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Chronic HMGCR/HMG-CoA reductase inhibitor treatment contributes to dysglycemia by upregulating hepatic gluconeogenesis through autophagy induction

Hye Jin Wang,[1,2,3,#] Jae Yeo Park,[4,5,#] Obin Kwon,[3,6] Eun Yeong Choe,[1,2] Chul Hoon Kim,[3,6] Kyu Yeon Hur,[7] Myung-Shik Lee,[7,8] Mijin Yun,[9] Bong Soo Cha,[1,2,3] Young-Bum Kim,[10] Hyangkyu Lee,[4,5,*] and Eun Seok Kang[1,2,3,*]

[1]Division of Endocrinology and Metabolism; Department of Internal Medicine; Yonsei University College of Medicine; Seoul, Korea; [2]Institute of Endocrine Research; Yonsei University College of Medicine; Seoul, Korea; [3]Brain Korea 21 PLUS Project for Medical Science; Yonsei University College of Medicine; Seoul, Korea; [4]Department of Clinical Nursing Science; Yonsei University College of Nursing; Seoul, Korea; [5]Nursing Policy and Research Institute; Biobehavioral Research Center; Yonsei University; Seoul, Korea; [6]Department of Pharmacology; Yonsei University College of Medicine; Seoul, Korea; [7]Department of Medicine; Samsung Medical Center; Sungkyunkwan University School of Medicine; Seoul, Korea; [8]Samsung Advanced Institute for Health Sciences and Technology; Sungkyunkwan University School of Medicine; Seoul, Korea; [9]Department of Nuclear Medicine; Yonsei University College of Medicine; Seoul, Korea; [10]Division of Endocrinology, Diabetes, and Metabolism; Department of Medicine; Beth Israel Deaconess Medical Center and Harvard Medical School; Boston, MA USA

[#]These authors contributed equally to this work.

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Abbreviations: ACTB, actin beta; AKT1, v-akt murine thymoma viral oncogene homolog 1; ATG7, autophagy-related 7; Baf A1, bafilomycin A_1; BECN1, Beclin 1 autophagy related; CQ, chloroquine; FOXO1, forkhead box O1; G6PC, glucose-6-phosphatase catalytic subunit; GCK, glucokinase (hexokinase 4); GFP, green fluorescent protein; HMGCR/HMG-CoA reductase, 3-hydroxy-3-methylglutaryl-CoA reductase; MAP1LC3A/LC3A, microtubule-associated protein 1 light chain 3 alpha; MTOR, mechanistic target of rapamycin (serine/threonine kinase); O-GluNAc, O-linked β-N-acetyl glucosamine; PCK1, phosphoenolpyruvate carboxykinase 1 (soluble); PIK3C3, phosphatidylinositol 3-kinase catalytic subunit type 3; PKLR, pyruvate kinase liver and RBC; qRT-PCR, quantitative reverse transcription-polymerase chain reaction; RFP, red fluorescent protein; RPS6KB1, ribosomal protein S6 kinase; 70kDa, polypeptide 1; shRNA, short hairpin RNA; T2DM, type 2 diabetes mellitus; XBP1, X-box binding protein 1.

Statins (HMGCR/HMG-CoA reductase [3-hydroxy-3-methylglutaryl-CoA reductase] inhibitors) are widely used to lower blood cholesterol levels but have been shown to increase the risk of type 2 diabetes mellitus. However, the molecular mechanism underlying diabetogenic effects remains to be elucidated. Here we show that statins significantly increase the expression of key gluconeogenic enzymes (such as G6PC [glucose-6-phosphatase] and PCK1 (phosphoenolpyruvate carboxykinase 1 [soluble]) in vitro and in vivo and promote hepatic glucose output. Statin treatment activates autophagic flux in HepG2 cells. Acute suppression of autophagy with lysosome inhibitors in statin treated HepG2 cells reduced gluconeogenic enzymes expression and glucose output. Importantly, the ability of statins to increase gluconeogenesis was impaired when ATG7 was deficient and BECN1 was absent, suggesting that autophagy plays a critical role in the diabetogenic effects of statins. Moreover autophagic vacuoles and gluconeogenic genes expression in the liver of diet-induced obese mice were increased by statins, ultimately leading to elevated hepatic glucose production, hyperglycemia, and insulin resistance. Together, these data demonstrate that chronic statin therapy results in insulin resistance through the activation of hepatic gluconeogenesis, which is tightly coupled to hepatic autophagy. These data further contribute to a better understanding of the diabetogenic effects of stains in the context of insulin resistance.

Introduction

Statins (HMGCR inhibitors) are the most widely prescribed drugs for the primary and secondary prevention of cardiovascular diseases. Although these drugs effectively reduce circulating cholesterol levels, numerous studies have demonstrated that statin therapy is linked to the development of type 2 diabetes mellitus (T2DM). Increased incidence of diabetes was observed in clinical trials evaluating pravastatin (Pravastatin or Atorvastatin Evaluation and Infection Therapy),[1] simvastatin (Heart

*Correspondence to: Eun Seok Kang; Email: edgo@yuhs.ac or Hyangkyu Lee; Email: hkyulee@yuhs.ac
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Protection Study),[2] atorvastatin (Anglo-Scandinavian Cardiac Outcomes Trial—Lipid-Lowering Arm),[3] and rosuvastatin (Controlled Rosuvastatin Multinational Trial in Heart Failure; Justification for the Use of Statins in Prevention: an Intervention Trial Evaluating Rosuvastatin).[4,5] Recent metaanalyses of major statin trials also demonstrated that the risk for T2DM is higher in statin users than in nonusers.[6,7] This effect is dose-dependent[8]: the risk of developing diabetes is 12% higher in patients on intensive-dose statin therapy than those on moderate-dose therapy, and this effect is likely to be class-dependent rather than drug-specific. Given that numerous well-designed clinical studies have reported the diabetogenic effect of statins, elucidating the underlying mechanism is of great importance.

The major site of therapeutic action for statins is the liver. Studies in rats have demonstrated that statins are selectively taken up by the liver[9] and transported into hepatocytes by a high-affinity process.[10] The hepatic specificity of statins may be due to efficient first-pass metabolism.[11] The liver plays a critical role in regulating blood glucose levels, especially under a fasting state, maintaining glucose homeostasis through glycogenolysis and gluconeogenesis. Statins have been shown to increase fasting plasma glucose levels in individuals with or without diabetes[12] and induce gluconeogenic gene expression in primary cultured human hepatocytes.[13] These results suggest that statins raise fasting blood glucose levels in vivo by stimulating gluconeogenesis in the liver.

Autophagy is the catabolic mechanism by which cells regulate the turnover of cellular organelles and proteins. This process also supplies various substrates for energy generation, leading to alterations in cell metabolism.[14] In carbohydrate metabolism, autophagy contributes to glycogen breakdown in lysosomes[15] and the maintenance of pancreatic β-cell mass and function.[16] In the liver, autophagy appears to play an important role in glucose homeostasis by promoting the conversion of amino acids to glucose.[17] Statins induce autophagy in various cell types (e.g., macrophages, cancer cells, coronary arterial myocytes),[18-21] therefore, we postulate that statins induce autophagy in the liver, thereby stimulating hepatic gluconeogenesis, which manifests clinically as diabetes. In this study we investigated this potential molecular mechanism underlying the diabetogenic effect of statins. Our results show that induction of liver autophagy is integral to statin-induced upregulation of hepatic gluconeogenesis, leading to dysglycemia in mice.

