Chronic HMG-CoA Reductase Inhibitor Treatment Contributes to Dysglycemia by Upregulating Hepatic Gluconeogenesis through Autophagy Induction

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Directed by Professor Eun Seok Kang

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ABSTRACT

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Statins (3-hydroxy-3-methyl-glutaryl-CoA [HMG-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 glucose 6-phosphatase and phosphoenolpyruvate carboxykinase 1) *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, statins' ability to increase gluconeogenesis was impaired when autophagy-related 7 protein and beclin 1 were 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.

Key words: HMG-CoA reductase inhibitor, statin, gluconeogenesis, diabetes, autophagy

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I. INTRODUCTION

Statins (3-hydroxy-3-methyl-glutaryl-CoA [HMG-CoA] reductase inhibitors) are the most widely prescribed drugs for the primary and secondary prevention of cardiovascular diseases. Statins are a class of cholesterol lowering drugs that inhibit the enzyme HMG-CoA reductase which plays an important role in the production of cholesterol. Hypercholesterolemia have been related with cardiovascular disease.[1] Statins have been discovered to prevent cardiovascular disease.

Although these drugs effectively reduce circulating cholesterol levels, numerous studies have demonstrated that statin therapy is linked to side effects include muscle pain, increased risk of diabetes mellitus, and abnormalities in liver enzyme tests.[2] In this study, we focused on how statin can develop of type 2 diabetes mellitus (T2DM). Increased incidence of diabetes was observed in clinical trials evaluating pravastatin (Pravastatin or Atorvastatin Evaluation and Infection Therapy),[3] simvastatin (Heart Protection Study),[4] atorvastatin (Anglo-Scandinavian Cardiac Outcomes Trial—Lipid-Lowering Arm),[5] and rosuvastatin (Controlled Rosuvastatin

Multinational Trial in Heart Failure; Justification for the Use of Statins in Prevention: an Intervention Trial Evaluating Rosuvastatin).[6,7] Recent meta-analyses of major statin trials also demonstrated that the risk for T2DM is higher in statin users than in non-users.[8,9] This effect is dose-dependent[10]: 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[11] and transported into hepatocytes by a high-affinity process.[12] The hepatic specificity of statins may be due to efficient first-pass metabolism.[13] 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[14] and induce gluconeogenic gene expression in primary cultured human hepatocytes.[15] 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. Also autophagy is a scavenging process that disassembles through a tightly regulated pathway, unnecessary or dysfunctional cellular components. During this process, targeted cytoplasmic ingredients are isolated from the rest of the cell within a double-membrane vesicle known as an autophagosome. The autophagosome then fuses with a lysosome and the contents are degraded and recycled.[16] There are three different forms of autophagy that are commonly described, namely macroautophagy, microautophagy and chaperone-mediated autophagy.[17] In the context of disease, autophagy has been seen as an adaptive response to stress which promotes survival, whereas in other cases it

appears to promote cell death and morbidity.[16] This process also supplies various substrates for energy generation, leading to alterations in cell metabolism.[18] In carbohydrate metabolism, autophagy contributes to glycogen breakdown in lysosomes[19] and the maintenance of pancreatic β-cell mass and function.[20] In the liver, autophagy appears to play an important role in glucose homeostasis by promoting the conversion of amino acids to glucose.[21]

Statins have been shown to induce autophagy in various cell types (e.g., macrophages, cancer cells, coronary arterial myocytes),[22-25] 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.

II. MATERIALS AND METHODS

1. Cell culture and drug treatments

Primary hepatocytes were isolated from male liver-specific *Atg7* knockout mice[26] and wild type mice (9 weeks old) using a previously described method.[27] The primary hepatocytes and hepatocellular carcinoma HepG2 cell lines were cultured in Dulbecco's modified Eagle's medium (Thermo Scientific Inc., San Jose, CA) containing 10% fetal bovine serum (Thermo Scientific Inc., San Jose, CA), 100 U/ml penicillin, and 100 μg/ml streptomycin (Thermo Scientific Inc., San Jose, CA) in a 5 %CO_2 incubator at 37°C. The statin drugs rosuvastatin (Sigma-Aldrich Co., St. Louis, MO), fluvastatin (Sigma-Aldrich Co., St. Louis, MO), pravastatin (Sigma-Aldrich Co., St. Louis, MO), and atorvastatin (Sigma-Aldrich Co., St. Louis, MO) 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 ≤ 0.1%. Chloroquine (Sigma-Aldrich Co., St. Louis, MO) and Bafilomycin A1 (Sigma-Aldrich Co., St. Louis, MO) were dissolved in distilled water before treatments. The final concentrations were 50 uM and 20 nM, respectively.

2. Analysis of autophagy by confocal microscopy

HepG2 cells were transfected with the expression vector GFP-LC3 and mRFP-GFP-LC3 using Lipofectamine 2000 (Invitrogen, CA) for 48 hours. The cells were then treated with statins for 24 hours 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 three times for 5 min and then observed using an LSM 700 and LSM780 confocal microscopes (Zeiss, Gottingen, Germany).

3. RNA extraction and real-time RT-PCR (quantitative PCR)

Total RNA was extracted from HepG2 cells using the RNeasy Mini Kit (Qiagen, CA, United States) and from primary hepatocytes using TRIzol (Invitrogen, CA, United States). Reverse transcription was carried out with 2 μg total RNA using the QuantiTect Reverse Transcription kit (Qiagen, CA, United States) 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, USA) 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.

Table 1. Primer sequences for qPCR of G6PC, PCK1, GCK, PKLR and ATG7

Target	Species	Direction	Sequence
G6PC	Human	Forward	5'-GGGTGTAGACCTCCTGTGGA-3'
		Reverse	5'-GAGCCACTTGCTGAGTTTCC-3'
PCK1	Human	Forward	5'-AGGCGGCTGAAGAAGTATGA-3'
		Reverse	5'-ACGTAGGGTGGATCCGTCAG-3'
GCK	Human	Forward	5'-GCAGAAGGGAACAATGTCGTG-3'
		Reverse	5'-CGTAGTAGCAGGAGATCATCGT-3'
PKLR	Human	Forward	5'-TCAAGGCCGGGATGAACATTG-3'
		Reverse	5'- CTGAGTGGGGAACCTGCAAAG-3'
G6pc	Mouse	Forward	5'-ACTCCAGCATGTACCGGAAG-3'
		Reverse	5'-AAGAGATGCAGGAGGACCAA-3'
Pck1	Mouse	Forward	5'-TGACAGACTCGCCCTATGTG-3'
		Reverse	5'-TGCAGGCACTTGATGAACTC-3'
Gck	Mouse	Forward	5'-AAAGATGTTGCCCACCTACG-3'
		Reverse	5'-TCTGGTGTTTCGTCTTCACG-3'
Pklr	Mouse	Forward	5'-GTTTGTGCCACACAGATGCT-3'
		Reverse	5'-ATACAGTCAGCCCCATCCAG-3'
Atg7	Mouse	Forward	5'-TGGAGCAGTCAGCAAATGAG-3'
		Reverse	5'-CGAAGGTCAGGAGCAGAAAC-3'

4. 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, 1 % Nonidet P-40, 0.1 % sodium dodecyl sulfate, 1 mM phenylmethylsulfonyl fluoride, 1 mM sodium fluoride, 1 mM sodium orthovanadate, and protease inhibitor cocktail (Roche, Mannheim, Germany). Equivalent amounts of each protein extract were separated on 10 % polyacrylamide gels and electrophoretically transferred onto polyvinylidene fluoride membrane (Millipore, Darmstadt, Germany). After blocking, the membranes were incubated with primary antibodies against PEPCK (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), G6Pase (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), FoxO1 (Cell Signaling Technology, Inc., Danvers, MA), phospho-FoxO1 (S256) (Cell Signaling Technology, Inc., Danvers, MA), Akt (Cell Signaling Technology, Inc., Danvers, MA), phospho-Akt (S473) (Cell Signaling Technology, Inc., Danvers, MA), LC3B (Sigma-Aldrich Co., St. Louis, MO), β-actin (Sigma-Aldrich Co., St. Louis, MO), Beclin 1 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) mTOR (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), phospho-mTOR (S2448) (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), p70 S6 kinase α (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), and phospho-p70 S6 kinase α (T389) (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) followed by horseradish peroxidase-conjugated IgG (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) and anti-rabbit IgG (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). The blots were developed using an enhanced chemiluminescent detection kit.

