20. Immunoreactive insulin was determined by Elisa kit from Crystal Chem (Crystal Chem, Chicago, IL). Rate of glucose appearance (Ra) and rate of glucose dissappearance (Rd) is determined at steady state. Endogenous glucose production (endoGP, given as g/kg/min) is determined by subtracting the glucose infusion rate (GIR; rate of cold glucose required to sustain glycemia) from total Ra.

Biochemical Assays
Animals were fasted for 6 hours prior to blood collection from the tail vein. Plasma cholesterol levels were determined using the Infinity Cholesterol Kit (Thermo Scientific Inc, Rockford, IL). Plasma FFA levels were determined using the NEFA HR (2) kit (Wako Chemicals USA, Inc., Richmond, VA). Plasma insulin levels were measured with the Ultra Sensitive Rat Insulin ELISA kit (Crystal Chem, Chicago, IL) using rat insulin as the standard. Plasma lactate levels were determined with the lactometer Lactate Pro (ARKRAY Inc., Kyoto, Japan). Intraperitoneal glucose tolerance test (ipGTT) was performed by injection of glucose (2 g/kg, 50% wt/vol. d-glucose [Sigma, St Louis, MO] in 0.9% wt/vol. NaCl) after a 5-h fast as described previously. Tail blood glucose levels [mg/dl] were measured with glucometer TheraSense Freestyle (Abbott Diabetes Care, Inc., Alameda, CA) before (0 min) and at 15, 30, 45, 60 and 120 min after injection.

Glycated hemoglobin was measured in whole blood using the mouse hemoglobin A1c (HbA1c) kit from Crystal Chem (Crystal Chem, Chicago, IL). Fibroblast growth factor (FGF21) levels were measured with the rat/mouse FGF21 ELISA kit (EMD Millipore Corporation, Billerica, MA) after an overnight fast of 15 hours. Samples were analyzed individually except for lipoprotein separation in which pooled samples were subjected to fast-performance liquid chromatography (FPLC) as described previously.

Glycogen content in snap-frozen quadriceps muscles of ad libitum fed mice was measured with the glycogen assay kit from Abcam (Abcam plc, Cambridge, UK) according to the manufacturer’s instructions. Liver glycogen content was determined with aminoglucosidase according to the method of Passoneau J. et al. Briefly, tissues were harvested and snap-frozen in liquid nitrogen and stored at -80C. 30 mg of powderized liver were homogenized in 1ml of ice-cold 0.3 M percloric acid. After centrifugation for 15 min at 3,000g, 50 ul of the homogenate was incubated with 500 ul of 50 mM sodium acetate containing 50ug/ml aminoglucosidase with shaking at room temperature for 2 hours. After centrifugation for 15 min at 3000g, 20 ul of samples were used for the determination of
glucose with the LabAssay Glucose kit from Wako (Wako Chemicals USA, Inc., Richmond, VA).

Liver lipid content of ad libitum fed mice was assessed by extraction as described previously \(^5\). In summary, 50 mg of snap-frozen liver was homogenated with 1 ml chloroform. Lipids were extracted by overnight shaking at room temperature. For phase separation, 700 ul of ddH2O were added, samples were then centrifuged at 2000 rpm for 20 min at 4\(^\circ\)C and organic layer was collected. For second extraction 700 ul chloroform/methanol (2:1 vol/vol) was added to the remaining homogenate and lipid extraction, phase separation and organic layer collection were performed as described above. 15 ul of both extractions were then transferred to glass tubes, evaporated and measured with Infinity Triglyceride Kit (Thermo Scientific Inc, Rockford, IL).

