Research Article

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Perilipin-5 is regulated by statins and controls triglyceride contents in the hepatocyte

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Background & Aims: Perilipin-5 (*PLIN5*) is a member of the perilipin family of lipid droplet (LD)-associated proteins. PLIN5 is expressed in oxidative tissues including the liver, and is critical during LD biogenesis. Studies showed that statins reduce hepatic triglyceride contents in some patients with non-alcoholic fatty liver disease and in rodent models of diet-induced hepatosteatosis. Whether statins alter triglyceride synthesis, storage, and/or utilization within the hepatocyte is unknown, though. Here we tested the hypothesis that statins alter the metabolism of LD in the hepatocyte during physiological conditions, such as fasting-induced steatosis.

Methods: Mice were gavaged with saline or atorvastatin, and the expression of LD-associated genes was determined in fed and fasted animals. The accumulation of triglycerides and LD was studied in mouse or human primary hepatocytes in response to statins, and following knock-down of SREBP2 or PLIN5.

Results: We show that statins decrease the levels of *PLIN5*, but not other LD-associated genes, in both mouse liver and mouse/human primary hepatocytes, which is paralleled by a significant reduction in both intracellular triglycerides and the number of LD. We identify an atypical negative sterol regulatory sequence in the proximal promoter of mouse/human *PLIN5* that recruits the transcription factor SREBP2 and confers response to statins. Finally, we show that the statin-dependent reduction of hepatocyte triglyceride contents is mimicked by partial knock-down of *PLIN5*; conversely, ectopic overexpression of *PLIN5* reverts the statin effect.

Conclusions: PLIN5 is a physiological regulator of triglyceride metabolism in the liver, and likely contributes to the pleiotropic effects of statins.

Keywords: PLIN5; Liver; Triglycerides; Fatty acid oxidation; Steatosis.
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Abbreviations: LD, lipid droplet; LDLR, low-density lipoprotein receptor; NAFLD, non-alcoholic fatty liver disease; FFA, free fatty acids; PLIN, perilipin; PPAR, peroxisome proliferator-activated receptor; SRE, sterol regulatory element; SREBP, sterol regulatory element binding protein; TG, triglycerides.

Introduction

Lipid droplets (LDs) are energy-storage organelles that play a remarkably complex role in triglyceride homeostasis. Thus, LDs both prevent the lipotoxic effects of non-esterified fatty acids (FFAs), and support cellular needs by releasing FFAs for β-oxidation and membrane synthesis. These organelles contain a core of neutral lipids (mostly triglycerides (TG) but also cholesteryl esters) surrounded by a phospholipid monolayer, and are lined with specific proteins that include members of the perilipin (*PLIN*) family [1]. Perilipins are grouped into two categories depending on their stability when not associated to LD: non-exchangeable and exchangeable. The former include PLIN1 and -2, which are only found in an LD-bound state and are rapidly degraded when not associated to lipids [1,2]. The latter include PLIN3, -4, and -5, which exist either in an LD-bound state or soluble in the cytosol [1,2]. PLIN1 and -4 are abundant in white adipose tissue [1], PLIN2 and -3 are expressed in many cell types [1,3], and PLIN5 is mainly expressed in oxidative tissues such as liver, heart, muscle, and brown adipose tissue [4–6].

PLIN5 was identified by three independent laboratories, which showed that both mRNA and protein are induced in the heart, liver, and skeletal muscle during fasting via the peroxisome proliferator-activated receptor α (PPARα) [4–6]. PPARγ and PPARδ were also reported to induce PLIN5 expression in adipose tissue [5] and skeletal muscle [7], respectively. These early studies established a role for PLIN5 on LD metabolism, likely inhibiting triglyceride lipolysis and/or decreasing fatty acid oxidation, and contributing to an overall intracellular lipid accumulation [4–6]. Mice deficient in *Plin5* showed a striking cardiac phenotype: they lacked detectable LDs, had decreased TG levels, increased β-oxidation, increased reactive oxygen species, and developed heart failure with age [8]. Conversely, cardiac-specific overexpression of *Plin5* resulted in severe TG accumulation and a robust increase in LDs [9,10]. These latter authors showed that PLIN5-coated LDs are resistant to TG hydrolysis and that mitochondrial function is decreased, suggesting that PLIN5 acts as a lipolytic barrier to prevent uncontrolled TG mobilization [9,10].

