

RESEARCH

Simvastatin profoundly impairs energy metabolism in primary human muscle cells

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Abstract

Objectives: Simvastatin use is associated with muscular side effects, and increased risk for type 2 diabetes (T2D). In clinical use, simvastatin is administered in inactive lipophilic lactone-form, which is then converted to active acid-form in the body. Here, we have investigated if lactone- and acid-form simvastatin differentially affect glucose metabolism and mitochondrial respiration in primary human skeletal muscle cells.

Methods: Muscle cells were exposed separately to lactone- and acid-form simvastatin for 48 h. After pre-exposure, glucose uptake and glycogen synthesis were measured using radioactive tracers; insulin signalling was detected with Western blotting; and glycolysis, mitochondrial oxygen consumption and ATP production were measured with Seahorse XF[®]96 analyzer.

Results: Lactone-form simvastatin increased glucose uptake and glycogen synthesis, whereas acid-form simvastatin did not affect glucose uptake and decreased glycogen synthesis. Phosphorylation of insulin signalling targets Akt substrate 160 kDa (AS160) and glycogen synthase kinase 3 β (GSK3 β) was upregulated with lactone-, but not with acid-form simvastatin. Exposure to both forms of simvastatin led to a decrease in glycolysis and glycolytic capacity, as well as to a decrease in mitochondrial respiration and ATP production.

Conclusions: These data suggest that lactone- and acid-forms of simvastatin exhibit differential effects on non-oxidative glucose metabolism as lactone-form increases and acid-form impairs glucose storage into glycogen, suggesting impaired insulin sensitivity in response to acid-form simvastatin. Both forms profoundly impair oxidative glucose metabolism and energy production in human skeletal muscle cells. These effects may contribute to muscular side effects and risk for T2D observed with simvastatin use.

Key Words

- ▶ ATP production
- ▶ glucose uptake
- ▶ glycogen synthesis
- ▶ glycolysis
- ▶ HMG-CoA reductase inhibitor
- ▶ insulin resistance
- ▶ mitochondrial respiration
- ▶ simvastatin
- ▶ skeletal muscle

Endocrine Connections
(2020) 9, 1103–1113

Introduction

HMG-CoA reductase inhibitors, statins, lower cholesterol levels by inhibiting mevalonate formation, the rate-limiting step in the cholesterol biosynthesis (1). These drugs are widely used and effective in primary (2, 3) and secondary (4) prevention of atherosclerotic events. However, data from several clinical studies have demonstrated that statin treatment increases the risk of type 2 diabetes (5). Statin use has been implicated with

decreases in insulin secretion and whole-body insulin sensitivity (6). A large observational study has revealed that statin use is associated with higher fasting glucose concentrations (7). Statin use has also been accompanied with impairments in glycemic control and insulin secretion in patients with type 2 diabetes (8). Regarding individual statins, simvastatin or rosuvastatin use was associated with the highest risk for type 2 diabetes,

whereas pravastatin use was associated with low risk of new-onset diabetes (9).

Statins may induce muscular side effects, ranging from mild myalgia to severe rhabdomyolysis, which may contribute to impaired glucose homeostasis (10). One proposed mechanism for muscle symptoms during statin use is the lipophilic nature of certain statins, which enables these to diffuse non-selectively into non-hepatic tissues such as skeletal muscle (11). This idea is supported by the PRIMO-study, which showed hydrophilic statins, such as pravastatin, causing less myalgia, whereas highly lipophilic simvastatin was most likely involved in muscular adverse effects (12).

The mechanisms whereby statins affect insulin sensitivity in human muscle are unclear. Simvastatin-induced insulin resistance has been proposed to result from decreased lipid synthetic capacity in skeletal muscle. This may lead to intracellular accumulation of toxic fatty acid metabolites, which impairs insulin sensitivity (13). This observation is supported by the finding of larger lipid droplets in muscle cells of patients treated with statins (14). An *in vitro* and *in vivo* study using rodent models revealed a link between statin-induced myotoxicity and impairments in mTOR/Akt signalling pathway, which led to negative consequences on important cellular processes, such as skeletal muscle protein synthesis and breakdown, as well as apoptosis (15). Inhibition of Akt phosphorylation was also responsible for simvastatin-induced mitochondrial dysfunction in mouse C2C12 muscle cells (16). Potential mechanisms for simvastatin-induced myotoxicity also include impaired mitochondrial function, increment in skeletal muscle catabolism due to increased expression of atrogen-1, reduction in skeletal muscle protein synthesis and inhibition of small GTPases due to impaired prenylation and/or impaired creatine synthesis (17).

