Cholesterol uptake into heart and skeletal muscle of lipoprotein lipase transgenic mice:

Evidence that statin therapy increases muscle lipid uptake

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Nonstandard abbreviations: triglyceride, TG; free fatty acids, FFA; LpL, lipoprotein lipase; cardiomyocyte anchored LpL, LpL⁶⁴; creatine phosphokinase, CPK; hydroxymethyl glutaryl CoA reductase, HMG-CoA-R; tyramine cellobiose, TC; low density lipoprotein receptor knockout, Ldlr⁻/⁻
Abstract

Regulation of cholesterol metabolism in cultured cells and in the liver is dependent on actions of the LDL receptor. However, non-hepatic tissues have multiple pathways of cholesterol uptake. One possible pathway is mediated by lipoprotein lipase (LpL), an enzyme that primarily hydrolyzes plasma triglyceride into fatty acids (FAs). In this study, LDL uptake and tissue cholesterol levels in hearts and skeletal muscles of wild type and transgenic mice with alterations in LpL expression was assessed. Overexpression of a myocyte-anchored form of LpL in heart muscle led to increased uptake of LDL and greater heart cholesterol levels. Loss of LDL receptors did not alter LDL uptake into heart or skeletal muscle. To induce LDL receptors, mice were treated with simvastatin. Statin treatment increased LDL receptor expression and LDL uptake by liver and skeletal, but not heart muscle. Plasma creatinine phosphokinase, and muscle mitochondria, cholesterol, and lipid droplet levels were increased in statin-treated mice overexpressing LpL in skeletal muscle. Thus, pathways affecting cholesterol balance in heart and skeletal muscle differ.

KEYWORDS: myopathy, hypercholesterolemia, statin
Introduction

Heart and skeletal muscle are among the lowest cholesterol biosynthetic tissues of the body (1) and, like for many tissues, circulating lipoproteins probably supply muscle cholesterol needs. Although this could occur via LDL receptor uptake, a curious aspect of regulation of the fibroblast LDL receptor is that the receptor is half maximally saturated by sub-physiologic levels (30 μg/ml) of LDL cholesterol (2). Thus, if the fibroblast is representative of muscles, the LDL receptor should be down-regulated and an alternative process must lead to acquisition of plasma LDL. Such a conclusion is consistent with studies showing that heart and skeletal muscle take up very little LDL from the circulation (3, 4).

There are likely to be other pathways mediating cellular cholesterol uptake by heart and skeletal muscle. These pathways could involve uptake of cholesterol from lipoproteins other than LDL or could include selective uptake of LDL cholesterol, i.e. acquisition of lipid exclusive of whole particles. In the case of HDL, selective uptake of lipoprotein lipids occurs via scavenger receptors (5). Selective uptake of cholesterol from LDL can be mediated by lipoprotein lipase (LpL) (6, 7), the primary enzyme responsible for intravascular hydrolysis of triglyceride. This process might be especially important in skeletal and heart muscle that have robust LpL expression.

Cells must modulate cholesterol content to prevent lipid intoxication. The liver eliminates excess cholesterol into the bile; adipose tissue can store excess cholesterol within lipid droplets. Muscles might need to more finely regulate cholesterol uptake. Inappropriate upregulation of LDL receptors leading to excess cellular cholesterol could be pathologic (8). Similarly, receptor mediated
increased muscle uptake of lipoproteins could lead to potentially toxic levels of phospholipids, triglycerides (TGs), and fatty acids (FAs).

Previously, we created mice that develop a dilated cardiomyopathy and excess cholesterol in the heart (9). These mice express a transgene for a cardiomyocyte-anchored form of human lipoprotein lipase (LpL); the mice are denoted LpL\textsuperscript{GPI} because the LpL has a glycosylphosphatidyl-inositol anchor. LpL is the rate-limiting enzyme responsible for plasma TG metabolism, but not LDL cholesterol metabolism. In the current study, we assessed whether expression of LpL and the LpL\textsuperscript{GPI} transgene altered LDL uptake into heart and skeletal muscles. The LpL\textsuperscript{GPI} transgene increased LDL uptake into the heart, but this was exclusive of the presence of LDL receptors. When mice were treated with high doses of a hydroxyl methyl glutaryl CoA reductase (HMG-CoA-R) inhibitor (statin), LDL receptor expression increased in skeletal but not heart muscle. In the presence of excess muscle LpL, statin treatment caused muscle toxicity.
Methods

Materials: Simvastatin as Zocor® (Merck, Whitehouse Station, NJ) was obtained in tablet form. The tablets were powdered and mixed with normal chow diet that contained 5.0% (w/w) fat (Research Diets, Inc, New Brunswick, NJ). The final concentration of simvastatin in the mixtures was 0.1% of the diet.