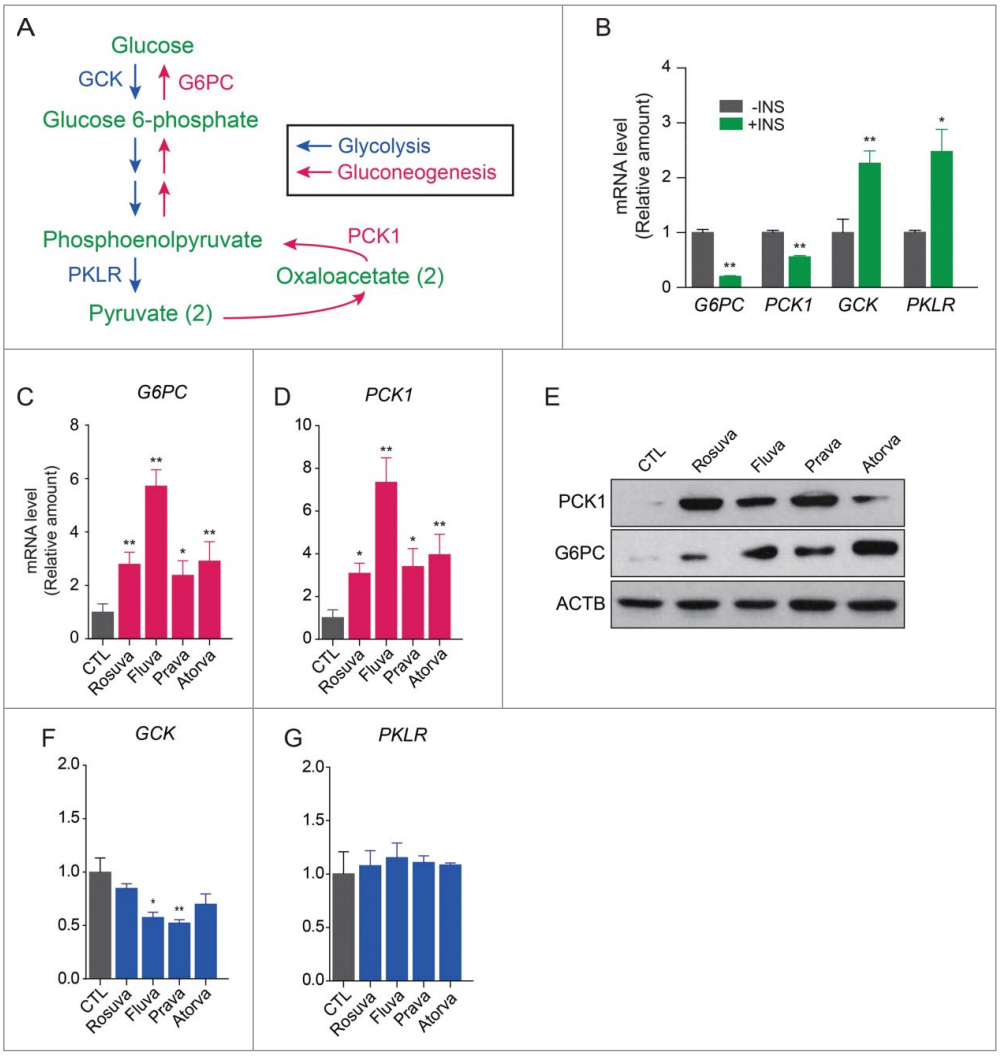

Figure 1. Statins upregulate the expression of gluconeogenic enzymes but not glycolytic enzymes in HepG2 cells. (**A**) Glycolysis and gluconeogenesis pathways. (**B**) Insulin inhibits gluconeogenesis and enhances glycolysis in this system. Statins (20 μM) upregulated not only mRNA levels of *G6PC* (**C**) and *PCK1* (**D**), but also protein levels of G6PC and PCK1 (**E**). However statins had little effect on mRNA levels of *GCK* (**F**) and *PKLR* (**G**) in HepG2 cells. *, $P < 0.05$; **, $P < 0.01$ compared with control (CTL).

Results

Statins increase gluconeogenic enzyme expression in HepG2 cells

To evaluate whether hepatic gluconeogenesis is involved in the diabetogenic effects of statins, we tested the effects of rosuvastatin, fluvastatin, pravastatin, and atorvastatin on the expression of key enzymes involved in gluconeogenesis and glycolysis in HepG2 hepatocellular carcinoma cells (**Fig. 1A**). To validate the system, we treated HepG2 cells with insulin to confirm that insulin promotes glycolysis in these cells.

Results of real-time quantitative reverse transcription-polymerase chain reaction (qRT-PCR) confirmed that insulin decreased expression of genes encoding the gluconeogenic enzymes G6PC (glucose-6-phosphatase, catalytic subunit) and PCK1 (phosphoenolpyruvate carboxykinase 1 [soluble]) and increased expression of genes encoding the glycolytic enzymes GCK (glucokinase [hexokinase 4]) and PKLR (pyruvate kinase, liver and RBC) (**Fig. 1B**). After 24-h treatment with rosuvastatin, fluvastatin, pravastatin, or atorvastatin (20 μM), mRNA levels of *G6PC* and *PCK1* increased in response to each statin (**Fig. 1C** and **Fig. 1D**) and protein levels of these enzymes also increased (**Fig. 1E**), suggesting increased gluconeogenesis. In contrast, statins had little or no effect on mRNA levels of *GCK* and *PKLR* (**Fig. 1F** and **Fig. 1G**). These data indicate that statins specifically affect the expression of gluconeogenic enzymes in HepG2 cells.

Statins induce autophagic flux leading to enhanced expression of gluconeogenic enzymes

Because autophagy promotes gluconeogenesis in the liver,[17] we tested whether statins increase autophagy in hepatocytes by transfecting HepG2 cells with a vector expressing the autophagy marker MAP1LC3A/LC3A (microtubule-associated protein 1 light chain 3 α) fused to green fluorescent protein (GFP). We found that statin treatment increased the number of GFP-LC3A fluorescent puncta representing autophagosomes in the cytosol (**Fig. 2A** and **Fig. 2B**). In contrast, puncta were barely discernible in control cells (**Fig. 2A** and **Fig. 2B**). To confirm this result, we evaluated LC3B-II expression by western blot analysis, which showed increased LC3B-II levels in statin-treated cells compared with controls, suggesting that statins promote autophagy in HepG2 cells (**Fig. 2C**). We treated cells with bafilomycin A_1 (Baf A1), a lysosomal blocker, to block autophagy and treated with statins to reveal whether increased LC3B with statin is due to autophagy induction or autophagy flow blockade. Statins additionally increased LC3B-II in cells pretreated with Baf A1, which suggests that statins induce autophagy rather than blocking autophagy flow (**Fig. 2C**). Then we transfected mRFP (monomeric red fluorescent protein)-GFP-LC3 tandem construct encoding LC3 fused to mRFP and GFP to HepG2 cells to evaluate autophagic flux.

The GFP protein is degraded in acidic conditions inside the lysosome, leading to loss of the green fluorescent signal whereas RFP is more stable in acidic condition, maintaining the red fluorescent signal. Therefore autophagosomes show the yellow fluorescent signal (merged signal of mRFP and GFP) and autolysosomes show only red signals (mRFP). The number of red and yellow puncta increased in HepG2 cells treated with statin, indicating that stains indeed induce autophagosome and autolysosome formation, representing an increase in autophagic flux (**Fig. 2D** and **Fig. 2E**). The possibility that the statins' effect is due to mere inhibition of lysosomal degradation is ruled out based on the result that Baf A1 increased number of yellow puncta without an increase in red puncta (**Fig. 2D** and **Fig. 2E**).

Blockage of autophagic flux attenuates the effect of statins on gluconeogenesis.

To see whether the gluconeogenic effect of statins is mediated by the autophagic process, we utilized a lysosomal inhibitor such as chloroquine (CQ) or Baf A1, with a statin treatment. Increased *G6PC* and *PCK1* expression with statin treatment were attenuated by CQ (**Fig. 3A** and **Fig. 3B**) and glucose production was also decreased (**Fig. 3C**). Inhibition of the autophagic process with Baf A1 treatment resulted in attenuation of increased *G6PC* and *PCK1* expression with statins (**Fig. 3D** and **Fig. 3E**). In addition glucose production was also decreased with Baf A1 (**Fig. 3F**).