5. Glucose output assay

Glucose output from HepG2 cells was quantified using a colorimetric glucose assay kit (BioVision Technologies, Inc, Exton, PA) according to the manufacturer's instructions. Briefly, HepG2 cells were treated with or without statin for 24 hours. 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).

6. *BECN1* RNA interference

Electroporation of shRNA-expressing plasmids in HepG2 cells was performed using the Neon® transfection system (Invitrogen, CA, USA) 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, Inc., Santa Cruz, CA) or a scrambled shRNA sequence (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) as a negative control and pulsed twice at 1200 V for 50 msec. After electroporation, cells were quickly seeded into six-well-plates and grown in culture medium for further experiments. Successful inhibition of *BECN1* expression was verified by Western blot analysis.

7. Animals

Four week-old male C57BL/6J mice were housed under controlled conditions (21 °C ± 2 °C, 60% ± 10% humidity, 12-hour light/12-hour dark cycle) with *ad libitum* access to food and water. After 1 week, the mice were divided into five groups according to treatment (untreated control, n=9; rosuvastatin, n=7; fluvastatin, n=7; pravastatin, n=11; atorvastatin, n=11). Beginning at 5 weeks of age, all mice were fed a high-fat diet that included 45 % lipids (Research Diets, Inc., New Brunswick, NJ). 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 two times a week at the same time of day. Fasting blood glucose level was measured weekly in the evening after an 8-hour fast. After 16 weeks, the mice were anesthetized with zolazepam and tiletamine (Zoletil, 50 mg/kg; Virbac France GTIN: 03597132126045), and blood was collected by cardiac puncture. The animal protocol was approved by the institutional animal care and use committee at Yonsei University College of Medicine.

8. 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-hour 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 hours after fasting, and then mice were intraperitoneally injected with 0.75 U/kg human insulin-R (Sigma-Aldrich Co., St. Louis, MO) 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 Co., St. Louis, MO) dissolved in phosphate buffered saline after an 18-hour fast. Blood was collected from the tail vein before pyruvate injection (0 hour) 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).

9. 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, Hayward, CA) according to the manufacturer's instructions.

10. Transmission electron microscopy

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

11. Statistical analysis

Data are presented as mean ± standard error of the mean. Groups were compared using Student's t test or one-way analysis of variance followed by Dunnett's 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).

III. RESULTS

1. 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. 1).

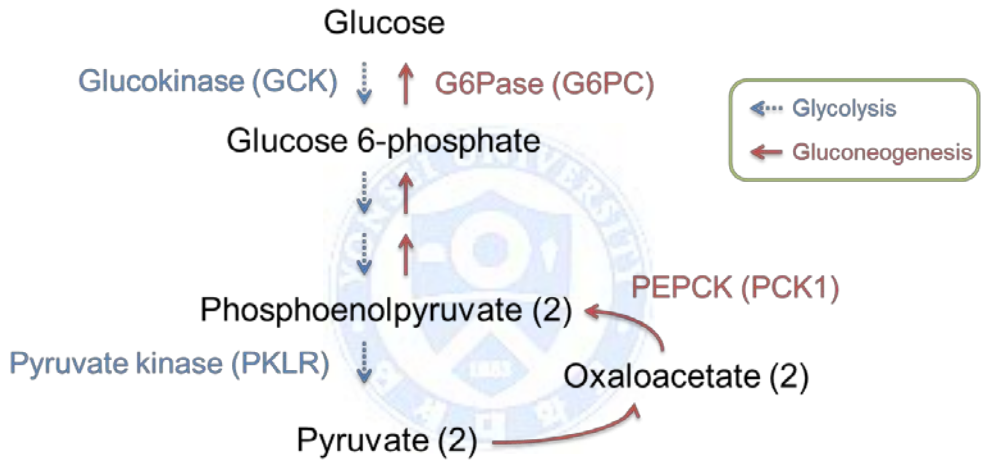

Figure 1. Schematic illustration of glycolysis and gluconeogenesis. Glycolysis is the metabolic pathway that converts glucose into pyruvate. Gluconeogenesis is the pathway that results in the generation of glucose from non-carbohydrate carbon substrates such as pyruvate.

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 glucose 6-phosphatase (*G6PC*) and phosphoenolpyruvate carboxykinase (*PCK1*) and increased expression of genes encoding the glycolytic enzymes glucokinase (*GCK*) and pyruvate kinase (*PKLR*) (Fig. 2).

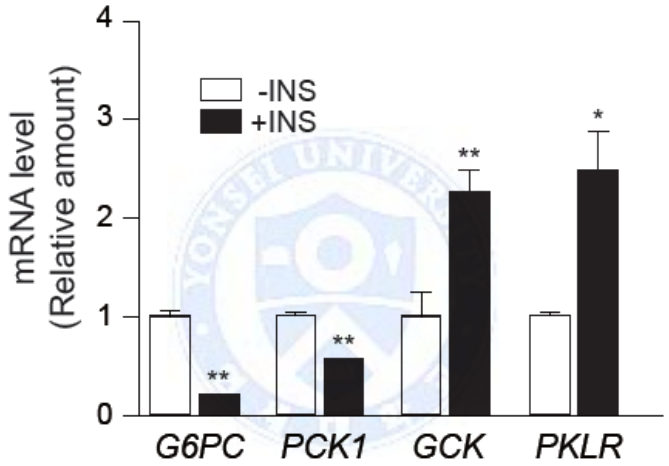

Figure 2. Insulin inhibits gluconeogenic gene expression and enhances glycolytic gene expression in HepG2 cells. HepG2 cells were treated with 100nM insulins for 6hr and harvested for RNA extraction. *p<0.05, **p<0.01 compared with control. (n=5)

After 24-hour treatment with rosuvastatin, fluvastatin, pravastatin, or atorvastatin (20 μM), mRNA levels of *G6PC* and *PCK1* increased in response to each statin (Fig. 3A and Fig. 3B) and protein levels of these enzymes also increased (Fig. 3C), suggesting increased gluconeogenesis.

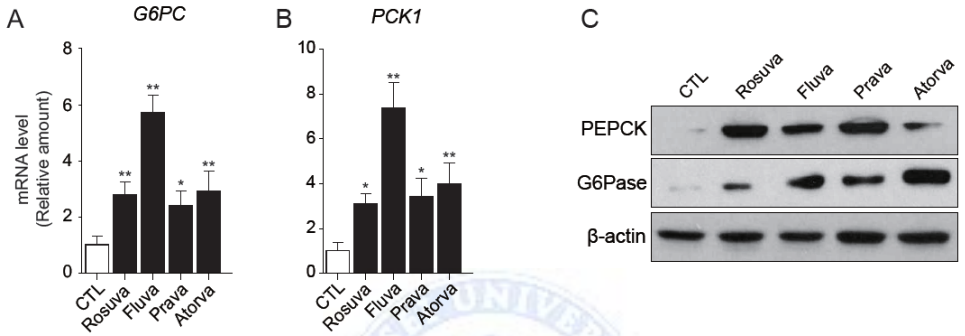

Figure 3. Statins increase gluconeogenic gene expression in HepG2 cells. HepG2 cells were treated with statins (20 μM) for 24hours. Statins upregulated not only mRNA levels of (A) *G6PC* and (B) *PCK1*, (C) but also protein levels of PEPCK and G6Pase in HepG2 cells. **p*<0.05, ***p*<0.01 compared with control. (n=5)

In contrast, statins had little or no effect on mRNA levels of *GCK* and *PKLR* (Fig. 4A and Fig. 4B). These data indicate that statins specifically affect the expression of gluconeogenic enzymes in HepG2 cells.