Simvastatin is administered in inactive lipophilic lactone-form, which is then converted to active acid-form in the body (10). Approximately equal amounts of lactone- and acid-forms are present in human plasma (18, 19). Lactone-form statins have been observed to be more prone to cause myotoxic effects (19), and thus may contribute to impairments in glucose metabolism. Glucose uptake was inhibited with lactone-form simvastatin in rat L6 myotubes via inhibition of IR/IRS-1/Akt signalling cascade (20). Moreover, a study with mouse C2C12 muscle cells demonstrated that translocation of GLUT4, an effect mediated by PI3K/Akt signalling, was inhibited and glucose uptake decreased in C2C12 cells treated with lactone-form simvastatin (21). Lactone-form statins have

also shown to inhibit mitochondrial complex III activity in C2C12 cells (22).

In this study, we examined the effects of lactone- and acid-form simvastatin on oxidative and non-oxidative glucose metabolism, mitochondrial respiration and insulin signalling in primary human skeletal muscle cells *in vitro*.

Materials and methods

Antibodies and reagents

The culture medias used for muscle cell proliferation and differentiation were high-glucose DMEM/F12 (Gibco cat. 31331) and low glucose DMEM/F12 (Gibco cat. 21885), respectively. Culture media used for statin pre-treatments and serum starvation was low glucose DMEM (Sigma cat. D5546). Culture media for glucose uptake assay was glucose-free DMEM (Gibco cat. 11966). Antibiotics used in proliferation and differentiation medias were Amphotericin B from Gibco and penicillin-streptomycin solution from Sigma. FBS and L-glutamine, glycogen carrier from rat liver and cytochalasin B were from Sigma. CD56-labeled magnetic beads were from Miltenyi Biotec (Gologne, DE). Simvastatin (in lactone form) was from Calbiochem/EMD Millipore. Pravastatin was from Tocris Bioscience (Bristol, UK). Fatty-acid free BSA was from Biowest (Nuaille, France). Actrapid Insulin was from Novo Nordisk (Bagsvaerd, DK). 2-[1,2-³H]-deoxy-D-glucose and D-[¹⁴C]-glucose were from Perkin Elmer. Seahorse XF Glycolysis Stress Test and Mito Stress Test kits, and XF Assay media were from Agilent Technologies Inc. The cOMplete EDTA-free Protease Inhibitor Cocktail tablets and PhosSTOP phosphatase inhibitor tablets were from Roche. BCA Protein Assay kit for protein concentration measurement and ECL2 Western blotting substrate for detection of ECL were from Pierce/Thermo Scientific. PVDF membrane was from Bio-Rad. All antibodies: pAkt-Ser⁴⁷³ (cat. #9271), pAkt-Thr³⁰⁸ (cat. #9275), total Akt (cat. #9272), pAS160-Thr⁶⁴² (cat. #4288), total AS160 (cat. #2447), pGSK3β-Ser⁹ (cat. #9336), total GSK3β (cat. #9315) and total AMPK (cat. #2532) were from Cell Signalling Technology, except pAMPK-Thr¹⁷² (cat. 07-626) was from Upstate (Lake Placid, NY, USA). Anti-rabbit HRP-conjugated secondary antibody was from Jackson ImmunoResearch Laboratories Inc.

Subjects and preparation of primary cell cultures

We studied 14 non-obese, non-smoking men (Table 1). Men were not using any medication, apart from one man

Table 1 Clinical characteristics (mean \pm s.e.m.) of the male participants ($n = 14$).