Animals and experimental treatments: LpLGPI transgenic mice (9) and muscle creatinine kinase (MCK)-LpL mice (10) have been described. LDL receptor knockout (Ldlr/-) mice on the C57BL/6 background mice were purchased from Jackson Laboratory and the MCK-LpL and LpLGPI mice were bred >6 generations onto this background. MCK-LpL mice were crossbred with Ldlr/- mice to obtain MCK-LpL/Ldlr/- mice. As was noted on the wild type background (10), the MCK-LpL transgene increased muscle TG (from 1-6 μg/mg) and fatty acids (from 0.25-0.4 nmol/mg) but led to smaller differences in tissue cholesterol in a small number of Ldlr/- mice. All mice were housed in a temperature-controlled (25°C) facility with a 12-h light/dark cycle.

For statin diet treatment, wild-type C57BL/6, Ldlr/-, MCK-LpL and MCK-LpL/Ldlr/-, 10-12 week old male mice were fed for 2 weeks with either the control chow diet or a diet that contained 0.1% simvastatin. Blood from fasted (24h) mice was collected from the retro-orbital plexuses into tubes containing EDTA. Enzymatic kits were used to determine plasma TG and total cholesterol (TC) (Thermo Electron Co., Ontario), free fatty acid (FFA) (Wako Chemicals USA, Richmond, VA), glucose (LIFESCAN Inc., Milpitas, CA), and CPK (Olympus Diagnostica GmbH, Germany) levels, all in duplicate (11).
**Northern blot analysis:** Total RNA (10 μg) was isolated from liver, heart and skeletal muscle using TRIzol reagent (Invitrogen, Carlsbad, CA), subjected to electrophoresis in a 1% agarose gel containing formamide and transferred to nylon filters (Hyboud N; Amersham, Piscataway, NJ). Northern blot analyses were performed using the radiolabeled cDNA probes for atrial natriuretic factor (ANF), brain natriuretic protein (BNP), LDL receptor and HMG-CoA-R. The data were normalized to the expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The blots were quantified using densitometric scanning.

**Real-Time Reverse Transcription PCR:** Sterol regulatory element binding protein 2 (Srebp2), HMG-CoA-R, LDL receptor (Ldlr), cyclophilin and β-actin mRNA levels were determined by real time reverse transcription PCR. RNA integrity was checked by ethidium bromide staining after electrophoresis. cDNA was produced from total RNA (1 μg) via reverse transcription using Superscript reverse transcriptase (Invitrogen) in a 20 μl reaction volume containing 1x Superscript buffer (Invitrogen), 1 mM dNTP, 20 ng of random hexamers, 10 mM dithiothreitol, and 20 units of RNase inhibitors. After 60 min at 55°C incubation, the reaction was stopped (10 min at 85°C). Ldlr, Srebp2, HMG CoA-R, cyclophilin and β-actin were amplified by PCR using Syber PCR master mix (Applied Biosystems, UK) and the following primer sequences: Ldlr (forward, 5'-GAAGTCGACACTGTACTGACCACCACC-3'; reverse 5'-CTCCTCATCCTCTGACCACCACC-3'), Srebp2 (forward, 5'-AGGTATAACCCCAGTATCC-3'; reverse, 5'-GATACCAGATTGTTTGG-3'), HMG-CoA-R (forward, 5'-CGCAACCTATATCCGT-3'; reverse, 5'-
GTAGCCGCTATGCTC-3'), cyclophilin (forward, 5'-ATGTGCCAGGGTGGTGACTT-3'; reverse 5'-GCCATCCAGGCCATTCTC-3'), β-actin (forward, 5'-TGAAGTGACGTGGACA-3'; reverse 5'-TAGAAGCACTTGCCGTGCACG-3'). Optimized PCR consisted of 40 cycles of amplification at 95°C for 15s followed by amplification at 60°C for 1 min. Significant PCR fluorescent signals were normalized for each sample to PCR fluorescent signals obtained from two independent controls, cyclophilin and β-actin genes.

**Electron microscopy:** Electron microscopy was performed on skeletal muscle (quadriiceps femoris) fixed with 2.5% glutaraldehyde in 0.1M Sorensen’s buffer (0.2M monobasic phosphate/0.2M dibasic phosphate 1:4 v/v, pH 7.2), post-fixed in osmium tetroxide, and embedded in Epon 812. Ultra-thin sections were stained with uranyl acetate and lead citrate and examined with a JEM-1200ExII electron microscope (JEOL Ltd., Tokyo, Japan).