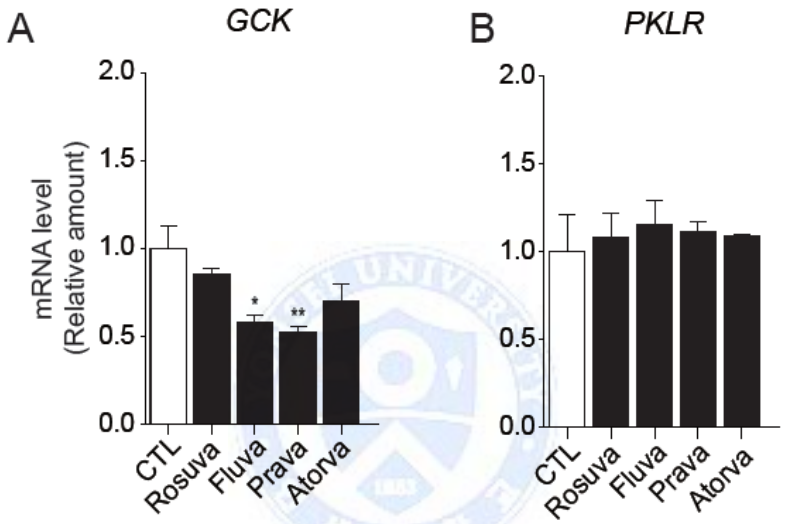

Figure 4. Statin has no direct effect on glycolytic gene expression in HepG2 cells. HepG2 cells were treated with statins 24hours. But statins little effected on mRNA levels of *GCK* (A) and *PKLR* (B) in HepG2 cells. **p*<0.05, ***p*<0.01 compared with control. (n=5)

2. Statins induce autophagic flux leading to enhanced expression of gluconeogenic enzymes

Because autophagy has been reported to promote gluconeogenesis in the liver,[21] we tested whether statins increase autophagy in hepatocytes by transfecting HepG2 cells with a vector expressing the autophagy marker, microtubule-associated protein 1 light-chain 3 isoform A (LC3A) 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. 5A and Fig. 5B). In contrast, puncta were barely discernible in control cells (Fig. 5A and Fig. 5B).

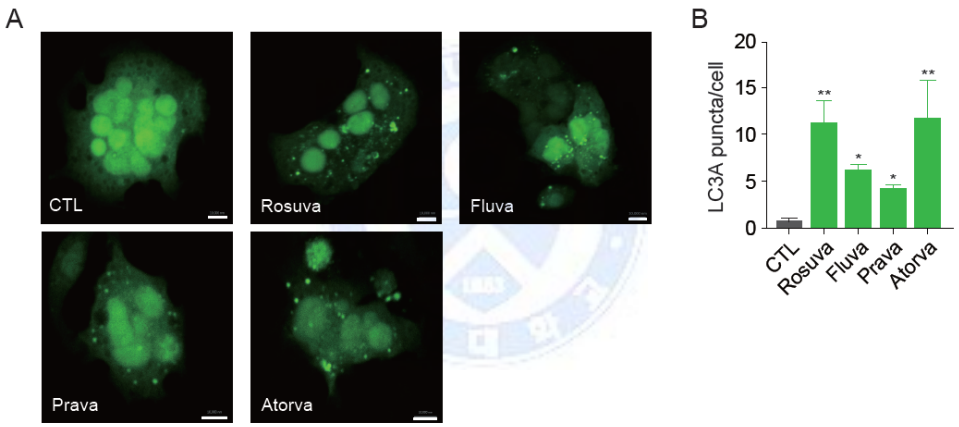

Figure 5. Statins induce autophagic vacuoles in HepG2 cells. HepG2 cells were transfected with the autophagy sensor GFP-LC3A then treated with rosuvastatin, fluvastatin, pravastatin, or atorvastatin (20 μM) for 24 hours. (A) Fluorescent images were obtained by confocal microscopy. Green dots indicate autophaic vacuoles; Scale bars indicate 1μm at x400 magnification. (B) Columns in the histogram represent the number of LC3A puncta per cell. At least six random fields were chosen from each sample. *p<0.05, **p<0.01 compared with control. (n=8)

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. 6). We treated cells with bafilomycin A1 (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. Statin additionally increased LC3B-II in cells pretreated with Baf A1 which suggests that statin induces autophagy rather than blocking autophagy flow (Fig. 6).

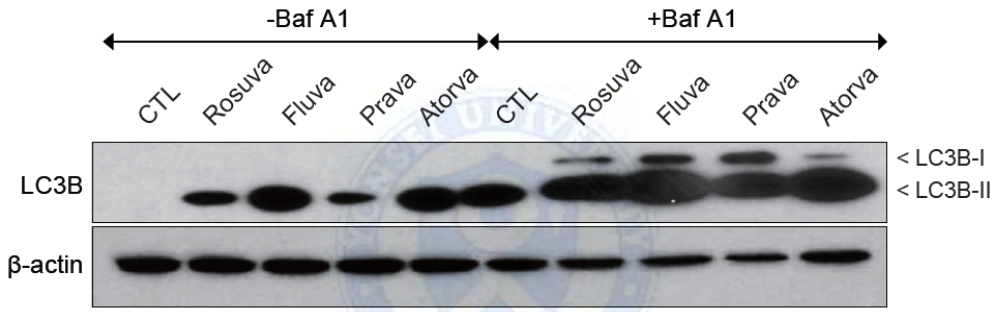

Figure 6. Statins increase autophagy flux in HepG2 cell. HepG2 cells were treated with statins for 22hours after then bafilomycin A1 for 2 hours with statins. Results of Western blot analysis showed increased LC3B-II levels in statin-treated cells. Bafilomycin-treated group showed that statins induced autophagy flux. (n=4)

Then we transfected mRFP (red fluorescence protein)-GFP-LC3 tandem construct encoding LC3 fused to mRFP and GFP to HepG2 cells to evaluate autophagic flux.

GFP protein is degraded in acidic condition inside the lysosome, leading to lose of green fluorescent signal whereas RFP is more stable in acidic condition, maintaining red fluorescent signal. Therefore autophagosomes show 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 increase in autophagic flux (Fig. 7A and Fig. 7B). 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 increase in red puncta (Fig. 7A and Fig. 7B).

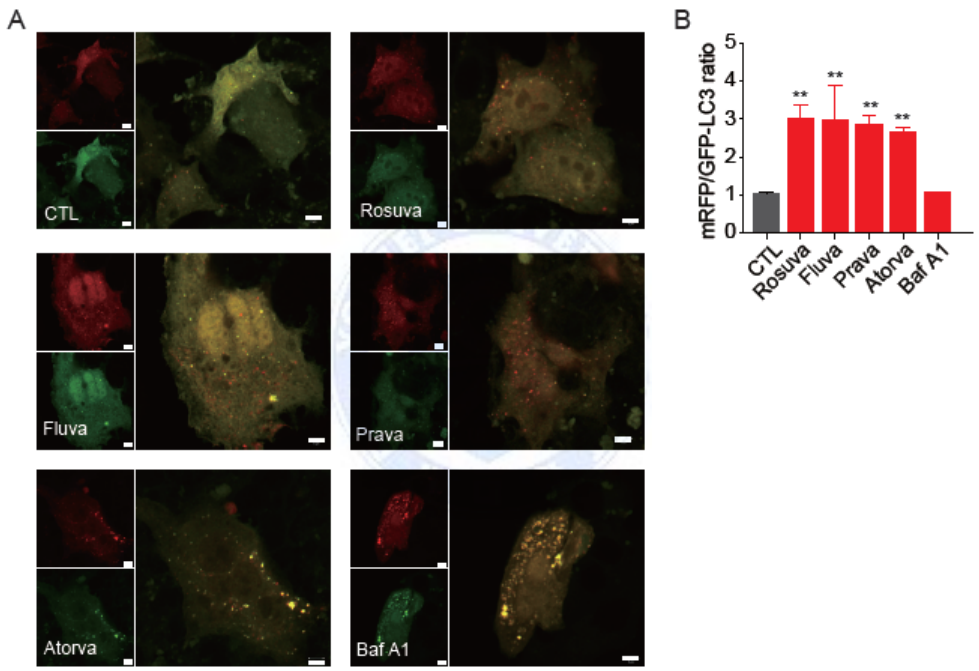

Figure 7. Stains increase autolysosome formation in HepG2 cells. After transfecting HepG2 cells with mRFP-GFP-LC3B, statins were treated for 24 hours. (A) Fluorescent images were obtained by confocal microscopy. GFP protein is unstable in low pH inside of lysosome thereby degraded. In contrast, RFP is more stable in acidic condition thereby could maintain red fluorescence; Scale bars indicate 5μm at x800 magnification. (B) Columns in the histogram represent the ratio of mRFP and GFP LC3B puncta. $^{**}p<0.01$ compared with control. (n=5)