Age (years)	56 \pm 3
BMI (kg/m ²)	24.4 \pm 0.5
Waist (cm)	93 \pm 2
Hip (cm)	97 \pm 1
Waist-to-hip ratio	0.96 \pm 0.02
Fasting plasma glucose (mmol/L)	5.5 \pm 0.1
HbA1c (%)	5.5 \pm 0.1
Serum cholesterol (mmol/L)	5.2 \pm 0.3
Serum triglyceride (mmol/L)	1.1 \pm 0.1
Serum HDL cholesterol (mmol/L)	1.6 \pm 0.1
Serum LDL cholesterol (mmol/L)	3.0 \pm 0.3

who used simvastatin and alfuzosine. All had normal glucose tolerance, based on WHO criteria, in standard oral glucose tolerance test performed after an overnight fast. Muscle biopsy was collected after an overnight fast from *vastus lateralis* muscle under local anaesthesia (10 mg/mL lidocaine hydrochloride). Satellite cells were isolated from the muscle biopsy by trypsinisation and maintained in high-glucose (3150 mg/L, 17.5 mmol/L) DMEM/F12 supplemented with 20% (v/v) FBS, 1% (v/v) penicillin-streptomycin and 1% (v/v) amphotericin B, as described (23). Primary myoblasts were purified from non-myogenic cells with CD56-labeled magnetic beads. To create mature myotubes, myoblasts were differentiated in low glucose (1000 mg/L, 5.6 mmol/L) DMEM/F12 containing 2% FBS (v/v) for 6–7 days prior experimental pre-treatments. For analyses performed with Seahorse XF^e96 analyser, primary muscle cells were used as non-differentiated myoblasts.

Preparation of statins

Simvastatin was provided as powder in inactive lactone-form and was dissolved in DMSO. In order to activate lactone-form into acid-form simvastatin, the powder was dissolved in 0.1 M NaOH-EtOH solution, incubated at 50°C for 2 h, and pH 7 was adjusted with 0.2 M HCl, as described (24, 25).

Pre-treatments with statins

Differentiated myotubes were pre-treated for 48 h with a final concentration of 6 μ g/mL (14.3 μ M) lactone- and acid-form simvastatin. This concentration was chosen based on the report by Yaluri *et al.* (20). As controls, myotubes or myoblasts were pre-treated with the corresponding solvent, 0.1% DMSO and EtOH-NaOH, for lactone- and acid-form simvastatin, respectively. In some experiments (Supplementary Fig. 1, see section on [supplementary](#)

[materials](#) given at the end of this article), pravastatin was used with a final concentration of 13 μ g/mL (28.5 μ M) (20). All pre-treatments were performed in low glucose DMEM containing 2% (v/v) FBS and 4 mM L-glutamine. After incubation for 46 h, the pre-treatment was switched to starvation medium (with respective statin or control solution): low-glucose DMEM without FBS and supplemented with 0.5% (w/v) fatty-acid free BSA and 4 mM L-glutamine. Cells were serum-starved for 2 h before insulin stimulations. All incubations were performed at +37°C, in 5% CO₂ incubator.

Glucose metabolism

Glucose uptake was investigated in triplicate by measuring intracellular accumulation of 2-[1,2-³H]-deoxy-D-glucose (final specific activity 100 mCi/mmol), as described (26, 27). In brief, statin-treated and serum-starved myotubes were stimulated with or without 100 nM insulin in starvation media for 60 min. Radioactive glucose tracer was added in glucose-free DMEM containing 0.5% fatty-acid free BSA for 20 min, without statins, and with or without insulin, followed by ice-cold PBS washes and freezing in –20°C. Non-specific glucose uptake was determined with cytochalasin B (50 μ M). The cells were lysed in 0.4 M NaOH for 3 h and radioactivity detected in scintillation counter. Values were normalized to protein concentration measured with BCA assay. Data (in pmol/mg protein/min) were normalized to the insulin-stimulated glucose uptake (control) of each subject.

Glycogen synthesis was measured in triplicate, as described (26, 27), by detecting D-[¹⁴C]-glucose (final specific activity 0.18 μ Ci/ μ mol) incorporation into glycogen during 90 min of incubation with or without statins in the presence or absence of 100 nM insulin added in starvation media. Glycogen was extracted from the cells lysed in 0.03% (w/v) SDS, by boiling 30 min at +100°C together with glycogen carrier (final concentration 2.5 mg/mL) and precipitated with 94% ethanol followed by overnight incubation in –20°C. Precipitated glycogen was resolved in purified H₂O and radioactivity detected in scintillation counter. Values were normalized to protein concentration measured with BCA assay. Data (in nmol/g protein/h) were normalized to the insulin-stimulated glycogen synthesis (control) of each subject.