**Lipoprotein uptake studies:** LDL were isolated from Ldlr-/- mice (n=25) by sequential ultracentrifugation at 1.025<d<1.063 for 30h. Immediately after isolation, LDL were labeled with 125I-labeled-tyramine cellobiose (TC)(35). 125I-TC was prepared using 1,3,4,6-tetrachloro-3α, 6α-diphenylglycouril (Iodogen)(Pierce Biotechnology Inc., Rockford, IL), and linked to LDL by activating the iodinated TC with cyanuric chloride as described previously (35). Labeled LDL were dialyzed extensively against saline containing 1 mg/ml EDTA, sterilized by filtration through an Acrodisc (0.45 μm), stored at 4°C, and used within 1 week. One hundred μg of 125I-TC-LDL (120 cpm/ng protein) was injected via saphenous vein. The mice remained fasting during the 20h study. At the end of the
experiment, the mice were anesthetized, exsanguinated, and then perfused with 10 ml of phosphate buffered-saline (PBS) containing 1 mg/ml EDTA. Isolated organs were quickly rinsed with ice-cold PBS, weighed, and radiiodine was quantified by an automatic gamma counter (WALLAC, 1470 wizard, Perkin-Elmer). To normalize for plasma pool size and the slower loss of labeled LDL from the circulation in \( Ldlr^{-/-} \) mice, \(^{125}\)I-TC-LDL uptake was corrected using the plasma decay curves.

**Tissue lipid analysis:** Hearts, liver and skeletal muscle were rapidly removed and homogenized in ice-cold 1M NaCl buffer containing protease inhibitors to prevent TG hydrolysis. Lipids were extracted from these tissues (50 mg) according to methods modified from that of Folch et al. (12). Briefly, snap frozen tissues were homogenized and extracted twice with chloroform/methanol (2:1 v/v) solution. The organic phase was dried under nitrogen gas and resolubilized in chloroform. An aliquot of the Folch extraction was resuspended in an aqueous solution containing 2% Triton X-100. Total and free cholesterol, TG and FFA levels were determined by enzymatic kits as described (35).

**Statistical analysis:** Student t-tests of group means were used to compare groups with a statistical significance at the level of \( p<0.05 \). Because of the variability of uptake, initial studies of TC-LDL uptake performed in statin-treated wild-type mice did not reach statistical significance and the experiment was repeated and the data were merged. Analysis of variance (ANOVA) was used for comparisons among different groups and to evaluate potential interactions between different groups.
Effects of LDL receptor deficiency on cardiac lipids and LDL uptake in LpL<sup>GPI</sup> mice: Mice with or without the LpL<sup>GPI</sup> transgene had identical plasma clearance of <sup>125</sup>I-TC-LDL (Figure 1A). As expected, liver uptake of LDL was not affected by the LpL<sup>GPI</sup> transgene (Figure 1B). However, the LpL<sup>GPI</sup> transgene doubled LDL uptake into the heart (Figure 1C).

LpL is not normally considered a major mediator of cellular cholesterol uptake. However, LpL can concentrate lipoproteins on the cell surface and increase their proximity to lipoprotein receptors. To test whether this process was occurring, we crossed the LpL<sup>GPI</sup> transgene onto the Ldlr<sup>-/-</sup> background. Ldlr<sup>-/-</sup> mice had 3.6-fold greater levels of plasma cholesterol than did wild-type mice (Table 1). The LpL<sup>GPI</sup> transgene did not alter plasma lipids when crossed onto the wild-type or Ldlr<sup>-/-</sup> background. However, Ldlr<sup>-/-</sup>/LpL<sup>GPI</sup> hearts had 70% more cardiac cholesterol and 20% more FFA than did Ldlr<sup>-/-</sup> mice (Table 1). Ldlr<sup>-/-</sup> mice, as expected, had delayed plasma LDL turnover (Figure 1A) and reduced liver LDL uptake (Figure 1B). However, loss of LDL receptors did not alter cardiac LDL uptake. Hearts from Ldlr<sup>-/-</sup>/LpL<sup>GPI</sup> mice still acquired twice as much TC-labeled LDL as did Ldlr<sup>-/-</sup> hearts (Figure 1C).

Heart failure markers ANF and BNP were not reduced in Ldlr<sup>-/-</sup>/LpL<sup>GPI</sup> hearts compared to LpL<sup>GPI</sup> hearts. Moreover, loss of the LDL receptor did not affect the mortality of the LpL<sup>GPI</sup> mice (Figure 2).

There are pathways other than via the LDL receptor whereby LpL could increase cellular uptake of LDL. LpL allows lipoproteins to associate with cell membrane proteoglycans and the lipoproteins can be internalized along with
recycling of the cell membrane or with other members of the LDL receptor superfamily (13). Our data demonstrate that cardiomyocyte cell surface LpL did not utilize the LDL receptor to augment LDL uptake.

**Effects of loss of LDL receptor on skeletal muscle uptake of TC-LDL:** It is possible that metabolism of cholesterol in the heart differs from cholesterol metabolism in other muscles. For this reason, we also determined the role of the LDL receptor in LDL uptake by skeletal muscle. Like for the heart, loss of LDL receptors had little effect on LDL uptake by skeletal muscle (**Figure 1D**).