3. Blockage of autophagic flux attenuates statin's effect on gluconeogenesis.

To see whether statin's gluconeogenic effect is mediated by autophagic process, we treated a lysosomal inhibitor such as chloroquine (CQ) or Baf A1 with statin. Increased *G6PC* and *PCK1* expression with statin treatment were attenuated by CQ (Fig. 8A and Fig. 8B) and glucose production was also decreased (Fig. 8C).

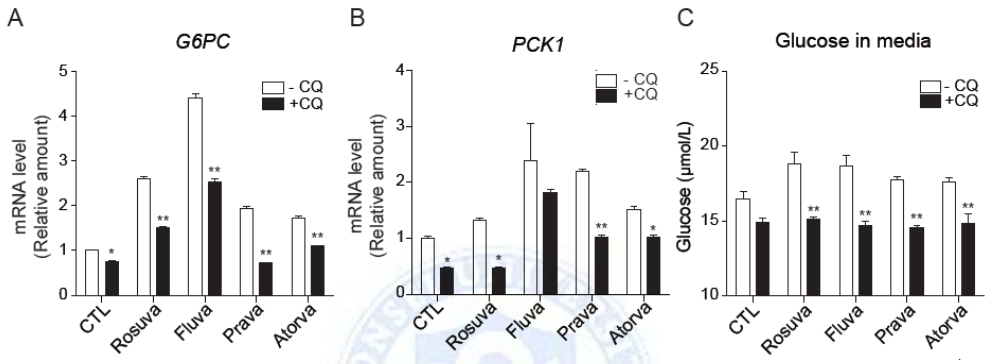

Figure 8. Chloroquine decrease gluconeogenic gene expression and glucose concentration in culture media. After treatment with 20 μM statins for 22 hours, 50μM chloroquine was treated to HepG2 cells for 2 hours with statins. HepG2 cells treated with statins and CQ (A) *G6PC* and (B) *PCK1* were analyzed with qRT-PCR. (C) Glucose production by HepG2 cells treated with statins and CQ. **p*<0.05, ***p*<0.01 compared with no CQ group. (n=5)

Inhibition of autophagic process with Baf A1 treatment resulted in attenuation of increased *G6PC* and *PCK1* expression with statin (Fig. 9A and Fig. 9B). In addition glucose production was also decreased with Baf A1 (Fig. 9C).

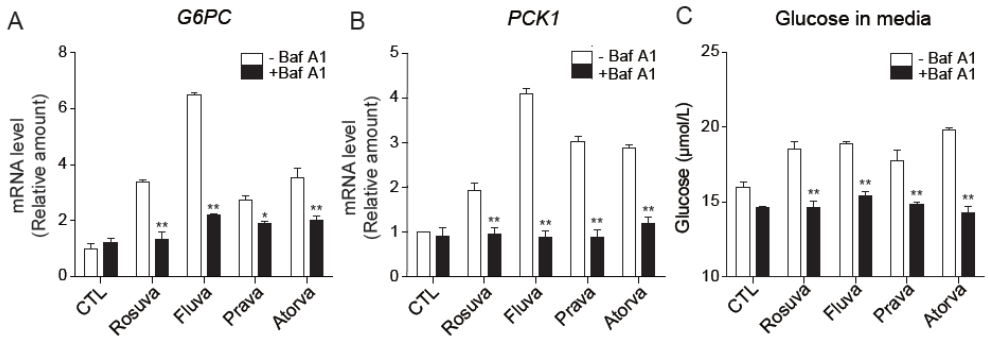

Figure 9. Bafilomycin A1 decrease gluconeogenic gene expression and glucose concentration in culture media. HepG2 cells treated with statins and 20 nM bafilomycin A1 for 2hours to confirm autophagy-dependent gluconeogenesis. (A) *G6PC* and (B) *PCK1* were analysed with qRT-PCR. (C) Glucose production was measured. **p*<0.05, ***p*<0.01 compared with no Baf A1 group. (n=5)

To better understand the effect of statins on autophagy, we transfected HepG2 cells with short hairpin RNAs (shRNAs) against the gene encoding beclin-1 (*BECN1*), which plays an important role in autophagy induction.[28] After confirming the knockdown of *BECN1* (Fig. 10A), we observed statin-dependent increases in *G6PC* and *PCK1* mRNA levels in transfected cells (Fig. 10B and Fig. 10C). These data support the role of autophagy in statin-induced gluconeogenesis.

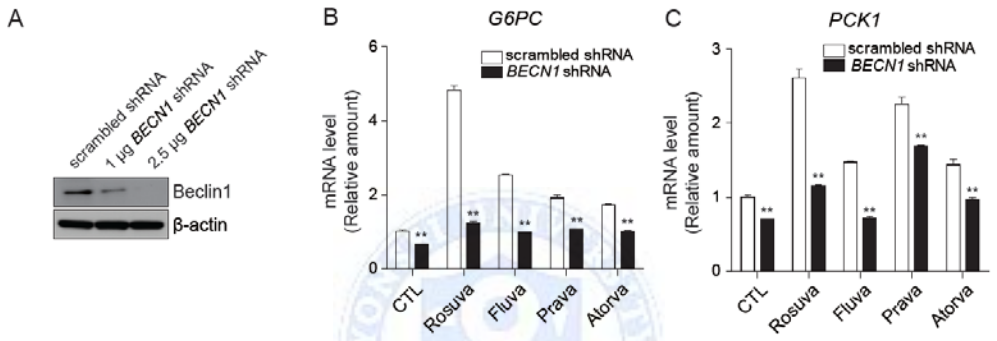

Figure 10. Statin has no effect on gluconeogenic gene expression without autophagic related beclin 1 expression. HepG2 cells were transfected with *BECN1* shRNA. (A) After 48hours, knockdown of *BECN1* was confirmed by Western blot analysis. Inhibition of autophagy by *BECN1* knockdown decreased expression of (B) *G6PC* and (C) *PCK1*. **p*<0.05, ***p*<0.01 compared with control cells transfected with scrambled shRNA. (n=5)

4. 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 autophagy-related 7 (*Atg7*) knockout mice. After the loss of *Atg7* in the liver was confirmed by RT-PCR (Fig. 11A), the cultured hepatocytes were treated with statins for 24 hours. Our results showed that the statin-induced increase in expression of gluconeogenic enzymes (*G6pc* and *Pck1*) was blocked in *Atg7* knockout hepatocytes (Fig. 11B). In addition, glucose output did not increase in response to statins in hepatocytes lacking *Atg7* (Fig. 11C). 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.

