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Simvastatin induces insulin resistance in L6 skeletal muscle myotubes by suppressing insulin signaling, GLUT4 expression and GSK-3β phosphorylation

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ABSTRACT

Simvastatin is a 3-hydroxy-3-methylglutaryl-CoA reductase inhibitor widely used for the treatment of hypercholesterolemia. Recent data indicates that simvastatin increases the risk of new-onset diabetes by impairing both insulin secretion and insulin sensitivity. However, systematic evaluation of mechanistic pathways is lacking. We aimed to explore the effects of simvastatin on glucose uptake and underlying mechanisms using L6 skeletal muscle myotubes. We performed our experiments at basal and insulin-stimulated conditions, at normal (5.5 mM) and high (16.7 mM) glucose. We showed that simvastatin inhibited glucose uptake at all conditions. We also found out that pravastatin, another widely used statin with different physicochemical properties, did not inhibit glucose uptake. The effect of simvastatin was reversed with geranylgeranyl pyrophosphate but not with farnesyl pyrophosphate, implying that reduced protein geranylgeranylation has a role in simvastatin-induced insulin resistance. Simvastatin also decreased phosphorylation of insulin receptor (IR), insulin receptor substrate 1 (IRS-1), AKT and glycogen synthase kinase 3β (GSK-3β), and downregulated GLUT4. In conclusion, our data indicate that simvastatin decreased both basal and insulin-stimulated glucose uptake through inhibiting the critical steps in IR/IRS-1/AKT signaling cascade, and by hindering GLUT4 function and normal regulation of glycogen synthesis, contributing to insulin resistance.

Keywords: Simvastatin; Glucose uptake; Glucose transporter 4; IR-dependent IRS-1/PI3K/Akt pathway; L6 myotubes

Abbreviations: FPP, farnesyl pyrophosphate; FTI, farnesyltransferase inhibitor; GGPP, geranylgeranyl pyrophosphate; GGTI, geranylgeranyltransferase 1 inhibitor; GLUT4, glucose transporter 4; GSK-3β, glycogen synthase kinase 3 beta; HMG-CoA, 3-hydroxy-3-methylglutaryl-coenzyme A; IR, insulin receptor; IRS1, insulin receptor substrate 1; PI3K, phosphoinositide 3-kinase
INTRODUCTION

Statins are the most effective drugs in lowering plasma cholesterol and prevention of cardiovascular disease [1]. They act by inhibiting 3-hydroxy-3-methyl-glutaryl-CoA (HMG-CoA) reductase, the rate-limiting enzyme in the cholesterol biosynthesis. Large scale meta-analyses showed that statins induce the new-onset type 2 diabetes (T2D) [2]. The risk varied between different statins, pravastatin having the lowest effect [3]. In a large population-based METSIm study, statin therapy was associated with a 46% increased risk of T2D, a reduction in insulin sensitivity and a decrease in insulin secretion during a 6 year follow-up [4]. Statins also affect glycemic control in patients with pre-existing T2D [5].

Although several studies have investigated the molecular mechanisms for increased risk of T2D with statin treatment, they are not fully understood. T2D is caused by impaired insulin secretion and insulin resistance [6]. Effects of statins on insulin sensitivity have been explored mainly in adipose cells. Skeletal muscle accounts for the majority of whole body glucose uptake (~80%) and insulin sensitivity [7], therefore understanding of the mechanisms of statin-induced insulin resistance in skeletal muscle is crucial for the prevention of T2D in individuals requiring statin treatment.

Glucose uptake in skeletal muscle is mediated by glucose transporter 4 (GLUT4) in both basal and insulin-stimulated conditions [8]. Statins were shown to inhibit GLUT4 expression and glucose uptake in adipocytes [9-12]. In muscle cells, simvastatin was also reported to decrease glucose uptake [13, 14], but no change in GLUT4 protein expression was observed [28]. Furthermore, inhibition of glucose uptake by simvastatin was observed in insulin-stimulated but not in basal conditions in L6 myotubes [14]. Some studies reported that the effect of statins on glucose uptake is mediated by cholesterol biosynthesis inhibition whereas others reported an independent mechanism [14]. Glucose uptake and GLUT4 translocation is stimulated through insulin receptor (IR) - insulin receptor substrate 1 (IRS1) - phosphoinositide 3-kinase (PI3K) - AKT kinase pathway [15]. Treatment with statins has been shown to suppress this pathway in adipocytes [9, 12], and in skeletal muscle cells under insulin-stimulated conditions [14]. There is still need for further investigation of insulin signaling pathway and its downstream targets.
We investigated systematically the effects of simvastatin and pravastatin, two widely used statins with different physicochemical properties, on basal and insulin-stimulated glucose uptake and insulin signaling in L6 skeletal muscle myotubes. We performed the experiments at normal (5.5 mM) and high (16.7 mM) glucose concentrations to investigate whether these effects are modulated by hyperglycemia.