**Effects of statins on muscle LDL uptake:** We tested whether increasing LDL receptors in the skeletal muscle would alter LDL cholesterol uptake. HMG-CoA-R inhibitors (statins) block the rate-limiting enzyme for cholesterol biosynthesis. By initially reducing cellular cholesterol, they lead to activation of SREBP2 and increase expression of genes within the cholesterol biosynthetic pathway and the expression of the LDL receptor (14). This latter action allows greater LDL uptake by the liver and lowers circulating concentrations of LDL (15).

We treated both wild-type and MCK-LpL mice with high doses of simvastatin. As has been seen by others, average plasma cholesterol was not significantly reduced by statin in wild-type mice (**Table 2**). Liver uptake of tracer TC-LDL was increased (**Figure 3A**). Heart LDL uptake was not altered by the statin (**Figure 3B**). In contrast, skeletal muscle LDL uptake increased in wild-type mice by ~30% from untreated controls (**Figure 3C**). Although statins block the rate-limiting enzyme required for cholesterol biosynthesis, muscle cholesterol was not decreased by this therapy (**Figure 3D**). These data are consistent with studies showing that muscles have little de novo cholesterol biosynthesis (1).
**Statin treatment increased LDL uptake in MCK-LpL mice:** Mice with gross overexpression of LpL in muscle via the MCK promoter develop a myopathy that is thought to reflect increased fatty acid flux into the tissue (10). Animals with less flagrant LpL overexpression appear normal, although the muscles of these mice accumulate more TG than do wild-type mice, and have some degree of insulin resistance (16, 17). Selective uptake of LDL cholesterol, i.e., uptake of cholesterol without the apoB representative of holo-LDL, is increased in skeletal muscle of MCK-LpL mice, however, holoparticle uptake is not (6). In our studies, the presence of the MCK-LpL transgene alone did not alter plasma turnover of TC-LDL. A statistically significant increase in LDL particle uptake into skeletal muscle was also not found (**Figure 3C**). Statin treatment increased LDL uptake in skeletal but not heart muscle of MCK-LpL mice (**Figures 3B and C**). Mice expressing the MCK-LpL transgene had increased muscle free and total cholesterol levels (**Figure 3D**). Statin treatment further increased muscle cholesterol content in these mice. Muscle LpL activity was not altered by statin treatment (data not shown).

**Regulation of LDL receptors in heart and skeletal muscle:** The major pharmacologic effect of statins in humans is an increase in clearance of LDL from the blood (15). We explored whether statin therapy induced LDL receptors in the liver, as expected, and in muscles of the treated mice. Both Ldlr and HMG-CoA-R mRNA levels were increased in the liver. Statin treatment also increased skeletal muscle Ldlr expression 2 fold; HMG-CoA-R mRNA increased 1.5 and 1.7 fold in wild-type and MCK-LpL mice, respectively (**Figure 4A and quantification**).
shown in Supplementary Figure 1). In contrast, expression of these two genes in hearts was unaltered.

**Skeletal muscle changes with statins:** Although statins were developed as cholesterol biosynthetic inhibitors, our data showed that statin treatment could actually increase muscle cholesterol in some strains of mice. We determined whether greater tissue cholesterol had altered the muscles of statin-treated mice. Control mice showed some increase in mitochondria after statin treatment (Figure 4B). Mitochondrial accumulation in the subsarcolemmal region was increased in non-treated MCK-LpL muscle, however, the increase was greatest in muscle from statin-treated MCK-LpL mice (Figure 4B). Statin treated MCK-LpL mice also had more and larger lipid droplets around the perinuclear region than those in muscles of untreated mice and statin-treated wild-type mice (Figures 4B and C). Further evidence of muscle damage was obtained by measuring plasma CPK; CPK increased 3.8-fold in statin-treated MCK-LpL mice (Figure 4D). *Every mouse* in this group had a greater plasma CPK level than that of untreated or non-transgenic mice.

**Statin effects in MCK-LpL/Ldlr-/- mice:** If statin-induced muscle toxicity results from excess lipoprotein uptake via stimulation of the LDL receptor pathway, then genetic loss of the LDL receptor should prevent this effect. To test this hypothesis we statin treated MCK-LpL/Ldlr-/- mice. Loss of the LDL receptor prevented the statin-mediated changes; muscle LDL uptake, muscle cholesterol levels, and plasma CPK elevation were unaltered (Supplementary Figures 2 A-C). Although it is most logical to explain the lack of toxicity in the Ldlr-/- mice as a result of the drug’s failure to induce excess lipoprotein uptake, these mice have
other metabolic alterations such as hyperlipidemia that might, by unknown mechanisms, have also prevented the statin myopathy.
Discussion

We studied the importance of LpL and its regulation on LDL uptake in cardiac and skeletal muscle using animals with tissue specific expression of LpL. Our data show the following: 1) LpL on the cardiomyocyte surface increased heart uptake of LDL. 2) Under control situations the LDL receptor had little effect on LDL uptake into muscle or heart. 3) With high doses of statin, LDL receptors were upregulated in the skeletal muscle and this was associated with increased LDL uptake. 4) Mice that had increased expression of muscle LpL had more muscle cholesterol, lipid droplets and mitochondria. Plasma CPK was elevated in these mice, indicating muscle damage. 5) This effect was not seen when LDL receptors were deleted. 6) Heart uptake of LDL was unaffected by loss of LDL receptors or statin treatment.