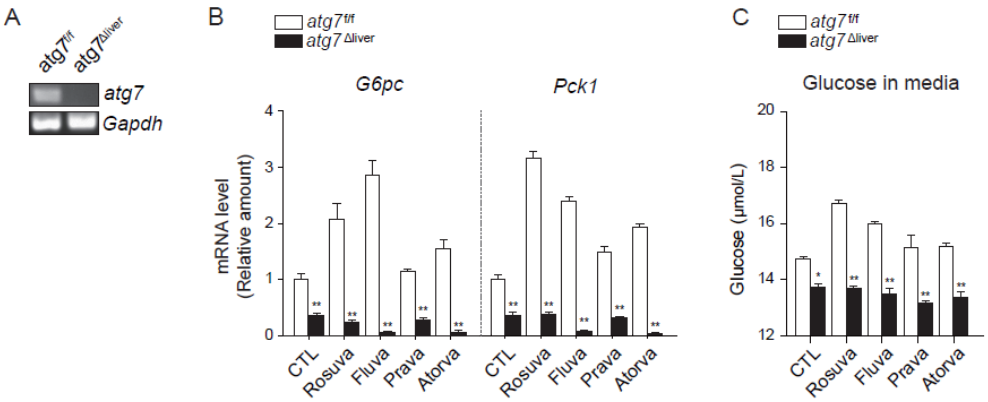

Figure 11. The gluconeogenic effect of statins is attenuated in primary hepatocytes derived from liver-specific *Atg7*-deficient mice. We isolated primary hepatocyte from *Atg7*-deficient mice. (A) Loss of *Atg7* in liver tissue of knockout mice was confirmed by qRT-PCR. (B) Primary hepatocytes derived from wild type and *Atg7* knockout mice were treated with statins (20 μM) for 24 hours. Increased expression of *G6pc a*nd *Pck1* was observed in wild type hepatocytes but not in *Atg7* knockout hepatocytes. (C) Glucose output by cultured hepatocytes from wild type or liver-specific *Atg7* knockout mice after 24-hour statin treatment. *p<0.05, **p<0.01 compared with wild type hepatocytes. (n=4)

5. Akt-FoxO1 pathway has no effect on statin induced gluconeogenesis but decrease mTOR phosphorylation.

To check gluconeogenesis was accelerated by statins, we tested 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. 12). These data suggested that statin-induced gluconeogenesis is working.

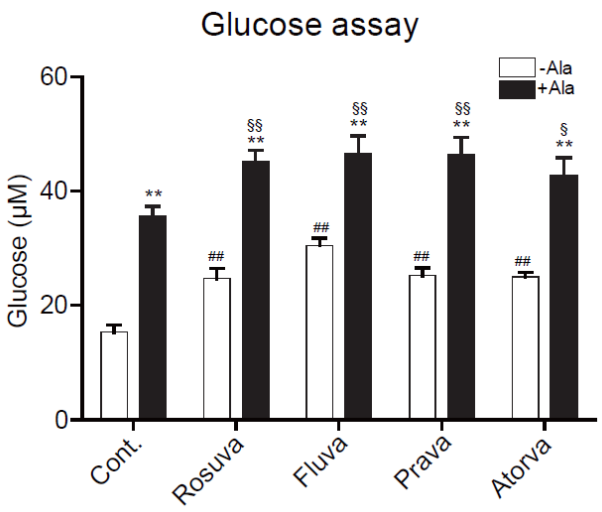

Figure 12. Glucogenic amino acid, alanine, enhances glucose output of HepG2 cell treated with statins. For glucose production assay, primary hepatocytes were incubated with statins for 18 hours in hepatocyte medium (DMEM with glucose and phenol red). Then we changed the medium with glucose production medium with/without alanine. After 6 hours, we collected glucose production medium and measured glucose concentration using glucose assay kit. Glucose production medium (DMEM without glucose and without phenol red; GIBCO A14430-01, 2 mM sodium pyruvate, 20 mM sodium lactate, 2 mM L-glutamine and 15mM HEPES) $^{**}P<0.01$ for –Ala vs. +Ala, $^{\#\#}P<0.01$ for control vs. statins in the no alanine treatment group, $^{\S}P<0.05$ and $^{\S\S}P<0.01$ for control vs. statins in the alanine treatment group (n=8).

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. 13).

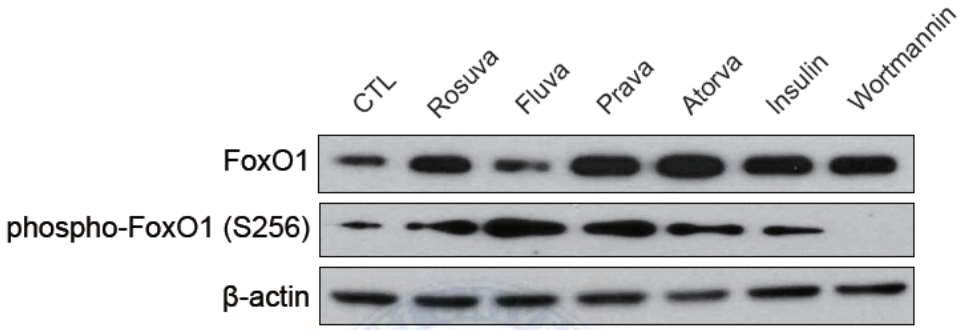

Figure 13. Elevated FoxO1 phosphorylation by statins in primary hepatocyte. FoxO1 phosphorylation level was increased with insulin treatment and decreased with Wortmannin, a PI3K inhibitor in primary mouse hepatocyte. Cells were treated with statins for 24 hours, and treated with insulin and Wortmannin for 6 hours.

The canonical insulin signaling pathway for regulation of glucose metabolism involves phosphorylation and export of the transcription factor FoxO1 out of the nucleus. Staying FoxO1 in the nucleus induces the transcriptional induction of gluconeogenic enzymes. Increased phosphorylation 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 accumulated in the cytoplasm. We found that Akt phosphorylation was decreased with by statin treatment in primary mouse hepatocyte (Fig. 14).

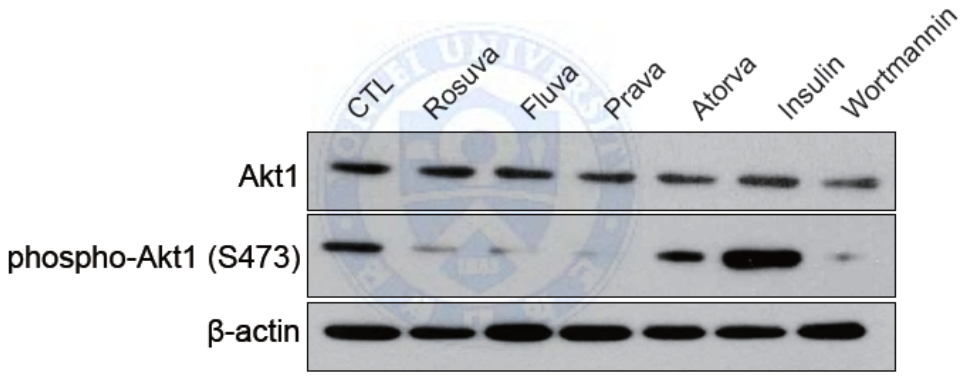

Figure 14. Akt phosphorylation was decreased with statin treatment in primary mouse hepatocyte. Akt phosphorylation level was increased with insulin treatment and decreased with Wortmannin, a PI3K inhibitor.

Because the role of mTOR in autophagy inhibition is well-established,[25,29] and simvastatin has been reported to suppress mTOR signaling in cardiomyocytes *in vitro* and *in vivo*,[25] we examined the effect of statins on mTOR complex 1and downstream target in HepG2 cells. Statins attenuated the mTOR and p70 S6 kinase α phosphorylation which are enhanced with insulin treatment in HepG2 cells (Fig. 15).