Glycolysis and glycolytic capacity were analysed by measuring extracellular acidification rate (ECAR) in myoblasts with Seahorse XF^e96 analyser (Seahorse Bioscience, a part of Agilent Technologies) by using XF Glycolysis Stress Test Kit, according to manufacturer's

instructions. Briefly, 20,000 cells/well were plated on XF96-well cell culture microplates 3 days before the assay, and on the next day pre-treated with 6 $\mu\text{g}/\text{mL}$ simvastatin (acid- and lactone-forms separately) or with their respective controls for 48 h, as described previously. The pre-treatment media was replaced with glucose-free XF Assay media and equilibrated for 60 min at 37°C in a CO₂-free incubator before being transferred to the XF^e96 analyser for the assay. During the assay, 10 mM glucose, 1 μM oligomycin and 50 mM 2-DG were injected sequentially to measure the glycolysis, glycolytic capacity and non-glycolytic acidification of the cells, respectively. Nuclear fluorescent stain Hoechst 33342 was added in the last injection to measure *in situ* nuclei count with BioTek Cytation 5 imager using the Montage function in the Gen5 software (28). Raw data were normalized to nuclei count and scaled up to 20 000 cells using the scale factor feature in Seahorse Wave software, as described in Kam *et al.* (29).

Protein extraction and Western blot analysis

Pre-treated myotubes, with or without insulin-stimulation (100 nM for 10 min), were lysed in NP40-lysis buffer (10 mM Tris, 150 mM NaCl, 7 mM EDTA and 0.5% NP-40) supplemented with protease and phosphatase inhibitors, sonicated and spun at 14,000 *g* for 15 min at +4°C. Protein concentration was determined with BCA Protein Assay kit. Proteins were separated by 10% SDS-PAGE, transferred to PVDF membrane by semi-dry electroblotting (Bio-Rad) and blocked with 5% (w/v) milk – 0.1% (v/v) Tween-TBS (TBST). Phosphorylated proteins were detected from membranes incubated with pAkt-Ser⁴⁷³, pAkt-Thr³⁰⁸, pAS160-Thr⁶⁴², pGSK3 β -Ser⁹ and pAMPK-Thr¹⁷² primary antibodies at a dilution of 1:1000 in 5% (w/v) BSA-TBST overnight at +4°C. Primary antibodies were probed with HRP-conjugated secondary antibody and visualized by enhanced chemiluminescence. Total target proteins were detected with corresponding antibodies after treating the membranes with stripping buffer (62.5 mM Tris pH 6.8, 2% (w/v) SDS, 0.7% (v/v) β -mercaptoethanol) for 45 min in +45°C shaker. Intensities of the proteins of interest were quantified using Fiji software (30) or Image Lab 5.1 software (Bio-Rad). Values of the intensities of the phosphorylated proteins were normalized to the intensity of their corresponding total protein. Data were normalized to the insulin-stimulated control sample of each subject.

Mitochondrial oxygen consumption

Mitochondrial respiration was analyzed by measuring oxygen consumption rate (OCR) with Seahorse XF^e96 analyzer by using XF Mito Stress Test Kit, according to manufacturer's instructions. Seeding the cells and all the pre-treatments were performed as described in the glycolysis assay. The pre-treatment media was replaced with XF Assay media supplemented with 5 mM glucose and equilibrated for 60 min at 37°C in a CO₂-free incubator prior to the assay. During the assay, basal oxygen consumption rate (OCR) was measured, followed by sequential injections of 1 mM oligomycin, 1.5 μM FCCP and 0.5 μM Rotenone and Antimycin C, to measure ATP production and proton leakage, maximal respiration and spare respiratory capacity, and finally non-mitochondrial respiration, respectively. Raw data were normalized as described in glycolysis assay.

Statistical analyses

Data are presented as mean \pm s.e.m. Statistical analyses were performed using GraphPad Prism (version 6.0h for Mac OS X). One- and two-way ANOVAs with repeated measurements followed by Sidak's *post hoc* test for multiple comparisons, were used to analyze data. $P < 0.05$ was considered statistically significant.