MATERIALS AND METHODS

Cell culture

L6 cells (CLR-1458, ATCC, Rockville, MD) were maintained in Dulbecco’s modified Eagle’s medium (DMEM, 4.5 g/l glucose, Lonza, Basel, Switzerland) supplemented with 10% fetal bovine serum (Gibco, Paisley, UK), 2 mM L-glutamine (Lonza), 100 units/ml penicillin (Lonza) and 100 µg/ml streptomycin (Lonza), at 37°C in an atmosphere of 5% CO₂. After the myoblasts reached confluence, the media was replaced with differentiation media containing DMEM (1 g/l glucose, Lonza), 2% horse serum (Gibco), 2 mM L-glutamine (Lonza), 100 units/ml penicillin (Lonza) and 100 µg/ml streptomycin (Lonza), and changed every 48 hours. After the differentiation the myotubes were treated with simvastatin (Merck Millipore, Darmstadt, Germany), pravastatin (Tocris Bioscience, Bristol, UK), insulin, mevalolactone (Sigma-Aldrich, Schnelldorf, Germany), GGPP (Geranylgeranyl pyrophosphate ammonium salt, Sigma-Aldrich), FPP (Farnesyl pyrophosphate ammonium salt, Sigma-Aldrich), FTI-277 (Tocris Bioscience) or GGTI-298 (Tocris Bioscience) for 24 hours, according to the experimental conditions.

Glucose uptake assay

L6 myotubes were exposed to test compounds for 24 hours in differentiation media supplemented with 5.5 mM or 16.7 mM glucose. The control cells were treated with DMSO. After treatments the myotubes were washed with PBS and incubated with HEPES-buffered saline (140 mM NaCl, 20 mM HEPES, 5 mM KCl, 2.5 mM MgSO₄, 1 mM CaCl₂, pH 7.4) for 1 hour with the test compounds. 100 nM human insulin (Sigma-Aldrich) was added to the cells for last 30 minutes. For uptake reaction, the cells were treated with HEPES-buffered saline containing
10 μM 2-deoxy-D-glucose (Sigma-Aldrich) and 1 μCi 2-Deoxy-D-[2,6-3H] glucose (PerkinElmer, Turku, Finland, Cat. No. NET549250UC) for 15 minutes. Cells were washed with ice cold PBS on ice. 500 μl of 0.2 N NaOH was added to each well and the plate was incubated for 90 minutes at room temperature with constant shaking. Collected samples were stored at -70°C. OptiPhase HiSafe 2 (PerkinElmer) was added to the samples and radioactivity was measured using 1450 MicroBeta Trilux (Wallac, Turku, Finland). The protein content was measured using the BCA Protein Assay (Pierce, Rockford, IL).