We had previously created a mouse model of lipotoxic cardiomyopathy that was associated with increased uptake of plasma lipoproteins (9). The most significantly increased lipid was cholesterol. Thus, we tested whether the transgene expressing cardiomyocyte-anchored LpL increased heart uptake of LDL. It did! This suggested that LpL on the cardiomyocyte surface could be an important mediator of heart cholesterol uptake. Surprisingly, the cardiomyopathy was not improved by crossing the LpL\textsuperscript{GPI} mice with Ldlr\textsuperscript{-/-} animals, i.e. loss of LDL receptors did not reduce uptake of plasma LDL. Therefore, heart uptake of LDL in LpL\textsuperscript{GPI} mice was exclusive of LDL receptors.

The heart is a major site of plasma lipoprotein metabolism; loss of only cardiac LpL leads to fasting and postprandial hyperlipidemia in mice (18) and cardiac specific LpL expression is sufficient to maintain normal plasma lipid
levels (19). Normally hearts acquire very little cholesterol from LDL. The heart does acquire cholesterol from chylomicron remnants via a pathway that is exclusive of LpL (20). Whether this process requires scavenger receptors, other members of the LDL receptor superfamily, or some novel process has not been determined.

As we had observed for the heart, LDL receptor expression did not alter muscle LDL uptake. This observation is consistent with other studies showing that LDL receptor mediated LDL uptake is lower for muscle than for any other tissue of the body (3). LpL overexpression in muscle did not lead to a significant increase in LDL protein uptake. However, skeletal muscle selective uptake of LDL cholesterol was increased in MCK-LpL mice (6).

We should note that in our studies the forms of LpL transgenes expressed in the heart and skeletal muscle differed: LpL\textsuperscript{GPI} was anchored to the myocyte surface, while non-mutated human LpL was expressed by skeletal muscle. In addition, the relative amounts of expression differed between these transgenes; MCK-LpL leads to an approximately 8-fold increase in muscle heparin-releasable activity (10), while the LpL\textsuperscript{GPI} transgene increased cardiac LpL activity ~3-fold (9). We suspect that the parenchymal cell-associated form of LpL is most effective for holo-LDL uptake. The mechanisms responsible for LpL-mediated uptake of LDL have been studied in cell culture and may involve LpL serving as a receptor ligand, LpL concentration of lipoproteins near classical lipoprotein receptors, and/or LpL associating with lipoproteins and mediating internalization along with the turnover of cell surface proteoglycans (21).
One might have expected that the MCK-LpL transgene would downregulate skeletal muscle LDL receptors. However, the uptake of cholesterol via muscle expression of LpL leads to only a small increase in basal cholesterol that may “prime” the muscle to the statin-mediated process, but not greatly alter intracellular metabolism. There are several reasons why the small change in muscle cholesterol may be insufficient to downregulate basal LDL receptor expression: 1) The change in cellular cholesterol concentration may be too small. 2) Acquisition of cholesterol via selective uptake of LDL cholesterol rather than receptor mediated endocytosis of holo-LDL might not be equivalent. 3) Skeletal muscle LDL receptors may be maximally downregulated and the residual expression controlled by other factors such as hormones (e.g., insulin and sex hormones), growth factors and peroxisomal proliferating activator receptor-gamma (22-25).

Another method to alter LDL receptor expression is via treatment of animals with statins. These drugs inhibit HMG-CoA-R, initially reduce cellular cholesterol, and then increase SREBP transfer to the nucleus leading to increased expression of genes for cholesterol biosynthesis and LDL receptors (26, 27). As expected, mice treated with simvastatin had increased LDL receptor expression in the liver and greater uptake of plasma LDL. Skeletal muscle in mice treated with simvastatin also had greater expression of LDL receptors and more LDL uptake than did skeletal muscle in untreated mice. Overall, muscle cholesterol content in wild-type mice was unchanged with statin treatment.

MCK-LpL mice with high level LpL expression develop myopathy even without addition of statins (10). This had been presumed to occur as a result of
excess uptake of fatty acids. The lower level MCK-LpL expressing mice used in this study did not have obvious muscle pathology. As shown in Figure 4B, they did have more lipid droplets in skeletal muscle. However, plasma CPK was not increased.