To better understand the effect of statins on autophagy, we transfected HepG2 cells with short hairpin RNAs (shRNAs) against the gene encoding BECN1, which plays an important role in autophagy induction.[22] After confirming the knockdown of *BECN1* (**Fig. 3G**), we observed statin-dependent increases in *G6PC* and *PCK1* mRNA levels in transfected cells (**Fig. 3H** and **Fig. 3I**). These data support the role of autophagy in statin-induced gluconeogenesis.

ATG7 is necessary for statin-induced gluconeogenesis in the liver

To further evaluate the role of autophagy in regulating the expression of gluconeogenic enzymes, ex vivo studies were performed with primary hepatocytes isolated from liver-specific *Atg7* (autophagy-related 7)-deficient mice. Deficiency of *Atg7* in the liver was confirmed by qRT-PCR (**Fig. 4A**), the cultured hepatocytes were treated with statins for 24 h. Our results showed that the statin-induced increase in expression of the genes encoding gluconeogenic enzymes (*G6pc* and *Pck1*) was blocked in *Atg7*-deficient hepatocytes (**Fig. 4B**). In addition, glucose output did not increase in response to statins in *Atg7*-deficient hepatocytes (**Fig. 4C**). Collectively, these data suggest that ATG7, the core autophagy regulator, is required for statin-induced gluconeogenesis in the liver, supporting the role of autophagy in statin-induced hepatic gluconeogenesis.

Statins increase hepatic gluconeogenesis, leading to hepatic insulin resistance in high-fat diet-fed mice

To evaluate the in vivo effects of statins on glucose homeostasis, beginning at 5 wk of age mice were fed a high-fat diet supplemented with rosuvastatin, fluvastatin, pravastatin, or atorvastatin for 16 wk. Mean body weight significantly increased in statin-treated mice compared with untreated mice (**Fig. 5A**), independent of food intake (**Fig. 5B**). We measured fasting blood glucose levels and performed the oral glucose tolerance test and insulin tolerance test at wk 15 and pyruvate tolerance test at wk 16. Our results showed that fasting blood glucose levels were higher in mice treated with pravastatin or atorvastatin compared with mice treated with rosuvastatin or fluvastatin (**Fig. 5C**). Results of the oral glucose tolerance test did not differ between pravastatin- and atorvastatin-treated mice and control mice (**Fig. 5D**), indicating that pancreatic β-cell function was not impaired at 15 wk. However, blood glucose levels failed to decrease upon insulin treatment in statin-treated mice, indicating

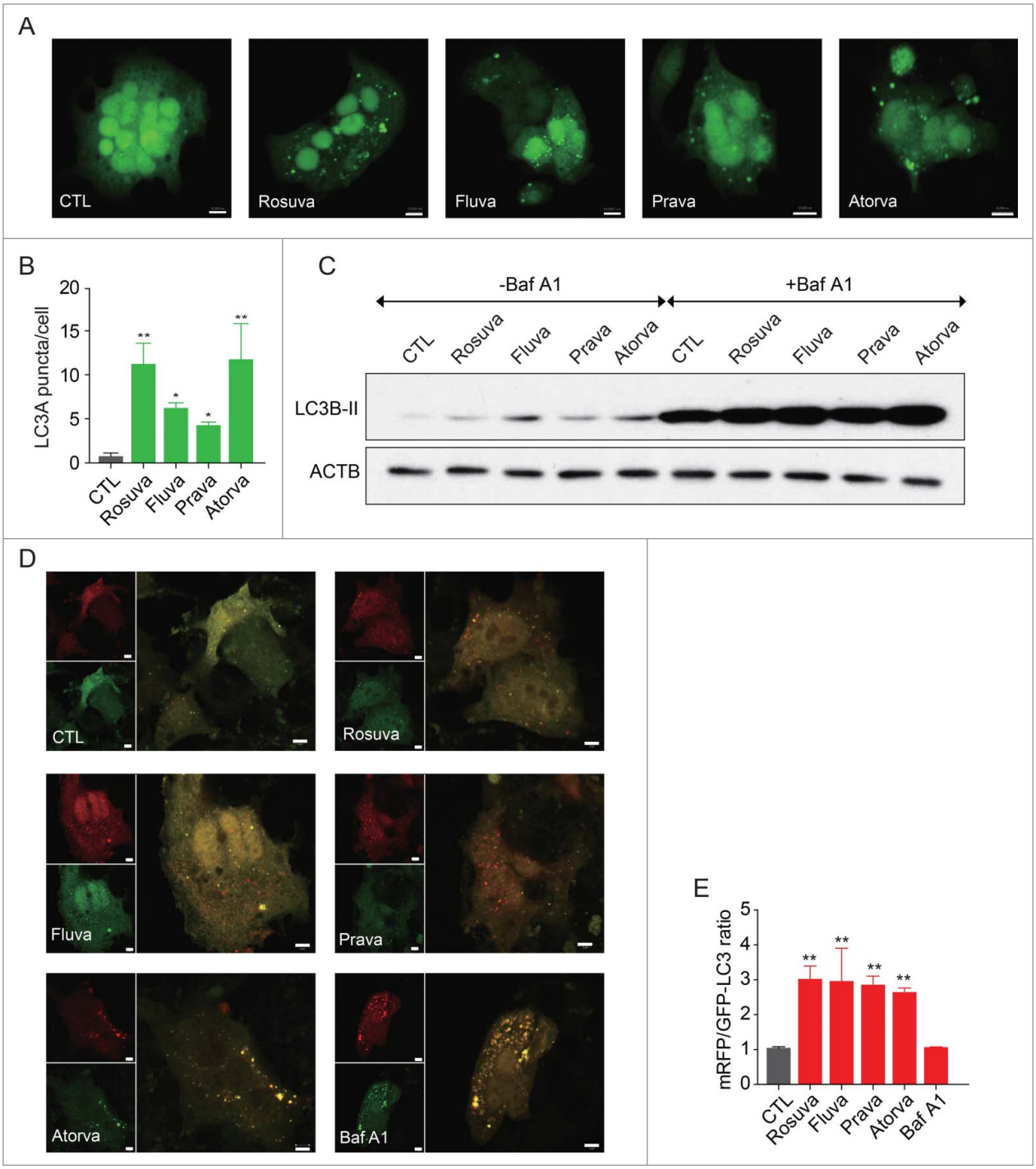

Figure 2. Statins induce autophagy in HepG2 cells. (**A**) HepG2 cells were transfected with the autophagy sensor GFP-LC3A then treated with rosuvastatin, fluvastatin, pravastatin, or atorvastatin (20 μM) for 24 h. Fluorescent images were obtained by confocal microscopy. (**B**) Columns in the histogram represent the number of LC3A puncta per cell. At least 6 random fields were chosen from each sample. (**C**) Results of western blot analysis showed increased LC3B-II levels in statin-treated cells. The Baf A1-treated group showed that statins induced autophagy flux. (**D**) After transfecting HepG2 cells with mRFP-GFP-LC3B, statins were added for 24 h. Fluorescent images were obtained by confocal microscopy. The GFP protein is unstable in low pH inside of the lysosome and thereby degraded. In contrast, RFP is more stable in acidic conditions and thereby it could maintain red fluorescence. (**E**) Columns in the histogram represent the ratio of mRFP and GFP LC3B puncta. *, $P < 0.01$ compared with control.

insulin resistance (**Fig. 5E**), and area under the curve for the insulin tolerance test differed significantly between the control group and the statin-treated groups (**Fig. S1**). To evaluate the possibility that statins induce hepatic insulin resistance by increasing hepatic glucose production, we performed the pyruvate tolerance test, which showed significantly