**Western blotting**

The cells were washed once with PBS and lysed with RIPA buffer (ThermoFisher Scientific, Waltham, MA) along with protease and phosphatase inhibitors (Roche, Basel, Switzerland). Protein concentrations were measured by BCA protein assay (Pierce). Protein samples (20-25 μg/lane) containing NuPAGE LDS sample buffer and reducing agent were loaded into 4-12% NuPAGE Bis-Tris gels (Life Technologies, Espoo, Finland), subjected to gel electrophoresis and transferred to polyvinylidene fluoride (PVDF) membranes (GE Healthcare, Uppsala, Sweden). Phospho-AKT (Ser473, Cat. No. 9271), AKT (Cat. No. 9272), GSK-3β (Cat. No. 9315), phospho-GSK-3β (Ser9, Cat. No. 9336), insulin receptor β-subunit (Cat. No. 3025), phospho-IRS1 (Ser307, Cat. No. 2381), phospho-IRS1 (Ser612, Cat. No. 3203) and IRS1 (Cat. No. 3407) antibodies were purchased from Cell Signaling Technology, Beverly, MA. Phospho-insulin receptor (Tyr1361, Cat. No. ab60946) and GAPDH (Cat. No. ab8245) antibodies were from Abcam, Cambridge, UK; GLUT4 antibody was from Santa Cruz Biotechnology, Heidelberg, Germany (Cat. No. sc-1606) and phospho-IRS1 (Tyr608) antibody was from Millipore (Cat. No. 09-432). The secondary antibodies anti-rabbit IgG-HRP (Cat. No. NA934V) and anti-mouse IgG-HRP (Cat. No. NA931V) were purchased from GE Healthcare and donkey anti-goat IgG-HRP (Cat. No. sc-2020) was from Santa Cruz Biotechnology. The membranes were blocked in 5% BSA in TBS-Tween-20 (TBS-T) for 1 hour at room temperature, washed with TBS-T and incubated overnight with primary antibodies. Later, the membranes were incubated with secondary antibodies for 1 hour at room temperature. For GAPDH detection, the membranes were blocked in 10% milk and for phospho-IRS1 (Tyr608), the membranes were blocked in 5% milk in TBS-T, incubated with primary antibody dilutions overnight and incubated with secondary antibodies for 1 hour at
room temperature. Membranes were washed with TBS-T between every step. Membranes were finally washed in TBS, the bands were visualized using chemiluminescence (ECL Plus, Pierce) and images were captured in Image Quant RT-ECL equipment (GE Healthcare). Bands were quantified with Quantity One software (Bio-Rad, Hercules, CA). Protein expressions were normalized with GAPDH or total protein levels.

**Statistical analysis**

Data were collected from independent experiments, with three replicates per experiment, and analyzed with one-way ANOVA with Bonferroni post-hoc test or Student’s t-test in GraphPad 5 (GraphPad Software, San Diego, CA). P < 0.05 was considered statistically significant. Error bars represent standard error of the mean (SEM).

**RESULTS**

**Simvastatin but not pravastatin decreases glucose uptake**

We treated L6 myotubes (Fig. 1A) with 6 µg/ml (14.3 µM) simvastatin (as in all simvastatin experiments) and 12 µg/ml (26.3 µM) pravastatin at normal (5.5 mM) and high (16.7 mM) glucose. Treatment with simvastatin decreased basal glucose uptake significantly (p<0.05) by 46% at normal glucose and 56% at high glucose (Fig. 1B) compared to control. Simvastatin decreased insulin-stimulated glucose uptake by 58% compared to insulin alone (p<0.001) (Fig. 1C). Treatment with pravastatin had a non-significant trend to increase the basal glucose uptake in both glucose concentrations (Fig. 1D).

**Effect of simvastatin on glucose uptake via the cholesterol biosynthesis pathway**

Treatment with 0.5 mM and 1 mM DL-mevalolactone, an activator of mevalonate pathway, did not change glucose uptake compared to control at normal glucose, but in combination with simvastatin DL-mevalolactone restored simvastatin-decreased glucose uptake almost to the level of control (p<0.01 compared to simvastatin treatment alone) (Fig. 2A).

We treated L6 myotubes with simvastatin and isoprenoids geranylgeranyl pyrophosphate (GGPP) and farnesyl pyrophosphate (FPP) (both 20 µM) at normal glucose.
Treatment with GGPP increased glucose uptake by 49% (p<0.01), whereas FPP did not have a significant effect. In treatments with simvastatin, GGPP restored simvastatin-decreased glucose uptake to the level 36% higher than control (p<0.001 compared to simvastatin alone), whereas FPP had no significant effect (Fig. 2B).

Treatments with two selective inhibitors of prenylation, farnesyltransferase inhibitor FTI-277 and geranylgeranyltransferase 1 inhibitor GGTL-298 (both 20 µM), at normal glucose decreased glucose uptake by 15% (p<0.01) and 38% (p<0.05), respectively, compared to control. Simvastatin decreased basal glucose uptake by 53% (p<0.001) (Fig. 2C).