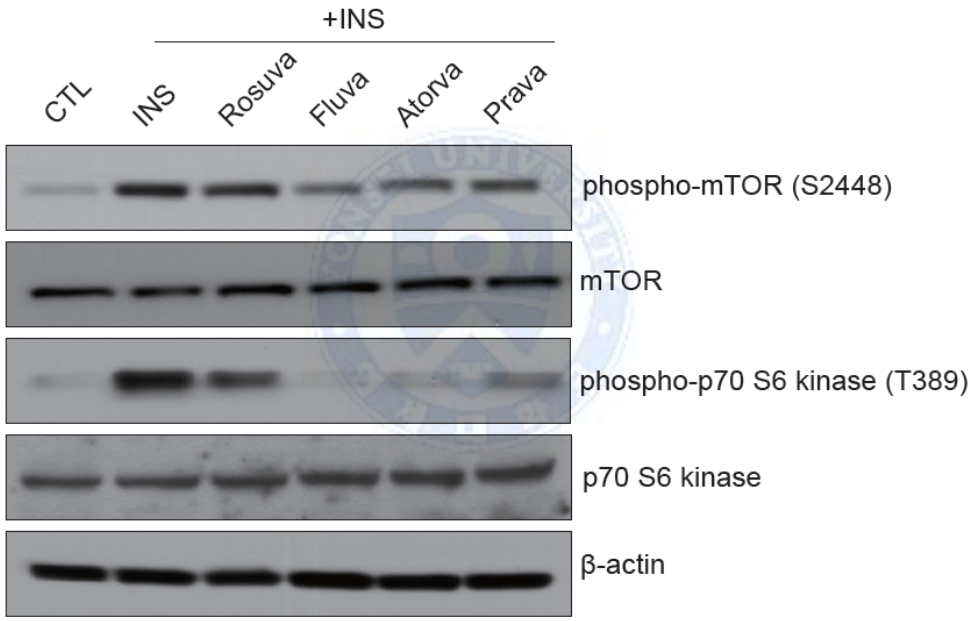

Figure 15. Down-regulation of mTOR signaling by statin treatment. Insulin treatment increased the levels of phospho-mTOR (S2448) and phospho-p70 S6 kinase α (T389). However, these were blocked by statin treatment in HepG2 cells.

6. 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 weeks of age mice were fed a high-fat diet supplemented with rosuvastatin, fluvastatin, pravastatin, or atorvastatin for 16 weeks. Mean body weight significantly increased in statin-treated mice compared with untreated mice (Fig. 16A), independent of food intake (Fig. 16B).

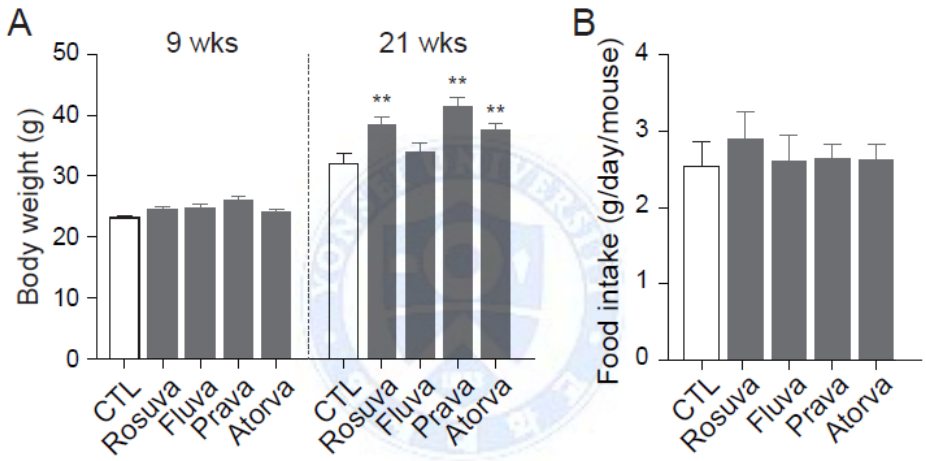

Figure 16. Statin treatment increased body weight of high fat diet fed mice whereas did not affect amount of food intake. 5 weeks old mice were fed a high-fat diet with or without a statin (0.01%, w/w) for 16 weeks. (A) Mean body weight gain was significantly higher in statin-treated mice compared with untreated control mice. (B) Food intake did not differ among the groups. *p<0.05, **p<0.01 compared with CTL group. (untreated control, n=9; rosuvastatin, n=7; fluvastatin, n=7; pravastatin, n=11; atorvastatin, n=11)

We measured fasting blood glucose levels and performed the oral glucose tolerance test and insulin tolerance test at week 15 and pyruvate tolerance test at week 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. 17).

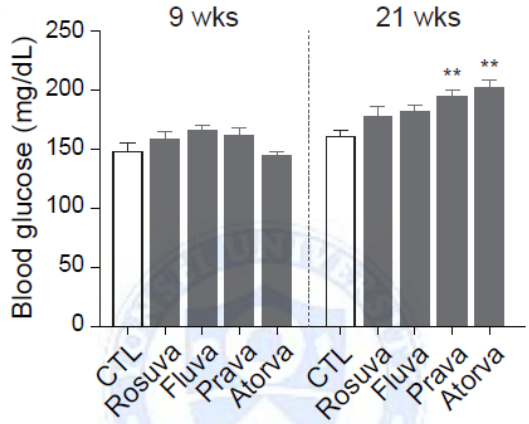

Figure 17. Fasting blood glucose levels were elevated in statin-treated mice. Mice were measured blood glucose after 8 hours fasting at each time points. **p*<0.05, ***p*<0.01 compared with CTL group. (untreated control, n=9; rosuvastatin, n=7; fluvastatin, n=7; pravastatin, n=11; atorvastatin, n=11)

Results of the oral glucose tolerance test did not differ between pravastatin- and atorvastatin-treated mice and control mice (Fig. 18A), indicating that pancreatic β-cell function was not impaired at 15 weeks. However, blood glucose levels failed to decrease upon insulin treatment in statin-treated mice, indicating insulin resistance (Fig. 18B), and area under the curve for the insulin tolerance test differed significantly between the control group and the statin-treated groups (Fig. 18D and 18E). To evaluate the possibility that statins induce hepatic insulin resistance by increasing hepatic glucose production, we performed the pyruvate tolerance test,

which showed significantly elevated blood glucose levels in pravastatin- and atorvastatin-treated mice over 3 hours (Fig. 18C and Fig. 18F). These data suggest that pravastatin and atorvastatin increase blood glucose levels *in vivo*, at least in part, by stimulating hepatic gluconeogenesis.