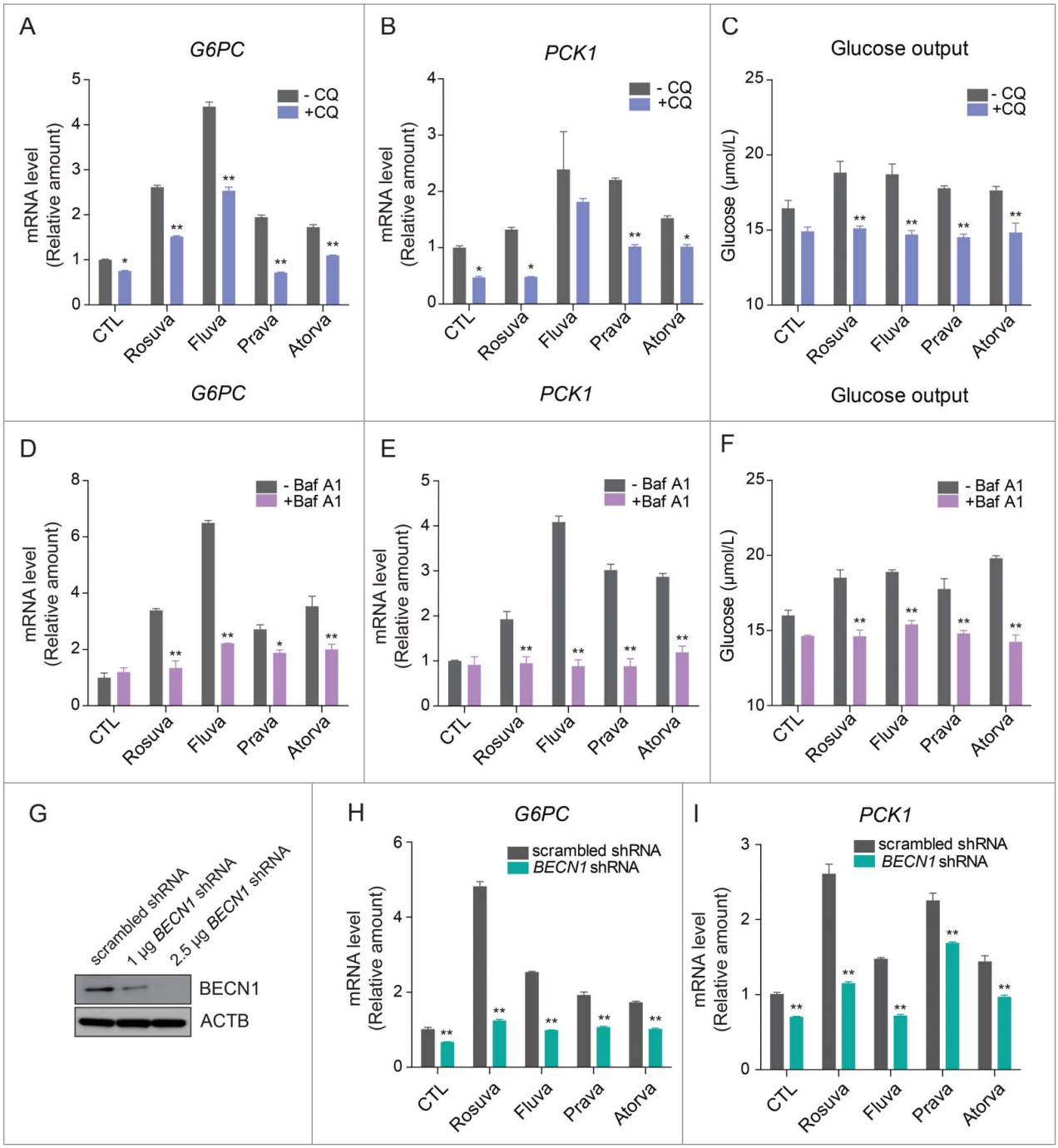

Figure 3. Statins increase autophagy-dependent gluconeogenesis in HepG2 cells. After treatment with 20 μM statins for 22 h, 50 μM chloroquine was added to HepG2 cells for 2 h, with statins. HepG2 cells treated with statins and CQ (**A**) *G6PC* and (**B**) *PCK1* were analyzed with qRT-PCR. (**C**) Glucose production by HepG2 cells treated with statins and CQ. HepG2 cells treated with statins and 20 nM Baf A1. (**D**) *G6PC* and (**E**) *PCK1* were analyzed with qRT-PCR. (**F**) Glucose production was measured. (**G**) After transfecting HepG2 cells with *BECN1* shRNA, knockdown of *BECN1* was confirmed by western blot analysis. Inhibition of autophagy by *BECN1* knockdown decreased expression of *G6PC* (**H**) and *PCK1* (**I**). *, $P < 0.05$, **, $P < 0.01$ compared with control cells or control cells transfected with scrambled shRNA.

elevated blood glucose levels in pravastatin- and atorvastatin-treated mice over 3 h (**Fig. 5F** and **Fig. S1**: area under the curve). These data suggest that pravastatin and atorvastatin increase blood glucose levels in vivo, at least in part, by stimulating hepatic gluconeogenesis.

To determine whether statins function as HMGCR inhibitors under our experimental conditions, we measured serum levels of cholesterol, triglycerides, and free fatty acids in the high-fat diet-fed mice. Serum cholesterol levels were only marginally decreased by rosuvastatin and fluvastatin (**Fig. 5G**); however, serum

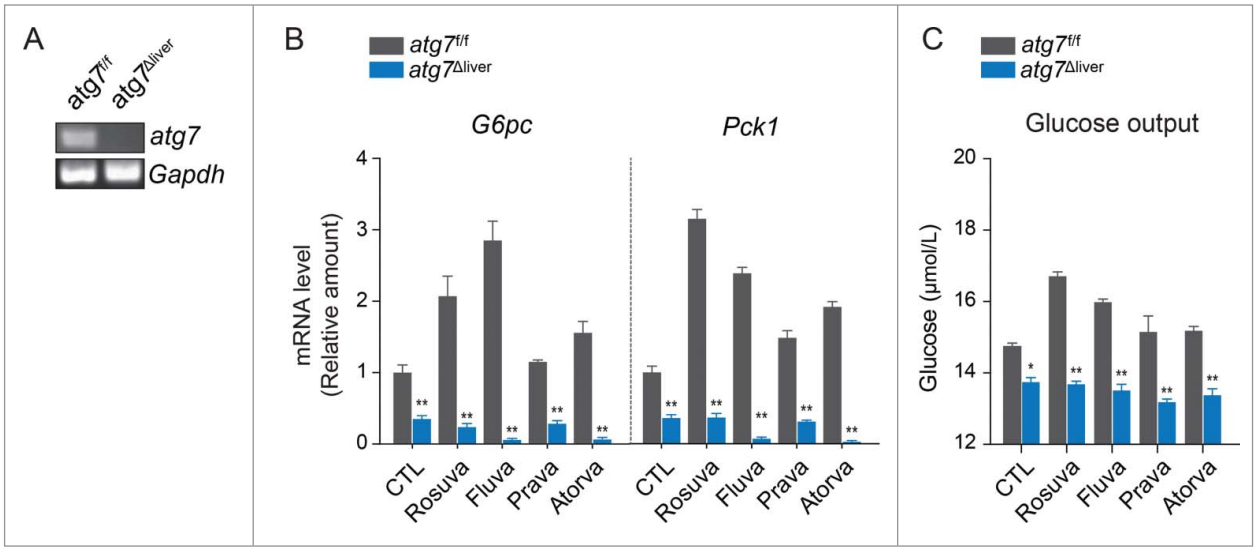

Figure 4. The gluconeogenic effect of statins is attenuated in primary hepatocytes derived from liver-specific *atg7*-deficient mice. (**A**) Loss of *Atg7* in liver tissue was confirmed by qRT-PCR. (**B**) Primary hepatocytes derived from wild-type and liver specific *atg7*-deficient mice were treated with statins (20 μM) for 24 h. Increased expression of *G6pc* and *Pck1* was observed in wild-type hepatocytes but not in *atg7*-deficient hepatocytes. (**C**) Glucose output by cultured hepatocytes from wild-type and liver specific *atg7*-deficient mice after 24-h statin treatment. *, $P < 0.05$; **, $P < 0.01$ compared with wild-type hepatocytes.

triglyceride and free fatty acid levels were significantly decreased in the statin-treated groups compared with the control group (**Fig. 5H** and **Fig. 5I**). These data suggest that under conditions in which statins increase hepatic gluconeogenesis, they function as HMGCR inhibitors within hepatocytes, and these statin-induced metabolic changes may be related to the inhibition of endogenous cholesterol synthesis.