**Effect of simvastatin on insulin signaling pathway**

Treatment with simvastatin decreased phosphorylation of insulin receptor (pIR) non-significantly by 34% at normal glucose (Fig. 3A) and by 35% (p<0.05) at high glucose (Fig. 3B). At normal glucose, treatment with simvastatin decreased phosphorylation of insulin receptor substrate 1 (pIRS1) at Tyr608 (Fig. 3C), Ser307 (Fig. 3D), and Ser612 (Fig. 3E) by 59% (p<0.01), 50% (p<0.001), and 40% (p<0.01) compared to control at basal conditions, and by 11% (non-significantly), 49% (p<0.01), and 33% (p<0.05) compared to insulin at insulin-stimulated conditions. At high glucose, treatment with simvastatin decreased pIRS1 at Tyr608 (Fig. 3F), Ser307 (Fig. 3G), and Ser612 (Fig. 3H) by 67% (p<0.01), 44% (p<0.05), and 40% (p<0.01) compared to control at basal conditions, and by 32% (p<0.05), 42% (p<0.05), and 32% (p<0.05) compared to insulin at insulin-stimulated conditions. Tyrosine phosphorylation of IRS1 was strongly stimulated by insulin treatment.

**Effect of simvastatin on AKT signaling and glycogen synthesis pathway**

At normal glucose (Fig. 4A) treatment with simvastatin decreased Ser473 phosphorylation of AKT (pAKT) by 29% at basal conditions (p<0.01 compared to control) and non-significantly by 27% at insulin-stimulated conditions (compared to insulin). At high glucose (Fig. 4B) treatment with simvastatin decreased pAKT(Ser473) by 30% at basal conditions (p<0.05 compared to control) and by 28% at insulin-stimulated conditions (p<0.05 compared to insulin). Phosphorylation of AKT was strongly stimulated by insulin treatment.
At normal glucose (Fig. 4C) treatment with simvastatin decreased phosphorylation of GSK-3β (pGSK-3β) by 50% at basal conditions (p<0.05 compared to control) and non-significantly by 12% at insulin-stimulated conditions (compared to insulin). At high glucose (Fig. 4D) treatment with simvastatin decreased pGSK-3β by 30% at basal conditions (p<0.05 compared to control) and non-significantly by 11% at insulin-stimulated conditions (compared to insulin). Treatment with insulin increased pGSK-3β by 73% and 62% (both p<0.05) compared to control at normal and high glucose, respectively.

**Effect of simvastatin on GLUT4 protein expression**

At normal glucose (Fig. 4E) treatment with simvastatin decreased GLUT4 protein expression by 28% at basal conditions (p<0.01 compared to control), and non-significantly by 20% at insulin-stimulated conditions (compared to insulin). At high glucose (Fig. 4F) treatment with simvastatin decreased GLUT4 protein expression by 21% at basal conditions (p<0.001 compared to control), and non-significantly by 28% at insulin-stimulated conditions (compared to insulin). Treatment with insulin alone did not have a significant effect on GLUT4 protein expression.

**DISCUSSION**

Statins have been shown to increase the risk of T2D through their effects on insulin sensitivity as well as insulin secretion. We show that simvastatin but not pravastatin altered glucose uptake in L6 myotubes, similarly at basal and insulin-stimulated conditions. The effect of simvastatin on glucose uptake was partially mediated by its inhibitory effect on cholesterol biosynthesis pathway and could be reversed by mevalonate and GGPP, but not FPP. Simvastatin inhibited insulin signaling pathway and GSK-3β phosphorylation, and decreased GLUT4 protein expression in L6 myotubes. These effects were independent of glucose concentration.

**Simvastatin and glucose uptake**

We show that a 24-hour simvastatin treatment decreased basal and insulin-stimulated glucose uptake in L6 myotubes both at normal and high glucose. In an earlier report [14] simvastatin and atorvastatin were shown to decrease only insulin-stimulated glucose uptake with no significant effect on basal glucose uptake in L6 myotubes. This difference might be explained by variations in treatment time (24 h vs. 48 h), glucose uptake method (radiolabeled glucose vs.
fluorescent glucose analog) and by a higher concentration of simvastatin used in our study (14.3 µM vs. 1 µM). Accordingly, a dose-dependent effect of simvastatin on glucose uptake in the absence of insulin has been reported in human myotubes [13].