Statin-treated wild-type mice, like most humans, did not experience adverse effects due to muscle upregulation of LDL uptake. However, statin-treated MCK-LpL mice had significantly increased muscle cholesterol concentrations. Electron microscopy of muscles from statin-treated MCK-LpL mice showed increased mitochondria and lipid droplets and plasma CPK levels were increased. Although this muscle toxicity could have resulted from greater tissue cholesterol levels, increased LDL receptors also mediate uptake of lipoproteins expressing apoE. Thus, in addition to cholesterol, muscles of the statin-treated MCK-LpL mice are likely to have been exposed to greater fluxes of fatty acids and phospholipids, both of which could be toxic.

The effects of simvastatin in our study are similar to the effects of statins in humans. Muscle biopsies from human patients with statin-associated muscle pain show lipid accumulation (28). Moreover, a recent study showed that subjects on high dose simvastatin actually had increased cholesterol and plant sterols in their muscles (29).

The reasons for statin-induced human muscle toxicity are unknown. Statins may be directly toxic to muscles, if for example they block the production of lipid precursors other than LDL. Much of the literature has speculated that statin-induced muscle dysfunction results from a defect in prenylation (30) or farnesylation (31) of proteins. However, patients on long-term statin treatment
have unaltered cholesterol biosynthesis (32, 33) and peripheral cells also compensate for the drug-induced block in synthesis (34). Thus, it is possible that upregulation of LDL receptors, as illustrated in our animal experiments, is responsible for the abnormal concentrations of muscle lipids in symptomatic statin-treated patients.

Because statin-induced myopathy occurs in a minority of patients, there may be some genetic or physiological predisposition to this side effect. Situations that are associated with elevated expression of LpL, and therefore more muscle cholesterol uptake via this pathway, might also increase the risk of this myopathy. Chronic exercise (35), fasting (36) and use of fibric acid drugs (37) increase muscle LpL. These conditions might increase side effects/myopathy in muscle because they create a milieu that is similar to that in the MCK-LpL mice. Competitive athletes, who have increased muscle LpL expression (38), are well known to have difficulty with statin therapy (39).

Although statins cause skeletal muscle side effects, there is no evidence that these drugs alter cardiac function, and in our studies statin treatment did not alter heart LDL receptor expression in the mice. This is not due to a defect in statin uptake into cardiac tissue (40). Moreover, in a preliminary experiment with a small group of mice, statin treatment did not exacerbate the heart dysfunction found in the LpL\textsuperscript{GPI} mice and may have led to some benefit. On-going experiments will study this in more detail.

In summary, our data demonstrate that cardiomyocyte anchored LpL increased LDL uptake. Thus, the site of LpL might markedly affect LpL’s function; endothelial cell LpL primarily hydrolyzes circulating TG while parenchymal cell
surface LpL mediates holo-lipoprotein uptake. Neither cardiac nor skeletal muscle uptake of LDL is, to a major degree, via the LDL receptor pathway in chow-fed mice. These two muscles differ in their response to statin therapy and loss of LpL. Only skeletal muscle LDL receptors were upregulated by this drug and by LpL deficiency. In mice with greater LDL uptake resulting from muscle overexpression of LpL, statin therapy increased LDL uptake and tissue cholesterol content, and caused muscle damage. Elevation of cellular cholesterol can lead to dysfunction or even apoptosis (8). It is possible that a similar process is associated statin muscle dysfunction in humans. However, cardiac muscle dysfunction is not a known side effect of this class of drugs. This, we hypothesize, is due to differences in cholesterol delivery pathways utilized by these two forms of muscle. It should be noted that the levels of LpL elevation in our mouse model might not replicate conditions that occur in the unusual patient that develops myopathy. However because of the rarity of statin-induced myositis and the likelihood that the disease, which destroys muscle tissue, would alter LpL activity, there is no obvious method to test whether patients with greater LpL activity are more susceptible to this side effect. Thus, our experimental data develop a hypothesis rather than a model for statin-induced myositis.
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Table 1. Plasma lipids in LpL\textsuperscript{GPI} and Ldlr\textsuperscript{-/-}/LpL\textsuperscript{GPI} mice

Blood was taken from 24-h fasted mice. Values are expressed as mean ± S.D.