Statins increase hepatic gluconeogenesis and autophagy in high-fat diet-fed mice

To determine whether statins increase hepatic gluconeogenesis and autophagy in vivo, expression of key gluconeogenic enzymes in the livers of high-fat diet-fed mice was evaluated by qRT-PCR. Consistent with in vitro results, statin treatment caused a significant increase in the expression of hepatic gluconeogenic genes (*G6pc* and *Pck1*) in mice (**Fig. 6A** and **Fig. 6B**). However, expression of glycolytic genes (*Gck* and *Pklr*) was not affected by statins (Fig. 6C and **Fig. 6D**).

To determine whether statins induce autophagy in mouse livers in vivo, electron microscopy analysis was performed. Transmission electron microscopy analysis revealed prominent vacuolization and autophagosomes in the hepatocytes of statin-treated mice (**Fig. 7A**). Autophagic vacuoles are increased in statin treated mouse livers (**Fig. 7B**). Collectively, these data demonstrate that statin treatment leads to insulin resistance by increasing gluconeogenesis, which is tightly coupled to autophagy.

Discussion

Although numerous clinical trials and epidemiologic studies have demonstrated that statin therapy increases the risk of T2DM,[1-7] the molecular mechanism underlying this unexpected drug action has not been elucidated. In this study, we showed that statin treatment leads to insulin resistance by activating hepatic gluconeogenesis, which is tightly coupled to hepatic autophagy.

One of this study's most important findings is that statins induce autophagy in hepatocytes both in vitro and in vivo. Although a recent study shows that statin treatment blocks autophagy flux in skeletal muscle by inhibiting PRKD (protein kinase D) activity,[23] most previous studies have described the effects of statins on autophagy in cultured cells.[18-21] In human prostate cancer and rhabdomyosarcoma cells, statins induce autophagy by blocking geranylgeranyl biosynthesis through the inhibition of HMGCR.[19,20] In coronary artery myocytes, RAC1 GTPase overexpression, which activates MTOR (mechanistic target of rapamycin [serine/threonine kinase]), blocks simvastatin-induced autophagy.[21] Because the role of MTOR in autophagy inhibition is well established,[21,24] and simvastatin suppresses MTOR signaling in cardiomyocytes in vitro and in vivo,[21] we examined the effect of statins on MTOR complex 1and its downstream target in HepG2 cells. Statins attenuated the MTOR and RPS6KB phosphorylation, which are enhanced with insulin treatment in HepG2 cells (**Fig. S2**).

G6PC and *PCK1* are FOXO1 target genes and they could be regulated by FOXO1. Therefore we examined whether FOXO1 phosphorylation is altered by statins. We found that statin increased FOXO1 phosphorylation in primary mouse hepatocytes (**Fig. S3**). The canonical insulin signaling pathway for regulation of glucose metabolism involves phosphorylation and export of the transcription factor FOXO1 out of the nucleus. Keeping FOXO1 in the nucleus induces the transcriptional induction of gluconeogenic enzymes. Increased phosphorylation

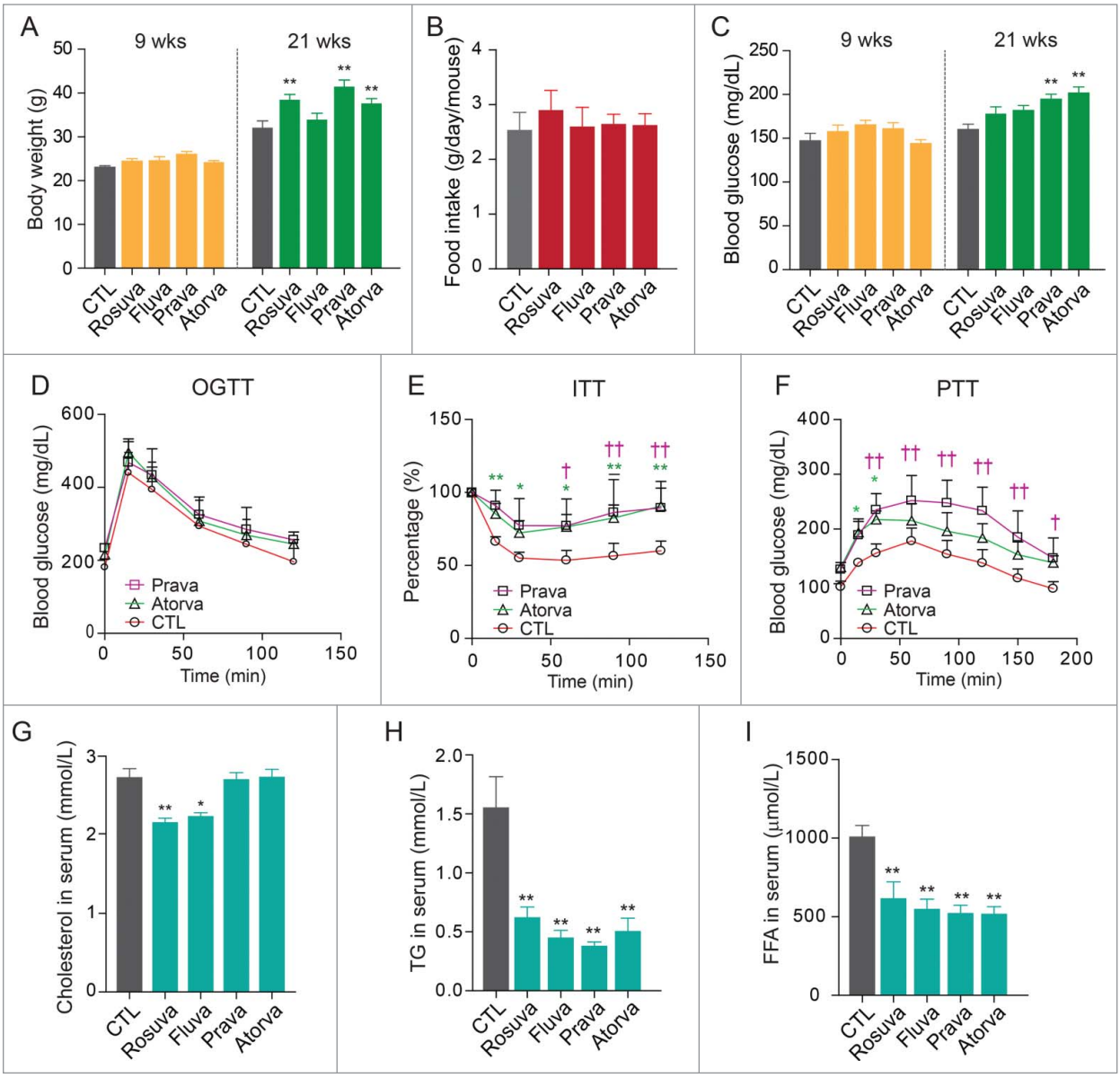

Figure 5. Statin treatment increases body weight and fasting blood glucose levels and impairs pyruvate tolerance in high-fat diet-fed mice. (**A**) Mice were fed a high-fat diet with or without a statin (0.01%, w/w) for 16 wk. Mean body weight gain was significantly higher in statin-treated mice compared with untreated control mice. (**B**) Food intake did not differ among the groups. (**C**) Fasting blood glucose levels were elevated in statin-treated mice. (**D**) Results of the oral glucose tolerance test performed at 20 wk of age showed no differences among groups (pravastatin-treated, atorvastatin-treated, and untreated control mice). (**E**) Results of the insulin tolerance test showed attenuated insulin responses in statin-treated mice. (**F**) Results of the pyruvate tolerance test performed at 21 wk of age showed elevated blood glucose levels in pravastatin- and atorvastatin-treated mice. Statin treatments significantly decreased serum cholesterol (**G**), triglyceride (TG) (**H**), and free fatty acid (FFA) (**I**) levels. *, $P < 0.05$; **, $P < 0.01$ compared with untreated mice (control, n = 9 ; rosuvastatin, n = 7 ; fluvastatin, n = 7 ; pravastatin, n = 11 and atorvastatin, n = 11).