**Pravastatin and glucose uptake**

In our study, treatment with pravastatin had no significant effect on glucose uptake in L6 myotubes. Similarly in adipocytes, others have reported no effect of pravastatin on glucose uptake [12], GLUT4 levels [11, 12] or insulin signaling pathway [11]. It has been hypothesized that the difference is most likely due to the hydrophilic nature of pravastatin compared to lipophilic nature of simvastatin [16]. Nonetheless, rosvustatin is a hydrophilic statin but substantially increases the risk of new-onset T2D [3].

**Cholesterol biosynthesis pathway**

Statins inhibit the conversion of HMG-CoA to mevalonate, and exogenous mevalonate is known to restore statin-decreased cholesterol biosynthesis [17]. We show that treatment with mevalolactone restored simvastatin-decreased glucose uptake, in accordance with the report in human myotubes [13], but in contrast with the report in L6 myotubes [14]. Mevalonate is a precursor for isoprenoids FPP and GGPP, which act as substrates for protein prenylation [18]. We further confirmed the involvement of cholesterol biosynthesis pathway in glucose uptake using farnesyltransferase and geranylgeranyltransferase 1 inhibitors, which also decreased glucose uptake significantly. Furthermore, GGPP, but not FPP, restored simvastatin-decreased glucose uptake, and GGPP was able to stimulate glucose uptake by itself. FPP is a precursor of GGPP and for conversion to GGPP requires isopentenyl pyrophosphate (the mevalonate pathway intermediate), which is reduced during the inhibition of HMG-CoA reductase [19]. Therefore, our results suggest that alteration of post-translational prenylation of proteins by GGPP contributes to simvastatin-decreased glucose uptake.

**Insulin signaling pathway**

Insulin signaling cascade is activated by tyrosine phosphorylation of IRS1 by activated IR which also tyrosine autophosphorylates itself. We found that simvastatin inhibited tyrosine phosphorylation of IR (Tyr1361) and IRS1 (Tyr698) at basal and insulin-stimulated conditions,
indicating that simvastatin has a direct inhibitory effect on the canonical insulin signaling pathway. Furthermore, simvastatin inhibited also serine phosphorylation of IRS1 (at Ser307 and Ser612). While the importance of IRS1 tyrosine phosphorylation in insulin signaling is undisputed, serine phosphorylation of IRS1 has been linked to both insulin resistance [20] and insulin sensitivity [21]. Moreover, it can be regulated by several kinases involved in other pathways such as JNK1 (c-Jun N-terminal kinase 1), mTOR (mammalian target of rapamycin) - S6K1 (ribosomal protein S6 kinase beta-1) kinase pathway, GRK2 (G protein-coupled receptor kinase 2) and some PKC (protein kinase C) isoforms [20]. Therefore, our results indicate that simvastatin treatment could impair also other cellular pathways involving IRS1 and negatively affect the IRS1 downstream signaling.

The IRS1 acts as a mediator transmitting signals from IR to AKT, which is phosphorylated and activated through the PI3K-mediated pathway [22]. We show that simvastatin decreased AKT phosphorylation at both basal and insulin-stimulated conditions. In previous reports, atorvastatin was shown to decrease IR beta-subunit, IRS1 and AKT in dose dependent manner in adipocytes [9, 12] and lovastatin decreased the phosphorylation of IR beta-subunit in fibroblasts [23]. In L6 myotubes [14] simvastatin decreased phosphorylation of IRS1 and pAKT at insulin-stimulated conditions, and our results extend these findings to basal conditions as well.