Results

Lactone-form simvastatin increases glucose uptake

Primary human myotubes were treated for 48 h with 6 $\mu\text{g}/\text{mL}$ (14.3 $\mu\text{mol}/\text{L}$) either lactone- or acid-forms of simvastatin. Exposure to lactone-form simvastatin led to a significant increase in basal (1.50-fold, $P < 0.0001$) and insulin-stimulated (1.40-fold, $P < 0.0001$) glucose uptake (Fig. 1A). Acid-form simvastatin did not affect glucose uptake (Fig. 1B). As the diabetogenic effect of hydrophilic pravastatin has been shown to be far less than with simvastatin (9), we next tested if pravastatin impacts glucose uptake. Exposure of myotubes for 48 h to 13 $\mu\text{g}/\text{mL}$ (28.5 $\mu\text{mol}/\text{L}$) pravastatin did not affect basal or insulin-stimulated glucose uptake (Supplementary Fig. 1A).

Lactone-form simvastatin increases, whereas acid-form simvastatin decreases glycogen synthesis

Exposure of primary human myotubes to lactone-form simvastatin led to a 1.51-fold increase in basal glucose

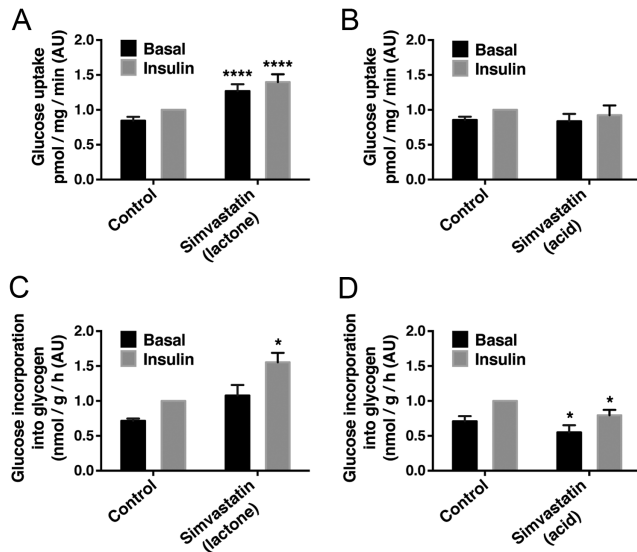


Figure 1 Glucose uptake (A and B) and glucose incorporation into glycogen (C and D) were measured using radioactive tracers. The effects of lactone- (A and C) and acid-form (B and D) simvastatin on glucose metabolism in primary human myotubes, in basal or insulin-stimulated conditions. Glucose uptake data (in pmol/mg/min) are expressed as mean \pm s.e.m. from 11 (lactone) and 9 (acid) men. Glycogen synthesis data (in nmol/g/h) are expressed as mean \pm s.e.m. from five (for both lactone and acid) men. * $P < 0.05$ and **** $P < 0.0001$ vs. respective control, two-way ANOVA with repeated measurements, Sidak's *post hoc* test, AU, arbitrary units.

incorporation into glycogen. However, this effect did not reach statistical significance ($P = 0.0606$). Exposure of myotubes to lactone-form simvastatin led to a significant 1.55-fold increase ($P = 0.0150$) in insulin-stimulated glucose incorporation into glycogen (Fig. 1C). In contrast to lactone-form, exposure to acid-form simvastatin led to a significant decrease in both basal ($P = 0.0344$) and insulin-stimulated ($P = 0.0148$) glucose incorporation into glycogen (Fig. 1D). Pravastatin exposure increased basal ($P = 0.0035$) and did not affect insulin-stimulated glycogen synthesis (Supplementary Fig. 1B).

Lactone-form simvastatin has a more profound effect on insulin signalling than acid-form simvastatin

Exposure of primary human myotubes to lactone-form simvastatin led to a non-significant reduction ($P = 0.0590$) in insulin-stimulated phosphorylation of Akt-Ser⁴⁷³ (Fig. 2A) and to a significant reduction ($P = 0.0303$) in insulin-stimulated phosphorylation of Akt-Thr³⁰⁸ (Fig. 2B). However, exposure to lactone-form simvastatin led to increased ($P = 0.0085$) insulin-stimulated phosphorylation of AS160 (Fig. 2C) and increased basal ($P = 0.0066$) and