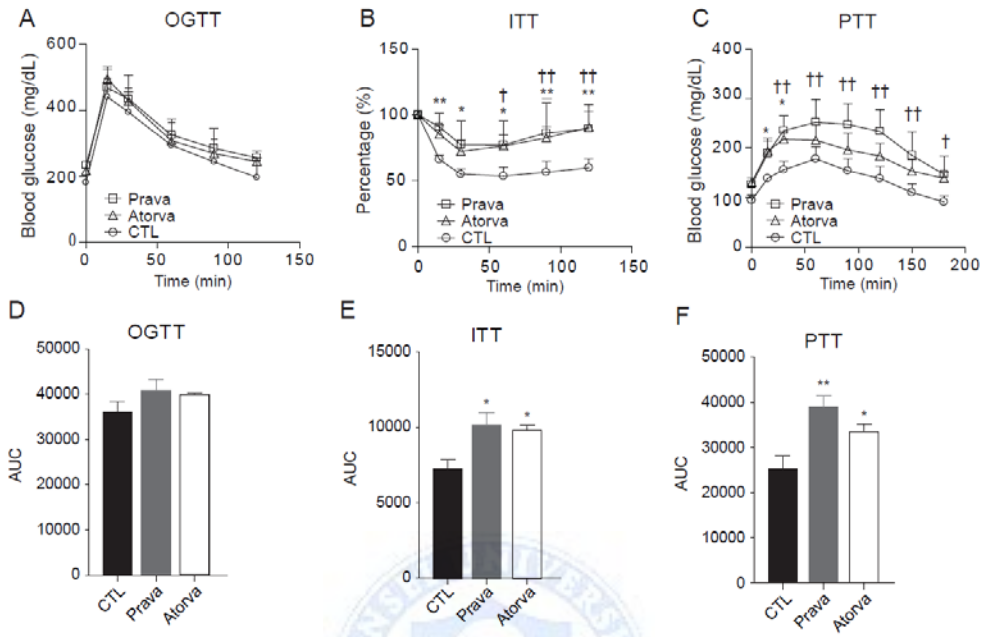

Figure 18. Statin treatment impairs insulin and pyruvate tolerance in high-fat diet-fed mice. We performed OGTT and ITT after 15 weeks of statin treatment, and PTT after 16 weeks of statin treatment. (A) Mice were fasted for 6-hour before oral glucose tolerance test. Results of the oral glucose tolerance test performed at 20 weeks of age showed no differences among groups (pravastatin-treated, atorvastatin-treated, and untreated control mice). (B) Mice were fasted for 4-hour before insulin tolerance test. Results of the insulin tolerance test showed attenuated insulin responses in statin-treated mice at 20 weeks. (C) Mice were fasted for 18-hour before pyruvate tolerance test. Results of the pyruvate tolerance test performed at 21 weeks of age showed elevated blood glucose levels in pravastatin- and atorvastatin-treated mice. Calculated areas under the curves of insulin tolerance test (E), and pyruvate tolerance test (F) were significant differences between CTL and statin-treated groups, but not oral glucose tolerance test (D). Data are presented as means with SEM. *p<0.05, **p<0.01 compared with CTL group. (untreated control, n=9; pravastatin, n=11; atorvastatin, n=11)

To determine whether statins function as HMG-CoA reductase 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. 19A); however, serum triglyceride and free fatty acid levels were significantly decreased in the statin-treated groups compared with the control group (Fig. 19B and Fig. 19C). These data suggest that under conditions in which statins increase hepatic gluconeogenesis, they function as HMG-CoA inhibitors within hepatocytes, and these statin-induced metabolic changes may be related to the inhibition of endogenous cholesterol synthesis.

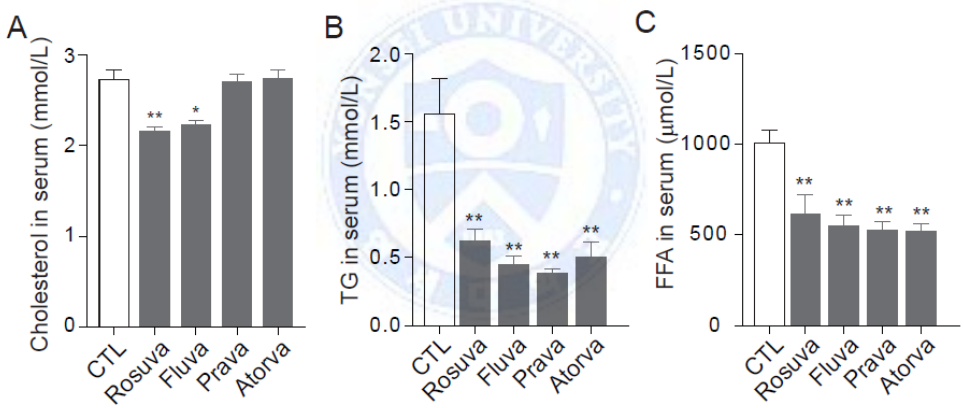

Figure 19. Statins decrease lipid profile in mouse serum. Statin treatments significantly decreased serum cholesterol (A), triglyceride (B), and free fatty acid (C) levels. *p<0.05, **p<0.01 compared with untreated mice (control, n=9; rosuvastatin, n=7; fluvastatin, n=7; pravastatin, n=11 and atorvastatin, n=11).

7. 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 real-time 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. 20A and Fig. 20B). However, expression of glycolytic genes (*Gck* and *Pklr*) was not affected by statins (Fig. 20C and Fig. 20D).

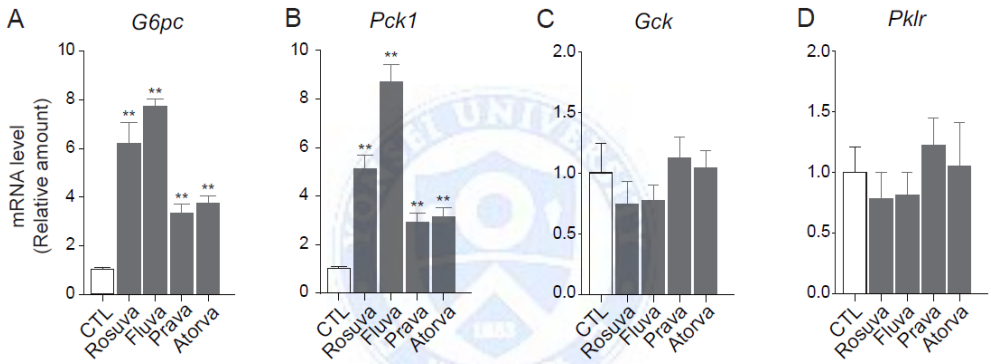

Figure 20. Gluconeogenic and glycolytic gene expression in mouse livers. Statin treatment elevates expression of gluconeogenic enzymes but not glycolytic enzymes in the livers of statin-treated mice. Results of qRT-PCR showed that statins increase expression of *G6pc* (A) and *Pck1* (B), which encode 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. (control, n=9; rosuvastatin, n=7; fluvastatin, n=7; pravastatin, n=11 and atorvastatin, n=11)

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. 21A). Autophagic vacuoles are increased in statin treated mouse livers (Fig. 21B). Collectively, these data demonstrate that statin treatment leads to insulin resistance by increasing gluconeogenesis, which is tightly coupled to autophagy.

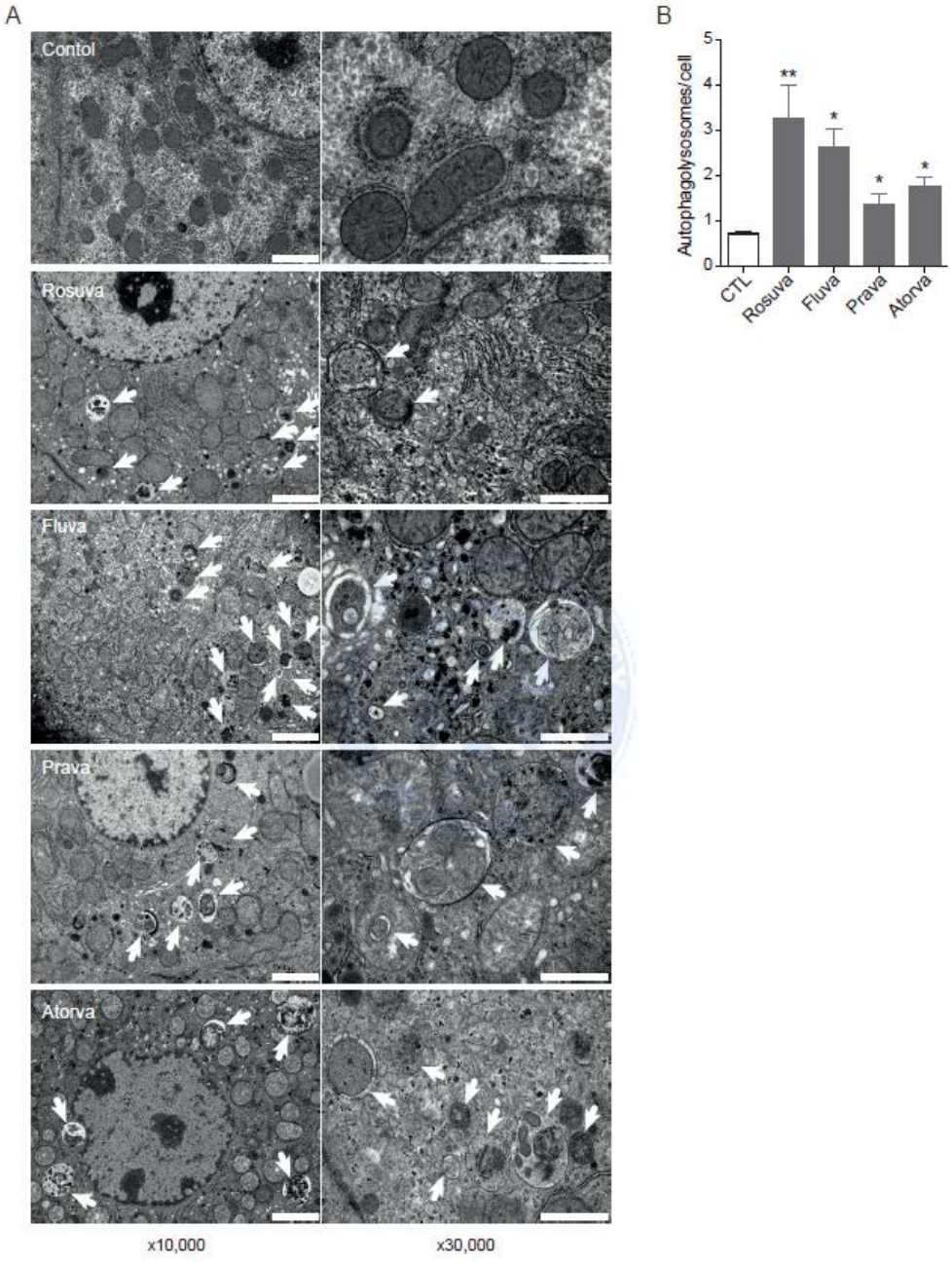
A
Contol
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Figure 21. Electron microscopy analysis of autophagosomes in the liver of statin-treated mice. (A) Hepatocytes of statin-treated mice showed prominent vacuolization and autophagosomes, as assessed by transmission electron microscopy. Arrows indicate double membranes of autophagosomes; scale bars indicate 2 μm at ×10,000 and 1 μm at ×30,000 magnification. (B) All statins significantly increase autophagic vacuoles formations in mouse livers. *p<0.05, **p<0.01 compared with control.