**Respiration studies in isolated skeletal muscle mitochondria and cultured muscle cells.**

After sacrifice, gastrocnemius muscles were excised and mitochondria were isolated immediately as described previously \(^6\). For isolated muscle mitochondria all respiration measurements were made in triplicate and followed this protocol: resting respiration (state II, absence of adenylates) was assessed by the addition of 5 mM glutamate and 2 mM malate as the complex I supply. State III respiration was assessed by the addition of 1 mM ADP and subsequent state IV (uncoupling respiration) was determined by adding 2 uM of oligomycin. The integrity of the outer mitochondrial membrane was established by the addition of 10 uM cytochrome c. Measurements of oxygen consumption rate (OCR) by the Seahorse 24XF analyzer (Seahorse Biosciences Inc., North Billerica, MA) were completed within a 8- to 12-min period.
To investigate whether HDL directly enhances glycolysis via the extracellular acidification rate (ECAR) and/or mitochondrial oxygen consumption rate (OCR), murine skeletal muscle C2C12 cells were incubated for 4 hours with increasing amounts of human HDL and 4.5 mg/ml glucose utilizing the Seahorse XF24 analyzer as described previously. 7. To determine whether the observed effect on glycolysis is specifically mediated by HDL and/or its major protein constituent apoA-I, we incubated C2C12 cells with glucose (4.5 mg/ml) and with or without HDL, LDL, phospholipid vesicles (all at the same phospholipid concentration 100 ug/ml), and apoA-I (which was used at the same protein concentration as for HDL, 50 ug/ml) for 4 hours prior to determining glycolysis (ECAR). HDL, LDL, phospholipid vesicles and human apoA-I were prepared as described previously.

**Immuno blot analysis:**

Whole gastrocnemius and quadriceps homogenates in lysis buffer from Pierce (Thermo Scientific Inc, Rockford, IL) containing protease and phosphates inhibitors were prepared with tissue lyzer from Qiagen (Qiagen Inc, Valencia, CA) and protein concentration was determined by BCA assay from Pierce. 80 ug of protein content from each sample were loaded on precast SDS-Tris gels from Pierce and separated by SDS-PAGE, transferred to polyvinylidene fluoride paper (EMD Millipore Corporation, Billerica, MA), and blotted with antibodies against the subunit a and b of the ATP synthase (Mitosciences, Eugene, OR), or with antibodies against all AKT isoforms (pan AKT) and against AKT phosphorylated around the Thr308 phosphorylation site (Cell Signaling Technology, Inc, Danvers, MA). Immunoreactive proteins were detected by incubating the blots with flourescently labeled species-specific secondary antibodies (Molecular Probes; Life Technologies, Grand Island, NY) and visualized by the Odyssee Infrared Imaging System (Li-Cor Biosciences, Eugene, NE) as described previously.
**Statistical analysis:**

Unpaired student-t test with two-tailed P values was used for analysis of 1-group variables and one-way ANOVA was used for comparison between 3 or more groups followed by Bonferroni or Dunnet’s post hoc tests. Genotype differences in glycogen levels between wt mice, apoA-I tg and apoA-I ko mice within one activity level of the two possible conditions (sed or exe) were analysed by one-way ANOVA followed by Bonferroni’s multiple comparisons tests (Fig 1I and J). Multiplicity adjusted P values were calculated with 3 comparisons for figure 1I and J per family and a family-wise significance and confidence level of 0.05. The effect of exercise on glycogen levels (Fig 1I and J) within one genotype of mice (e.g. sed wt mice vs exe wt mice) was determined by unpaired student t test with two-tailed P values. The effect of HDL and apoA-I on ECAR and OCR (Figure 3 A, B and C) was analysed using one-way ANOVA followed by Dunnett’s Multiple Comparison Test comparing cells incubated with glucose only (0+) vs. cells incubated with glucose and HDL, LDL, PL, and ApoA-I. Multiplicity adjusted P values were calculated with 6 comparisons for figure 3A and B and 4 comparisons for figure 3C per family and a family-wise significance and confidence level of 0.05. All data presented are raw values and are expressed as mean ± SEM and the 0.05 level of probability was accepted to indicate statistical significance. Statistical analyses were performed with GraphPad Prism version 6.0.

**Supplemental References:**


Supplemental Figures:

Figure 1S: Hepatic mitochondrial function is not altered by circulating HDL levels.

Oxygen consumption rate (OCR) in mitochondria isolated from liver in basal state (State II), after addition of ADP (State III), and after addition of the ATP synthase inhibitor oligomycin (State IVo) of chow fed wt (open bars), apoA-I tg (filled bars) and apoA-I ko mice (hatched bars) (n = 4). Data are expressed as means ± SEM.
**Figure 2S: Circulating HDL levels do not modulate insulin sensitivity:** Absolute glucose levels (A) and change in glucose levels during basal clamp period (B), glucose infusion rates (GIR) (C) and hepatic glucose production (Endo GP) (D) during hyperinsulinemic-euglycemic clamp studies of chow fed and age-matched male wt (open bars, filled circles), apoA-I tg (filled bars; open squares) and apoA-I ko mice (hatched bars; open triangles) (n= 6-8). Data are expressed as means ± SEM ***P < 0.0005.