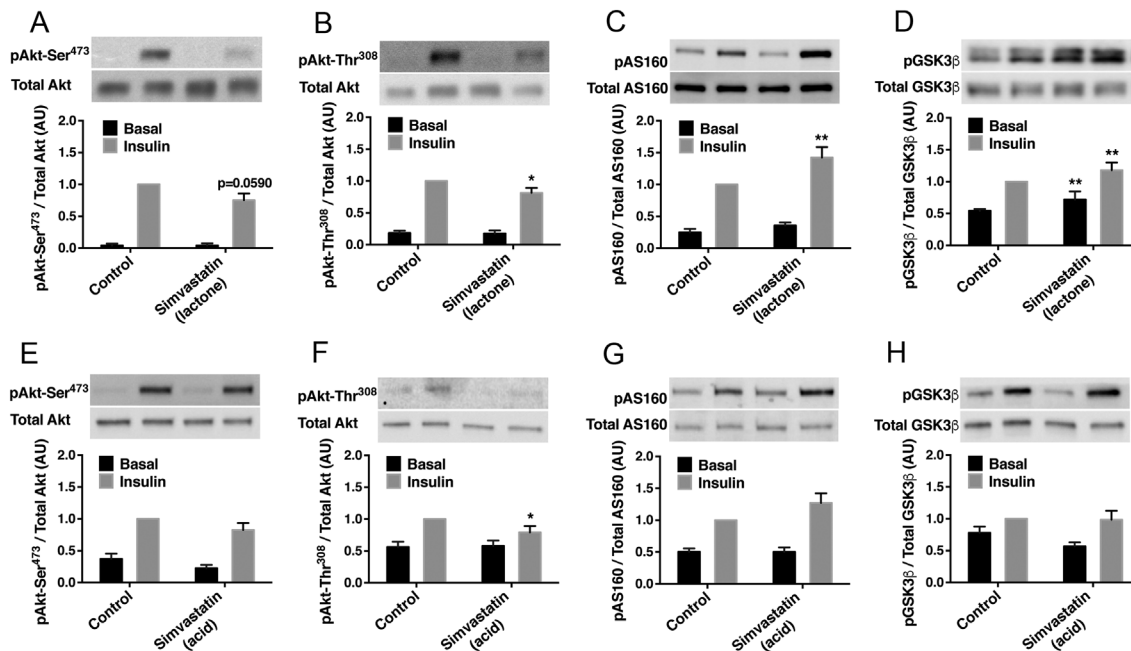


Figure 2 Activation of insulin signalling pathway. Western blot analysis showing the effect of lactone- (A, B, C and D) and acid-form (E, F, G and H) simvastatin in primary human myotubes, with or without insulin-stimulation. Representative blots and quantification of phosphorylated (p) Akt-Ser⁴⁷³ (A, E), pAkt-Thr³⁰⁸ (B, F), pAS160-Thr⁶⁴² (C, G), and pGSK3 β -Ser⁹ (D, H), and of their respective total proteins. Data are expressed as mean \pm s.e.m. from five men, except $n = 9$ for pAS160-Thr⁶⁴² (C) and $n = 7$ for pGSK3 β -Ser⁹ (D) in lactone-treated group. * $P < 0.05$ and ** $P < 0.01$ vs respective control, two-way ANOVA with repeated measurements, Sidak's *post hoc* test, AU, arbitrary units.

insulin-stimulated ($P = 0.0060$) phosphorylation of GSK3 β (Fig. 2D). Exposure of primary human myotubes to acid-form simvastatin led to reduced ($P = 0.0438$) insulin-stimulated phosphorylation of Akt-Thr³⁰⁸ (Fig. 2F), whereas phosphorylation of Akt-Ser⁴⁷³ (Fig. 2E), AS160 (Fig. 2G) or GSK3 β (Fig. 2H) was not affected by acid-form simvastatin.

Lactone- and acid-form simvastatin reduce glycolysis and mitochondrial respiration, and lactone-form simvastatin activates AMPK

Exposure of human myoblasts to lactone- or acid-form simvastatin led to a profound reduction in both glycolysis ($P = 0.0004$ for lactone-, and $P = 0.0004$ for acid-form) and glycolytic capacity ($P = 0.0009$ for lactone-, and $P = 0.0004$ for acid-form) (Fig. 3). Exposure to either form simvastatin led to a reduction also in mitochondrial oxygen consumption ($P = 0.0073$ for lactone-, and $P = 0.0001$ for acid-form) and ATP production ($P = 0.0002$ for lactone-, and $P < 0.0001$ for acid-form) (Fig. 4). Inhibition of mitochondrial ATP production leads to an increase in cellular AMP to ATP ratio, which activates AMPK, a sensor of cellular energy status (31). Thus, we next analyzed the phosphorylation of AMPK. Exposure of primary human myotubes to lactone-form simvastatin led to a significant increase in basal ($P = 0.0075$) and insulin-stimulated ($P = 0.0030$) AMPK phosphorylation at Thr¹⁷² (Fig. 5A). Acid-form simvastatin did not affect AMPK phosphorylation (Fig. 5B).