IV. DISCUSSION

Although numerous clinical trials and epidemiologic studies have demonstrated that statin therapy increases the risk of T2DM,[3-9] 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 showed that statin treatment blocks autophagy flux in skeletal muscle by inhibiting protein kinase D activity,[30] most previous studies have described the effects of statins on autophagy in cultured cells.[22-25] In human prostate cancer and rhabdomyosarcoma cells, statins were shown to induce autophagy by blocking geranylgeranyl biosynthesis through the inhibition of HMG-CoA reductase.[23,24] In coronary artery myocytes, Rac1-GTPase overexpression, which activates mammalian target of rapamycin (mTOR), blocked simvastatin-induced autophagy.[25]

However, paradoxically, statins decreased the phosphorylation of Akt whereas they increased the phosphorylation of FoxO1 in primary mouse hepatocytes. This suggests that statin-induced increase in FoxO1 phosphorylation is independent of insulin-Akt signaling. Insulin-PI3K-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. Other mechanism rather than phosphorylation, like FoxO1 acetylation,[31-34] XBP-1 mediated FoxO1 proteosomal degradation[35] and/or O-GlcNAc glycation[36-38] could overcome the effect of FoxO1 phosphorylation and enhance gluconeogenesis. Therefore our data 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 reported a link between mTOR signaling and intracellular cholesterol trafficking.[39] In this study, pharmacological depletion of cholesterol from the plasma membrane of endothelial cells inhibited mTOR activity, and this effect was partially reversed by restoration of cholesterol to the membrane, suggesting that mTOR is involved in sensing cellular membrane sterol concentrations.[39] Because statins block cholesterol synthesis in the liver,[40] 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.[41,42] Moreover, mevalonate, the cholesterol intermediate just downstream of HMG-CoA, reverses statin-induced inhibition of mTOR signaling,[43,44] indicating that HMG-CoA reductase 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.[17,45] Accordingly, autophagy-deficient *Atg5* knockout mice generally die from hypoglycemia within 24 hours after birth,[46] and liver-specific *Atg7* knockout mice fed a high-fat diet show improved insulin sensitivity and glucose tolerance compared to wild-type high-fat diet-fed mice.[26] 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 *Atg7* knockout mice, strongly support the idea that statins stimulate gluconeogenesis through autophagy. A previous study suggested the opposite role of autophagy in gluconeogenesis with the finding that virus-mediated overexpression of *Atg7* reduced expression of *G6pc* and *Pck1* in the livers of mice.[47] However, the induction of autophagy by *Atg7* overexpression in this study was not verified;

therefore, it is not clear that this effect was due to autophagy induction.

In addition, our study revealed a potential new mechanism underlying autophagy-induced gluconeogenesis: increased expression of the key gluconeogenic enzymes G6Pase and PEPCK.

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 indicate 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.[48-50] A previous study showed that pravastatin does not affect insulin secretion in a pancreatic β-cell line,[50] which is consistent with our result. In contrast, atorvastatin and simvastatin were reported to inhibit glucose-dependent insulin secretion by blocking calcium signaling in β-cells,[48,50] 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 elevated gluconeogenesis is another important aspect of diabetic pathophysiology.[51] 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 reported that statin can improve insulin sensitivity in liver of obese mice.[52] There are some differences between the studies. They studied with 4 week-old Wistar rat and used

lovastatin 6 mg/kg/day whereas we used C57BL/6J mice and the dose of statin was 0.01% weight of food. We administered statins for 16 weeks while they treated statin for a week. Our study represents the chronic administration of statins.

V. CONCLUSION

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.

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ABSTRACT (IN KOREAN)

HMG-CoA 환원효소 억제제의 장기적 사용에 따른 Autophagy의 유발에 의한 간의 당신생 증가와 고혈당

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왕 혜 진

스타틴 (3-hydroxy-3-methyl-glutaryl-CoA [HMG-CoA] 환원효소 억제제) 은 혈중 콜레스테롤을 낮추기 위해 널리 쓰이는 약이지만 최근 제 2형 당뇨병의 발병을 증가시키는 것이 문제가 되고 있다. 그러나 이러한 당뇨병을 일으키는 분자적 기전은 아직 알려져 있지 않다. 우리는 이번 연구를 통해 스타틴이 *in vitro*와 *in vivo* 에서 당신생에 관련된 중요한 효소들 (glucose 6-phosphatase; G6Pase 와 phosphoenolpyruvate carboxykinase 1; PEPCK) 의 발현을 증가 시키고 간에서 당생성을 증가 시킨다는 것을 발견하였다. HepG2 세포에 스타틴을 처리 했을 때 autophagy 흐름이 증가 하였다. 또한 HepG2 세포에 lysosome 억제제를 처리하여 autophagy 를 억제 한 경우 당신생 효소들의 발현과 당분비가 줄어들었다. 스타틴이 당생성을 증가시키는 작용은 Atg7 (autophagy-related protein 7)과 beclin 1이 없을 경우에는 나타나지 않았고, 이것은 autophagy가 스타틴의 당뇨병 유발 효과에 중요한

역할을 한다는 것을 의미한다. 식이 유발 비만 마우스에게 스타틴을 투여한 경우 마우스의 간에서 autophagic vacuoles 과 당신생 유전자의 발현이 증가하였고, 결국 간에서 당생성이 증가하고 고혈당증이 생기며 인슐린 저항성이 생기게 된다. 종합적으로, 이 결과들은 스타틴의 장기적인 사용이 간에서 autophagy 를 증가시켜 당신생을 증가시키는 것과 연관이 되어 있고, 그 결과 인슐린 저항성이 생긴다는 것을 증명하였다. 이 결과들은 스타틴의 당뇨병을 발병시키는 기전을 인슐린 저항성 측면에서 좀더 잘 이해 할 수 있게 기여할 것이다.

핵심되는 말: HMG-CoA 환원효소 억제제, 스타틴, 당신생, 당뇨병, autophagy

PUBLICATION LIST

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