of FOXO1 by statins in primary mouse hepatocytes suggests that statins promote gluconeogenesis enzymes independent of FOXO1 phosphorylation. AKT can induce the phosphorylation of FOXO1, making it accumulate in the cytoplasm. We found that AKT phosphorylation was decreased with statin treatment in primary mouse hepatocyte (**Fig. S4**). However, paradoxically, statins decreased the phosphorylation of AKT whereas they increased the phosphorylation of FOXO1 in primary mouse hepatocytes. This suggests that the statin-induced increase in FOXO1 phosphorylation is independent of insulin-AKT signaling. Insulin-PIK3C3-AKT-FOXO1 pathway itself is intact in primary cultured hepatocytes because insulin could increase the phosphorylation levels of FOXO1 and AKT, and wortmannin could decrease both the phosphorylation. Mechanisms other than phosphorylation, like FOXO1 acetylation,[25-28] XBP1 mediated FOXO1 proteasomal degradation[29] and/or O-GlcNAc glycation[30-32] could overcome the effect of FOXO1 phosphorylation and enhance gluconeogenesis. Therefore our data

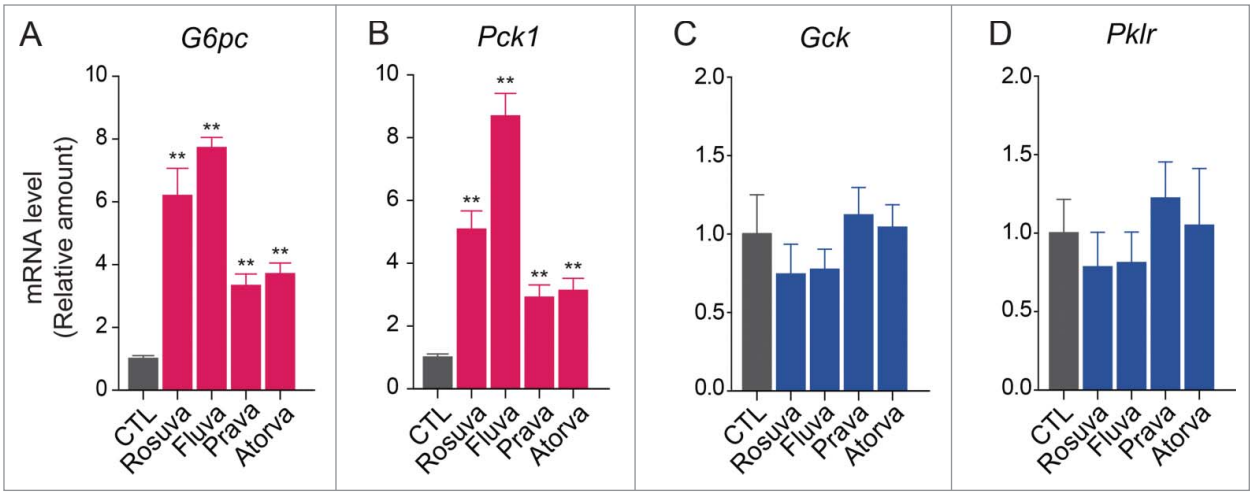

Figure 6. Statin treatment elevates expression of gluconeogenic enzymes but not glycolytic enzymes in the livers of statin-treated mice. Results of qRT-PCR showed that statins increase expression of *G6pc* (**A**) and *Pck1* (**B**), which encode gluconeogenic enzymes. In contrast, expression of *Gck* (**C**) and *Pklr* (**D**), which encode glycolytic enzymes, did not differ between statin-treated and untreated control mice. *, $P < 0.05$; **, $P < 0.01$ compared with control.

suggest that statin-induced elevation of gluconeogenesis is independent of FOXO1 phosphorylation.

Nevertheless, the mechanism by which statins affect MTOR activity is unknown. A recent study reports a link between MTOR signaling and intracellular cholesterol trafficking.[33] In this study, pharmacological depletion of cholesterol from the plasma membrane of endothelial cells inhibits MTOR activity, and this effect is partially reversed by restoration of cholesterol to the membrane, suggesting that MTOR is involved in sensing cellular membrane sterol concentrations.[33] Because statins block cholesterol synthesis in the liver,[34] these drugs may cause cholesterol depletion from the plasma membrane by suppressing de novo cholesterol synthesis. Indeed, statins have been reported to decrease membrane cholesterol levels in various cell types.[35,36] Moreover, mevalonate, the cholesterol intermediate just downstream of HMG-CoA, reverses statin-induced inhibition of MTOR signaling,[37,38] indicating that HMGCR inhibition is required for the suppression of MTOR activity. However, additional studies are needed to confirm that statins reduce membrane cholesterol level in hepatocytes and that statin-induced autophagy and gluconeogenesis are mediated through the suppression of MTOR activity.

The mechanism by which autophagy induces gluconeogenesis is also unclear. Autophagy is thought to be a survival mechanism during starvation that supplies amino acids for gluconeogenesis in the liver.[17,39] Accordingly, autophagy-deficient *atg5* knockout mice generally die from hypoglycemia within 24 h after birth,[40] and liver-specific *Atg7*-deficient mice fed a high-fat diet show improved insulin sensitivity and glucose tolerance compared to wild-type high-fat diet-fed mice.[41] Our results showing increased expression of *G6pc* and *Pck1* and elevated glucose output by statin-treated primary hepatocytes, and the blocking of these effects in the hepatocytes of liver-specific *Atg7*-deficient mice, strongly support the idea that statins stimulate gluconeogenesis through autophagy. A previous study suggests the opposite role of autophagy in gluconeogenesis with the finding that virus-mediated overexpression of *Atg7* reduces expression of *G6pc* and *Pck1* in the livers of mice.[42] However, the induction of autophagy by *Atg7* overexpression in this study is not verified; therefore, it is not clear that this effect is due to autophagy induction.

In addition, our study revealed a potential new mechanism underlying autophagy-induced gluconeogenesis: increased expression of the key gluconeogenic enzymes G6PC and PCK1.

We checked whether glucogenic amino acid, alanine, could enhance glucose output of HepG2 cell treated with statins. We found that treatment of HepG2 cells with alanine significantly increased glucose output in the absence of statin. In addition synergistic effects were seen when alanine and statins were treated simultaneously (**Fig. S5**). These data suggested that statin-induced gluconeogenesis is working.