<table>
<thead>
<tr>
<th></th>
<th>Cholesterol</th>
<th>TG</th>
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<tr>
<td>Wild</td>
<td>66.2 ± 4.4</td>
<td>66.8 ± 18.9</td>
</tr>
<tr>
<td>Ldlr\textsuperscript{-/-}</td>
<td>242.1 ± 15.2*</td>
<td>84.9 ± 21.5</td>
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<tr>
<td>LpL\textsuperscript{GPI}</td>
<td>66.5 ± 3.7</td>
<td>63.6 ± 17.5</td>
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<tr>
<td>Ldlr\textsuperscript{-/-}/LpL\textsuperscript{GPI}</td>
<td>241.2 ± 10.3**</td>
<td>83.5 ± 25.8</td>
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TG=triglyceride. Significant differences (*p<0.05 vs. Wild, **p<0.05 vs. LpL\textsuperscript{GPI})

Heart lipids in LpL\textsuperscript{GPI} and Ldlr\textsuperscript{-/-}/LpL\textsuperscript{GPI} mice

Cardiac lipids were measured with enzymatic tests. Values are the mean ± S.D. ww = heart wet weight

<table>
<thead>
<tr>
<th></th>
<th>TC (µg/mg)</th>
<th>TG (µg/mg heart)</th>
<th>FFA (nmol/mg heart)</th>
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<tbody>
<tr>
<td>Wild</td>
<td>1.08 ± 0.24</td>
<td>5.15 ± 0.32</td>
<td>1.32 ± 0.11</td>
</tr>
<tr>
<td>Ldlr\textsuperscript{-/-}</td>
<td>1.89 ± 0.22</td>
<td>4.90 ± 0.82</td>
<td>1.25 ± 0.14</td>
</tr>
<tr>
<td>LpL\textsuperscript{GPI}</td>
<td>3.45 ± 0.18*</td>
<td>5.70 ± 0.55</td>
<td>1.62 ± 0.10*</td>
</tr>
<tr>
<td>Ldlr\textsuperscript{-/-}/LpL\textsuperscript{GPI}</td>
<td>3.22 ± 0.15**</td>
<td>5.54 ± 0.78</td>
<td>1.59 ± 0.13**</td>
</tr>
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</table>

TC= total cholesterol; TG= triglyceride; FFA = free fatty acids. Significant differences (\*p<0.05 vs. Wild, **p<0.05 vs. Ldlr\textsuperscript{-/-})
Table 2. Plasma lipids in Wild-type and MCK-LpL mice with statin treatment

Blood was taken from 24-h fasted mice. Values are expressed as Mean ± S.D.

<table>
<thead>
<tr>
<th></th>
<th>Cholesterol</th>
<th>TG</th>
<th>FFA</th>
<th>Glucose</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild</td>
<td>102.9 ± 17.8</td>
<td>64.6 ±12.2</td>
<td>0.93 ± 0.18</td>
<td>100.5 ± 22.9</td>
</tr>
<tr>
<td>Wild+ST</td>
<td>92.6 ± 23.3</td>
<td>53.9 ± 4.6</td>
<td>0.75 ± 0.10</td>
<td>120.5 ± 26.0</td>
</tr>
<tr>
<td>MCK</td>
<td>97.1 ± 22.7</td>
<td>48.1 ± 7.1</td>
<td>0.69 ± 0.15</td>
<td>92.0 ± 16.0</td>
</tr>
<tr>
<td>MCK+ST</td>
<td>67.9 ± 8.9*</td>
<td>38.5 ± 3.7*</td>
<td>0.64 ± 0.05*</td>
<td>122.0 ± 39.6</td>
</tr>
</tbody>
</table>

TG= triglyceride; FFA = free fatty acids.

*Significant differences with vs. without statin treatment (p<0.05)
Figure legends

Figure 1. LDL kinetic studies in wild-type, Ldlr-/-, LpL<sup>GPI</sup> and Ldlr-/-/LpL<sup>GPI</sup> mice. (A) Plasma clearance of <sup>125</sup>I-TC-LDL in wild-type, LpL<sup>GPI</sup>, Ldlr-/- and Ldlr-/-/LpL<sup>GPI</sup> mice. Male mice (10-12 weeks) were used for these and subsequent studies with an additional two-week feeding period as described in "Methods". <sup>125</sup>I-TC-LDL was injected into wild-type (open circles, n = 4), LpL<sup>GPI</sup> (closed circles, n = 5), Ldlr-/- (open squares, n = 4) and Ldlr-/-/LpL<sup>GPI</sup> (closed squares line, n = 4). At the indicated times, blood was collected. Values are expressed as mean ± S.D. (*p < 0.05, versus Ldlr-/- background). B-D, Tissue <sup>125</sup>I-TC-LDL uptake into liver (B), heart (C), and skeletal muscle (D). Twenty h after injection, the mice were perfused with phosphate-buffered saline containing 1 mg/ml EDTA and indicated tissues were taken out, washed and counted. The data are shown as percentage of wild-type mice (cpm/g of tissues/plasma activity). Values are expressed as mean ± S.D. (*p < 0.05, versus wild-type mice, **p < 0.05, versus LpL<sup>GPI</sup> mice, †p < 0.05, versus Ldlr-/- mice). Wild-type are denoted Wild.