A downstream target of AKT playing role in muscle insulin resistance is glycogen synthase kinase 3 beta (GSK-3β), a serine/threonine kinase essential for the regulation of glycogen synthesis. Unphosphorylated GSK-3β inhibits the activity of glycogen synthase, and AKT directly phosphorylates and inactivates GSK-3 [24]. Here we report a novel finding that treatment with simvastatin decreased GSK-3β phosphorylation, thus activating GSK-3β, which would result in inhibition of glycogen synthase. GSK-3 overactivity was associated with defective IRS1 signaling resulting in reduced GLUT4 translocation and reduced glucose uptake in skeletal muscle [25]. Impaired insulin-stimulated glycogen synthesis has been found in patients with T2D and persons at the risk of the disease [26].
**GLUT4**

The phosphorylation of AKT is known to mediate glucose uptake by facilitating GLUT4 translocation from intracellular storages to the plasma membrane [27]. GLUT4 is also required for basal glucose uptake [8]. We show that treatment of L6 myotubes with simvastatin decreased protein expression of GLUT4 at both basal and insulin-stimulated conditions. Similar inhibitory effects on GLUT4 protein expression have been reported in 3T3-L1 adipocytes with simvastatin [11], lovastatin [10] and atorvastatin [9, 12]. Defects in GLUT4 trafficking or protein expression are known to cause insulin resistance [28]. Our results confirm that decreased protein expression of GLUT4 contributes to simvastatin-decreased glucose uptake in L6 myotubes.

**Glucose levels and the effects of simvastatin**

Statin treatment has been shown to affect glycemic control in patients with pre-existing diabetes, although the effect on HbA1c level seemed to be smaller than in individuals without T2D [5]. We performed our experiments at normal and high glucose and observed essentially similar results in both conditions, suggesting that simvastatin’s effect on glucose uptake and insulin signaling are independent of glucose levels.

In summary, our results in L6 myotubes show that simvastatin impaired glucose uptake by inhibiting insulin signaling pathway and GLUT4 protein expression. Simvastatin also activated GSK-3β, thus affecting glucose utilization. Simvastatin’s effect on glucose uptake was at least partly mediated by cholesterol biosynthetic pathway inhibition, most likely through reduced geranylgeranylation of proteins involved in glucose uptake. The effects of simvastatin were observed at both insulin-stimulated and basal conditions, independently of glucose concentration. Pravastatin did not affect glucose uptake. Our results extend the previous investigations on the molecular mechanisms of statin-induced insulin resistance. This understanding is crucial for the prevention and management of type 2 diabetes in individuals with cardiovascular disease requiring statin treatment.
Acknowledgements and conflict of interest

This work was supported by the Academy of Finland, the Finnish Diabetes Research Foundation, and Strategic Research Funding from the University of Eastern Finland. No potential conflicts of interest relevant to this article were reported.
REFERENCES


FIGURE LEGENDS

Fig 1. Effect of simvastatin on basal and insulin-stimulated glucose uptake.
(A) Phase contrast images of undifferentiated myoblasts and differentiated myotubes treated with DMSO (control) and simvastatin. (B) The effect of simvastatin (6 µg/ml) on basal glucose uptake at 5.5 and 16.7 mM glucose. (C) The effect of simvastatin on insulin-stimulated (100 nM insulin for 30 min) glucose uptake at 5.5 mM glucose. (D) The effect of pravastatin (12 µg/ml) on basal glucose uptake at 5.5 and 16.7 mM glucose. Glucose uptake was normalized with protein concentration, data are mean±SEM relative to control (100%). p values were calculated with the Student’s t-test or one-way ANOVA with Bonferroni post-hoc test, *p<0.05, **p<0.01, ***p<0.001 compared to control, ###p<0.001 compared to insulin treatment. Based on data from 2-3 independent experiments.

Fig 2. Effect of simvastatin and modulators of cholesterol biosynthesis pathway on basal glucose uptake at 5.5 mM glucose.
(A) The effect of simvastatin (Simva, 6 µg/ml) and DL-mevalolactone (Mvl, 0.5 mM and 1 mM) on glucose uptake. (B) The effect of geranylgeranyl pyrophosphate (GGPP, 20 µM), farnesyl pyrophosphate (FPP, 20 µM) and simvastatin on glucose uptake. (C) The effects of simvastatin, farnesyltransferase inhibitor FTI-277 (20 µM) and geranylgeranyltransferase 1 inhibitor GGTI-298 (20 µM) on glucose uptake. Glucose uptake was normalized with protein concentration, data are mean±SEM relative to control (100%). p values were calculated with the one-way ANOVA with Bonferroni post-hoc test, *p<0.05, **p<0.01, ***p<0.001 compared to control, ##p<0.001, ###p<0.001 compared to simvastatin. Based on data from 2 independent experiments.