Our results showing the attenuated glucose response to exogenous insulin and delayed glucose disposal after pyruvate loading in our mouse model provide additional evidence for elevated hepatic gluconeogenesis with statin treatment. No difference in oral glucose tolerance test results between treatment groups and controls indicates that chronic statin treatment did not impair the ability of pancreatic β-cells to secrete insulin under our experimental conditions. However, the effect of statins on insulin secretion has been controversial.[43-45] A previous study shows that pravastatin does not affect insulin secretion in a pancreatic β-cell line,[45] which is consistent with our result. In contrast, atorvastatin and simvastatin are reported to inhibit glucose-dependent insulin secretion by blocking calcium signaling in β-cells,[43,45] which differs from our result with atorvastatin. This discrepancy may be due to differences in the statin concentrations used, because pravastatin and atorvastatin clearly induced insulin resistance in high-fat diet-fed mice with normal pancreatic β-cell function. Although increased peripheral insulin resistance and impaired insulin secretion are considered the main pathophysiologic features of T2DM, hepatic insulin resistance manifested by

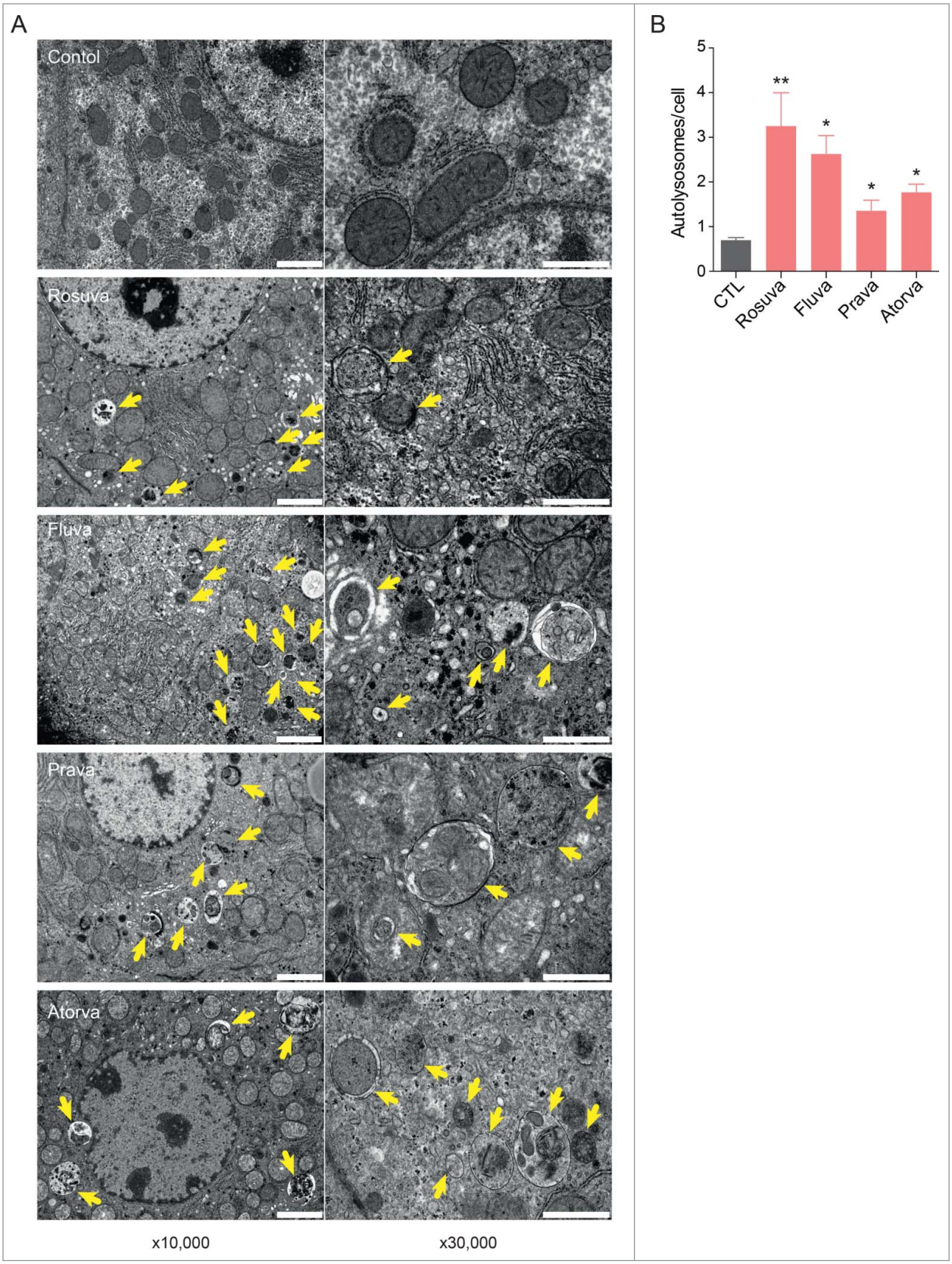

Figure 7. Electron microscopy analysis of autophagosomes in the livers of statin-treated mice. (**A**) Hepatocytes of statin-treated mice showed prominent vacuolization and autophagosomes, as assessed by transmission electron microscopy. Arrows indicate double membranes of autophagosomes; scale bars: 2 μm at ×10,000 and 1 μm at ×30,000 magnification. (**B**) All statins significantly increased autophagic vacuole formation in mouse livers. *, $P < 0.05$; **, $P < 0.01$ compared with control.

elevated gluconeogenesis is another important aspect of diabetic pathophysiology.[46] Consistent with this, our results showed that pravastatin and atorvastatin increased fasting glucose level, which is, at least in part, due to enhanced hepatic gluconeogenesis.

Collectively, these data showing statin-induced hepatic insulin resistance and gluconeogenesis in mice suggest that autophagy-induced hepatic gluconeogenesis is a potential mechanism of statin-induced T2DM in humans. In contrast to these results, one study reports that statin can improve insulin sensitivity in liver of obese mice.[47] There are some differences between the studies. Their report studies 4-wk-old Wistar rats and uses lovastatin (6 mg/kg/d) whereas we used C57BL/6J mice and the dose of statin was 0.01% of food weight. We administered statins for 16 wk while they included the statin treatment for a wk. Our study represents the chronic administration of statins.

In conclusion, our study found that chronic statin treatment contributes to the development of T2DM in mice. Statin treatment upregulated the gene expression of key enzymes involved in hepatic gluconeogenesis (*G6PC* and *PCK1*), increasing glucose production in the liver, and ultimately leading to hepatic insulin resistance. Our results showed that these effects are mediated through autophagy induction in the liver. This work advances our understanding of the mechanism underlying the effects of statins on insulin resistance and T2DM.

Materials and Methods

Cell culture and drug treatments

The primary hepatocytes and hepatocellular carcinoma HepG2 cell lines were cultured in Dulbecco's modified Eagle's medium (Thermo Scientific, SH30243.01) containing 10% fetal bovine serum (Thermo Scientific, SH30071.03), 100 U/ml penicillin, and 100 μg/ml streptomycin (Thermo scientific, SV30010) in a 5% CO_2 incubator at 37°C. The statin drugs rosuvastatin (Sigma-Aldrich, SML1264), fluvastatin (Sigma-Aldrich, Y0001090), pravastatin (Sigma-Aldrich, P4498), and atorvastatin (Sigma-Aldrich, PZ0001) were dissolved in dimethyl sulfoxide before dilution in the culture medium. In all experiments the final statin concentration was 20 μM, and final dimethyl sulfoxide concentration was $\leq$ 0.1%. Chloroquine (Sigma-Aldrich, C6628) and bafilomycin A_1 (Sigma-Aldrich, B1793) were dissolved in distilled water before treatments. The final concentration was 50 uM and 20 nM each, respectively.

Analysis of autophagy by confocal microscopy

HepG2 cells were transfected with the expression vector GFP-LC3 and mRFP-GFP-LC3 using Lipofectamine 2000 (Invitrogen, 11668–027) for 48 h. The cells were then treated with statins for 24 h and fixed in 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) for 10 min. After fixation, HepG2 cells were washed in phosphate-buffered saline (PBS; Amresco, E404–200TABS) 3 times for 5 min and then observed using LSM 700 and LSM780 confocal microscopes (Zeiss, Gottingen, Germany).