Figure 2. Effects of loss of LDL receptors on heart failure markers and mortality of LpL<sup>GPI</sup> mice. (A) ANF and (B) BNP mRNA levels in ventricular muscle of 3-4 month old male wild type, Ldlr-/-, LpL<sup>GPI</sup>, and Ldlr-/-/LpL<sup>GPI</sup> mice was measured by RT-PCR (n=3). Survival of LpL<sup>GPI</sup> and Ldlr-/-/LpL<sup>GPI</sup> mice is shown in (C). *= P<0.05 versus control; ** = P<0.05 versus Ldlr-/-

Figure 3. Effects of statin treatment on LDL kinetics and tissue uptake in wild-type and MCK-LpL mice. Tissue <sup>125</sup>I-TC-LDL uptake into (A) liver, (B)-heart, and (C) skeletal muscle. <sup>125</sup>I-TC-LDL was injected into male wild-type (Wild, n =11), wild-type treated with statin (Wild+ST, n = 12), MCK-LpL (MCK, n = 7),
MCK-LpL treated with statin (MCK+ST, n = 8). At the indicated times, blood was collected. Twenty h after injection, the mice were perfused with phosphate-buffered saline containing 1 mg/ml EDTA, and indicated tissues were taken out, washed and counted. The data are shown as percentage increase uptake compared to tissues of untreated wild-type mice (% increase cpm/g of tissues/initial plasma activity). Values are expressed as mean ± S.D. (*p < 0.05, versus wild-type mice, **p < 0.05, versus MCK-LpL mice). (D) - Muscle cholesterol contents were measured with enzymatic tests. Free cholesterol is shown in the shaded area and ester in the open areas. Values are the mean ± S.D. ww = muscle wet weight. (*p < 0.05, versus wild-type mice, **p < 0.05, versus MCK-LpL mice).

Figure 4. Effects of statin treatment on muscle gene expression and lipid content. (A) Gene changes with statin treatment in MCK-LpL. Ten μg of total RNA was isolated from liver, muscle and heart and northern blot analyses were performed using radio-labeled cDNA probes for Ldlr and HMG-CoA-R in wild-type (Wild), wild-type with statin (Wild+ST), MCK-LpL (MCK), MCK-LpL with statin (MCK+ST). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is shown as a control for loading. (B) Electron microscopy of skeletal muscle. Arrows show lipid droplets. M indicates mitochondria. (C) Number of lipid droplets within 150 μm² area (5 for each samples) (**p < 0.05, versus MCK-LpL mice) (D) Plasma CPK level in four groups measured with enzymatic tests. Values are expressed as mean ± S.D. (**p < 0.05, versus MCK-LpL mice).
Figure 1

(a) LDL uptake (% control) over time in muscle.
(b) LDL uptake (% control) in liver.
(c) LDL uptake (% control) in heart.
(d) LDL uptake (% control) in muscle.

Liver
- Wild
- hLpL<sup>GPI</sup>
- Ldr<sup>-/-</sup>
- Ldr<sup>-/-</sup>/hLpL<sup>GPI</sup>

Heart
- Wild
- hLpL<sup>GPI</sup>
- Ldr<sup>-/-</sup>
- Ldr<sup>-/-</sup>/hLpL<sup>GPI</sup>

Muscle
- Wild
- hLpL<sup>GPI</sup>
- Ldr<sup>-/-</sup>
- Ldr<sup>-/-</sup>/hLpL<sup>GPI</sup>
Figure 2

(a) ANF

(b) BNP

Survival (% total)

Wild hLpL<sup>GPI</sup> Ldlr<sup>-/-</sup> Ldlr<sup>-/-</sup>/hLpL<sup>GPI</sup>

* **

ANF/GAPDH (arbitrary units) BNP/GAPDH (arbitrary units)

Wild hLpL<sup>GPI</sup> Ldlr<sup>-/-</sup> Ldlr<sup>-/-</sup>/hLpL<sup>GPI</sup>

* **

C

Survival (% total)

0 10 20 30 40

weeks

hLpL<sup>GPI</sup> Ldlr<sup>-/-</sup>/hLpL<sup>GPI</sup>

NS.
Supplementary Figure 1. The blots in Figure 4A were scanned and quantified by densitometric analysis (n=6 in each group). *=p<0.05 by paired test versus non-statin treated mice.
Supplementary Figure 2. Statin effects on LDL turnover and tissue uptake in MCK-LpL/Ldlr-/- mice. A, Tissue $^{125}$I-TC-LDL uptake into skeletal muscle. Twenty h after injection, the mice were perfused with phosphate-buffered saline containing 1 mg/ml EDTA, and indicated tissues were taken out, washed and counted. The data are shown as percentage of MCK-LpL/Ldlr-/- mice without statin (cpm/g tissues/ plasma activity). Values are expressed as mean ± S.D. (*p < 0.05, versus MCK-LpL/Ldlr-/- mice without statin). B, Muscle cholesterol content and, C, plasma CPK levels in MCK-LpL/Ldlr-/- mice with and without statin treatment.