Patients with hypercholesterolemia are normally prescribed statins, competitive inhibitors of 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGCR, the rate-limiting enzyme of the *de novo* cholesterol synthesis pathway). Interestingly, several data suggest that statins can decrease hepatic TG contents in

patients [11–15] and rodents [16–18]. The mechanisms by which statins exert beneficial effects on pathological hepatosteatosis are not well understood, however. Likewise, whether statins control physiological hepatic TG homeostasis is unknown. Here we tested the hypothesis that statins alter the metabolism of LD in the hepatocyte by stimulating fatty acid β-oxidation, and show that the transcription of *PLIN5*, but not other perilipins, is controlled by statins via SREBP2.

Materials and methods

Mice

Male, 8–10 week-old C57BL/6 mice were maintained in a 12 h/12 h light/dark cycle with unlimited access to food and water. All studies were approved by the IACUC at SLU.

Primary hepatocytes

Normal human primary hepatocytes were obtained from Lonza (CC-2591) and cultured in hepatocyte basal medium (Lonza). Mouse primary hepatocytes were isolated using perfusion and digest buffers (Invitrogen) as described [19]. Cells were seeded in 12- or 6-well BioCoat Collagen I plates (BD), and incubated at 37 °C and 5% CO_2 in William's E media + Hepatocyte Supplements (Invitrogen). For siRNA, cells were transfected with anti-SREBP2 (M-050073-01-0005), anti-PLIN5 (M-0557756-01-05), or control (D-001210-01-05) oligonucleotides (siGENOME SMART pool, ThermoScientific), using Dharmafect 1 reagent (ThermoScientific). For adenovirus-mediated overexpression, cells were transduced with Adeno-SREBP2, Adeno-Plin5, or Adeno-empty vectors at moi = 3. Where indicated, cells were cultured in media supplemented with 5 μmol/L statins (Sigma) for 48 h. For oleate challenge, cells were pre-treated with simvastatin for 24 h, before addition of 600 μmol/L oleate:BSA (1:3) for an additional 24 h.

Additional materials and methods are provided as Supplementary data.

Results

Statins decrease hepatic PLIN5 levels and fasting-induced steatosis

To test the hypothesis that statins influence the metabolism of TG in the hepatocyte, we measured the effects of atorvastatin on hepatic TG contents and on the expression of selected genes encoding lipogenic and LD-associated proteins. We gavaged chow-fed mice with saline or 20 mg/kg/day atorvastatin for 10 days; then some mice were allowed access to food, while others were fasted overnight, before sacrifice. We found no significant changes in body weight, plasma lipids, and transaminases between groups (data not shown). As expected, fasting induced significant hepatic TG accumulation, compared to feeding, with no change in hepatic cholesterol levels (Fig. 1A, light blue bars); and atorvastatin induced the hepatic expression of *Srebp-2* and SREBP-2 targets (*Hmgcr*, *Pcsk9*) (Supplementary Fig. 1A). Our data show that both groups of mice (fasted and non-fasted) had a significant decrease (~35%) in liver triglycerides when treated with atorvastatin, compared to those gavaged with saline (Fig. 1A, dark blue *vs.* light blue bars), which was independent on changes in *Pparα* and PPARα target genes (Supplementary Fig. 1B), and *Srebp1c* and SREBP1C target genes (Supplementary Fig. 1C). Analysis of selected LD-associated genes is presented in Fig. 1B and C. Data show that the levels of *Plin2*, *Plin5*, *Atgl*, and *Hsl* were induced in fasted, compared to fed animals, as expected [3,5,20,21]. However, only the expression of *Plin5* was differentially regulated by the statin: a 25% and 30% decline in fed and fasted mice, respectively, compared to saline (Fig. 1B). These latter changes were more pronounced at the protein level (Fig. 1C). Our data are also consistent with those of other investigators who showed that fasting induces both PLIN2 and PLIN5, but not PLIN3, in the livers of mice [3,5,22]. The reduction in PLIN2 in the livers of fasted, atorvastatin-treated mice (Fig. 1C) is also consistent with the degradation of this protein following the decrease in intracellular TG, since this perilipin is unstable in the cytosol when not bound to LDs [8].