Fig 3. Effect of simvastatin on insulin signaling pathway.
The effects of simvastatin (Simva, 6 µg/ml) on the levels of phospho-insulin receptor (pIR) at 5.5 mM (A) and 16.7 mM (B) glucose; phospho-insulin receptor substrate 1 (pIRS1) at Tyr608 at 5.5 mM (C) and 16.7 mM (D) glucose; pIRS1 at Ser307 at 5.5 mM (E) and 16.7 mM (F) glucose; pIRS1 at Ser612 at 5.5 mM (G) and 16.7 mM (H) glucose. Insulin receptor (IR), insulin receptor
substrate 1 (IRS1) and GAPDH were used as loading controls. When indicated, insulin was used at 100 nM concentration for 30 min. (I) Representative western blots for figures A-H. p values were calculated with the Student’s t-test or one-way ANOVA with Bonferroni post-hoc test, *p<0.05, **p<0.01, ***p<0.001 compared to control, "p<0.001, ""p<0.001, "###p<0.001 compared to insulin. Based on data from 2-5 independent experiments.

**Fig 4. Effect of simvastatin on phosphorylation of AKT, GSK-3β and protein expression of GLUT4.**

The effects of simvastatin (Simva, 6 µg/ml) on protein expression of phospho-AKT (pAKT) at Ser 473 at 5.5 mM (A) and 16.7 mM (B) glucose; phospho-GSK-3β (pGSK-3β) at 5.5 mM (C) and 16.7 mM (D) glucose, and GLUT4 at 5.5 mM (E) and 16.7 mM (F) glucose. AKT, GSK-3β and GAPDH were used as loading controls. In A-D, 100 nM insulin was used for 30 min. (G) Representative western blots for figures A-F. p values were calculated with the one-way ANOVA with Bonferroni post-hoc test, *p<0.05, **p<0.01, ***p<0.001 compared to control, "p<0.001 compared to insulin. A-D are based on data from 4-7 independent experiments, E-F on 3 independent experiments.
Figure 1

A

Undifferentiated myoblasts

Differentiated myotubes

B

5.5 mM glucose

16.7 mM glucose

Glucose uptake (%)

Control
Simvastatin
Control
Simvastatin

C

5.5 mM glucose

Glucose uptake (%)

Control
Simvastatin
Insulin
Insulin+Simvastatin

D

5.5 mM glucose

16.7 mM glucose

Glucose uptake (%)

Control
Pravastatin
Control
Pravastatin
Figure 2

A

B

C

Glucose uptake (%)

Control
Simv
MV (0.5 mM)
MV (1 mM)
MV (1 mM)/Simv

Glucose uptake (%)

Control
Simv
GOFR
GOFR-Simv
FPF
FPF-Simv

Glucose uptake (%)

Control
Simvastatin
FTL/277
GCT/298
Figure 3

A

5.5 mM glucose

B

16.7 mM glucose

C

5.5 mM glucose

D

16.7 mM glucose

E

5.5 mM glucose

F

16.7 mM glucose

G

5.5 mM glucose

H

16.7 mM glucose

I

5.5mM glucose

1 2 3 4

16.7mM glucose

1 2 3 4

pSer307-IRS

pSer612-IRS1

pTyr608-IRS1

Total IRS1

GAPDH

5.5mM glucose

C S C S

16.7mM glucose

C S C S

pIR

1. DMSO

2. Simvastatin

3. Insulin

4. Simvastatin +Insulin

C. Control

S. Simvastatin

GAPDH
Figure 4

A 5.5 mM glucose

B 16.7 mM glucose

C 5.5 mM glucose

D 16.7 mM glucose

E 5.5 mM glucose

F 16.7 mM glucose

G 5.5 mM glucose

1. DMSO
2. Simvastatin
3. Insulin
4. Simvastatin+Insulin

MANUSCRIPT 
ACCEPTED 
ACCEPTED MANUSCRIPT
• Simvastatin but not pravastatin inhibits glucose uptake in L6 myotubes
• Mevalonate and GGPP but not FPP restore simvastatin-decreased glucose uptake
• Simvastatin suppresses IR-IRS1-AKT signaling cascade
• Simvastatin activates GSK-3β to inhibit glycogen synthesis
• Simvastatin downregulates GLUT4