RNA extraction and qRT-PCR

Total RNA was extracted from HepG2 cells using the RNeasy Mini Kit (Qiagen, 74104) and from primary hepatocytes using TRIzol (Invitrogen, 15596–018). Reverse transcription was carried out with 2 μg total RNA using the QuantiTect Reverse Transcription kit (Qiagen, 205311) according to the manufacturer's instruction. Expression of target genes *G6PC*, *PCK1*, *GCK*, and *PKLR* was analyzed by qPCR using SYBR Premix Ex Taq (Clontech, RR420A) and gene-specific primers designed from sequences submitted to the NCBI nucleotide sequence database. Amplification was carried out using the Takara Thermal Cycler Dice® Real-Time system (Otsu, Shiga, Japan) and the following cycling conditions: 40 cycles of 95°C for 5 sec, 58°C for 10 sec, and 72°C for 20 sec. All reactions were performed in triplicate, and target gene expression was normalized to that of the internal control glyceraldehyde 3-phosphate dehydrogenase.

Immunoblotting

Cells were lysed in buffer consisting of 50 mM Tris-HCl, pH 8.0, 5 mM EDTA, 150 mM NaCl, 0.5% sodium deoxycholate (Sigma-Aldrich, D6750), 1% Nonidet P-40 (Sigma-Aldrich, 74385), 0.1% sodium dodecyl sulfate (Sigma-Aldrich, L3771), 1 mM phenylmethylsulfonyl fluoride, 1 mM sodium fluoride, 1 mM sodium orthovanadate, and protease inhibitor cocktail (Roche, 11 836 153 001). Equivalent amounts of each protein extract were separated on 10% polyacrylamide gels and electrophoretically transferred onto polyvinylidene fluoride membrane (Millipore, IPVH00010). After blocking, the membranes were incubated with primary antibodies against PCK1 (Santa Cruz Biotechnology, sc-32879), G6PC (Santa Cruz Biotechnology, sc-25840), FOXO1 (Cell Signaling Technology, 2880), phospho-FOXO1 (S256; Cell Signaling Technology, 9461), AKT (Cell Signaling Technology, 9272), phospho-AKT (S473; Cell Signaling Technology, #9271), LC3B (Sigma-Aldrich, L7543), ACTB (Sigma-Aldrich, A1978), BECN1 (Santa Cruz Biotechnology, sc-11427), MTOR (Santa Cruz Biotechnology, sc-8319), phospho-MTOR (S2448; Santa Cruz Biotechnology, sc-101738), RPS6KB (Santa Cruz Biotechnology, sc-230), and phospho-RPS6KB (T389; Santa Cruz Biotechnology, sc-11759) followed by horseradish peroxidase-conjugated IgG (Santa Cruz Biotechnology, sc-2371) and anti-rabbit IgG (Santa Cruz Biotechnology, sc-2030). The blots were developed using an enhanced chemiluminescent detection kit.

Glucose output assay

Glucose output from HepG2 cells was quantified using a colorimetric glucose assay kit (BioVision, K686–100) according to the manufacturer's instructions. Briefly, HepG2 cells were treated with or without statin for 24 h. The conditioned medium was then collected and incubated with the reaction mix for 30 min at room temperature. Absorbance at 450 nm was measured in a 96-well plate reader (Molecular Devices, Sunnyvale, CA).

BECN1 RNA interference

Electroporation of shRNA-expressing plasmids in HepG2 cells was performed using the Neon® transfection system (Invitrogen, MPK10096) according to the manufacturer's protocol. Briefly, trypsinized HepG2 cells (1×10^6 cells) were washed in PBS and then resuspended in Neon Resuspension Buffer R. The cell suspension was mixed with 2 μg shRNA against *BECN1* (Santa Cruz Biotechnology, sc-29797-SH) or a scrambled shRNA sequence (Santa Cruz Biotechnology, sc-108060) as a negative control and pulsed twice at 1200 V for 50 msec. After electroporation, cells were quickly seeded into 6-well plates and grown in culture medium for further experiments. Successful inhibition of *BECN1* expression was verified by western blot analysis.

Animals

Four-wk-old male C57BL/6J mice were housed under controlled conditions (21°C ± 2°C, 60% ± 10% humidity, 12-h light/12-h dark cycle) with ad libitum access to food and water. After 1 wk, the mice were divided into 5 groups according to treatment (untreated control, n = 9 ; rosuvastatin, n = 7 ; fluvastatin, n = 7 ; pravastatin, n = 11 ; atorvastatin, n = 11). Beginning at 5 wk of age, all mice were fed a high-fat diet that included 45% lipids (Research Diets, Inc., D12451). The food given to each treatment group was supplemented with 0.01% (w/w) of the appropriate statin. Food intake and body weight of the mice were evaluated 2 times a wk at the same time of day. Fasting blood glucose level was measured weekly in the evening after an 8-h fast. After 16 wk, the mice were anesthetized with zolazepam and tiletamine (Zoletil, 50 mg/kg; Virbac France GTIN: 03597132126045), and blood was collected by cardiac puncture. Primary hepatocytes were isolated from male liver-specific *atg7*-deficient mice[41] and wild-type mice (9 wk old) using a previously described method.[48] Atg7f/f mice were crossed with albumin promoter-driven Cre mice to generate liver-specific *atg7*-deficient mice[41] (atg7f/f;alb-Cre mice). The animal protocol was approved by the institutional animal care and use committee at Yonsei University College of Medicine.

Oral glucose tolerance, insulin tolerance, and pyruvate tolerance tests

To perform the oral glucose tolerance test, 40% glucose (2 g/kg body weight) was administered via oral gavage after a 6-h fast. Blood was collected from the tail vein at 0, 30, 60, 90, and 120 min after glucose administration. To assay insulin tolerance, fasting glucose was measured 4 h after fasting, and then mice were intraperitoneally injected with 0.75 U/kg human insulin-R (Sigma-Aldrich, I9278) dissolved in PBS. Blood glucose was measured at 15, 30, 60, 90, and 120 min after injection. To assay pyruvate tolerance, mice were intraperitoneally injected with 2 g/kg sodium pyruvate (Sigma-Aldrich, P2256) dissolved in PBS after an 18-h fast. Blood was collected from the tail vein before pyruvate injection (0 h) and at 15, 30, 60, 90, and 120 min after injection. Glucose levels were determined using an Accu-Chek Performa® glucometer (Boehringer-Mannheim, Indianapolis, IN).

Plasma glucose, cholesterol, triglyceride, and free fatty acid measurement

Blood was collected in microcentrifuge tubes and centrifuged to obtain serum, which was divided into aliquots and stored at -80°C for subsequent assays. Serum glucose, cholesterol, triglyceride, and free fatty acid levels were measured with the respective assay kits (BioAssay Systems, EBGL-100 for glucose, ECCH-100 for cholesterol, ETGA-200 for triglyceride and EFFA-100 for free fatty acid) according to the manufacturer's instructions.

Transmission electron microscopy

Autophagic vacuoles in the liver were visualized by transmission electron microscopy. Glutaraldehyde-fixed mouse liver tissues were postfixed in 2% osmium tetroxide, dehydrated in graded alcohol, and flat embedded in Epon 812 (Electron Microscopy Sciences, 100503–876). Ultrathin tissue sections (300 nm) were stained with uranyl acetate and lead citrate and examined with an electron microscope (JEM-1011, JEOL/Mega-View III, Olympus, Tokyo, Japan).

Statistical analysis

Data are presented as mean ± standard error of the mean. Groups were compared using the Student *t* test or one-way analysis of variance followed by the Dunnett multiple comparison test, where appropriate; $P < 0.05$ was considered significant. Data analysis was carried out using Prism 5.0 software (GraphPad Software, La Jolla, CA).

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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