To confirm that the repressing effect on PLIN5 was shared by different statins, we tested simvastatin, pravastatin, and atorvastatin in mouse primary hepatocytes. Data in Fig. 1D show that all three statins reduced the mRNA levels of *Plin5*, but not *Plin2* or *Plin3*, compared to vehicle. The transcript levels of other LD-related genes were unaffected by either statin (Supplementary Fig. 2A). As expected, all statins induced the expression of classic SREBP2 targets (Fig. 1D and Supplementary Fig. 2A). In agreement with these results, immunoblots performed in a different set of primary hepatocytes revealed that the abundance of PLIN5, but not PLIN2 or PLIN3, was significantly decreased following incubation with simvastatin (Fig. 1E). Finally, experiments in human primary hepatocytes showed that the regulation of *PLIN5*, but not other LD-related genes, by statins is conserved in humans (Fig. 1F and Supplementary Fig. 2B). Collectively, the data suggest that statins alter LD metabolism via down-regulation of *PLIN5*, likely limiting hepatic TG accumulation in both mice and humans.

Statins decrease the expression of PLIN5 through SREBP2

To test whether the decrease in *PLIN5* following treatment with statins is SREBP2-dependent, we assessed the impact of either SREBP2 knock-down or overexpression in mouse primary hepatocytes (Fig. 2A–D). Hence, we transfected cells with either non-targeting (siNT) or anti-*Srebp2* (siSREBP2) oligonucleotides, and later cultured them in the presence or absence of simvastatin. Data in Fig. 2A show that, as expected, knock-down of *Srebp2* prevented the induction of both *Ldlr* and *Hmgcr* by simvastatin. Importantly, the statin-dependent repression of *Plin5* was also abolished in siSREBP2-transfected cells at both the mRNA and protein levels (Fig. 2A and C), with no change in the expression of *Plin2* or *Plin3* (Fig. 2A and C). Gain-of-function studies by ectopically expressing a constitutively active SREBP2 show that the levels of *Plin5* mRNA and protein were significantly decreased following SREBP2 overexpression, while known SREBP2 targets (*Ldlr* and *Hmgcr*) were induced, and the expression of *Plin2* and *Plin3* did not change under the same conditions (Fig. 2B and D). Taken together, these data demonstrate that statins control the expression of *PLIN5* through an SREBP2-dependent mechanism.

Analysis *in silico* of both mouse and human *PLIN5* proximal promoters with the TF-Search algorithm [23], identified a conserved putative sterol regulatory element (SRE), suggesting that SREBP2 binds to the *PLIN5* promoter. Fig. 2E shows that this SRE differs only 1 nucleotide from the SRE in the human *LDLR* promoter. Luciferase reporter constructs containing a 3.5 kb fragment (which includes the potential SRE) of the mouse or human *PLIN5* promoter where tested in transfection experiments. Data in Fig. 2E show that the activity of both mouse and human *PLIN5* reporters was reduced following co-transfection with a plasmid encoding a constitutively active SREBP2, while an empty reporter did not respond to SREBP2 co-transfection. Importantly, site-directed mutagenesis of the putative SRE abolished the effect of