Discussion

Statins are widely used to lower plasma cholesterol levels. They inhibit the mevalonate pathway that mediates cholesterol synthesis (2, 3, 4). While use of statins effectively reduces the risk of atherosclerotic events, several studies have demonstrated that statin treatment may have adverse effects including myotoxicity, impaired insulin sensitivity and increased risk for type 2 diabetes (6, 12, 14, 32). Direct molecular mechanisms leading to statin-induced myotoxicity and insulin resistance in skeletal muscle are yet to be comprehensively reported. However, one issue may relate to the lipophilic nature of certain statins, such as the lactone-form simvastatin, which are more prone to cause myotoxicity (19). Lactone-form simvastatin has also been suggested to induce insulin resistance by suppressing insulin signalling and GLUT4 expression (20).

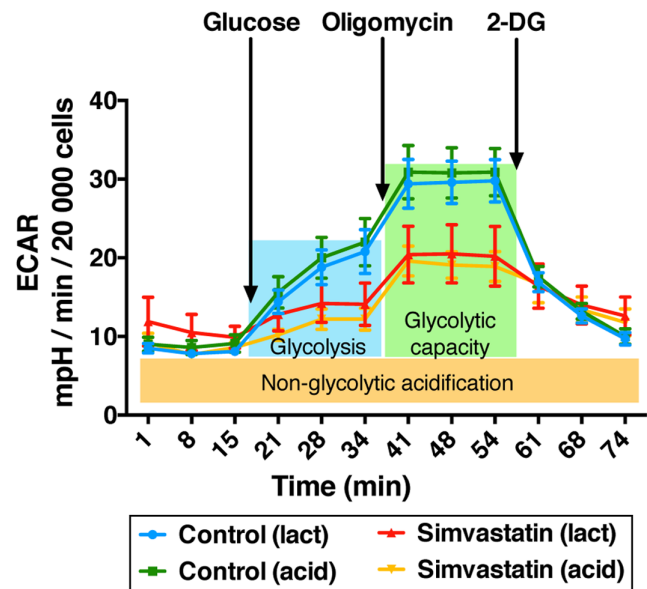
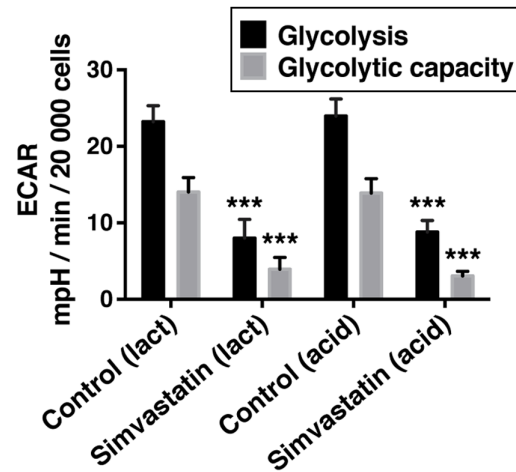


Figure 3 Glycolysis and glycolytic capacity in response to pre-exposure to lactone- and acid-form simvastatin were measured by detecting extracellular acidification rate (ECAR) in primary human myoblasts with Seahorse analyzer. Data (in mpH/min/20,000 cells) are expressed as mean \pm S.E.M. from six men. *** $P < 0.001$ vs respective control, one-way ANOVA with repeated measurements, Sidak's *post hoc* test.

Here, we compared the effects of lactone- and acid-forms simvastatin on glucose metabolism in primary human skeletal muscle cells. To the best of our knowledge, the effects of simvastatin on insulin-stimulated glucose metabolism have not been previously reported in human skeletal muscle cells. In our study, lactone-form simvastatin increased both basal and insulin-stimulated glucose uptake. This finding was quite unexpected and in contradiction with previous data using L6 (rat) or C2C12 (mouse) myotubes, where both basal and

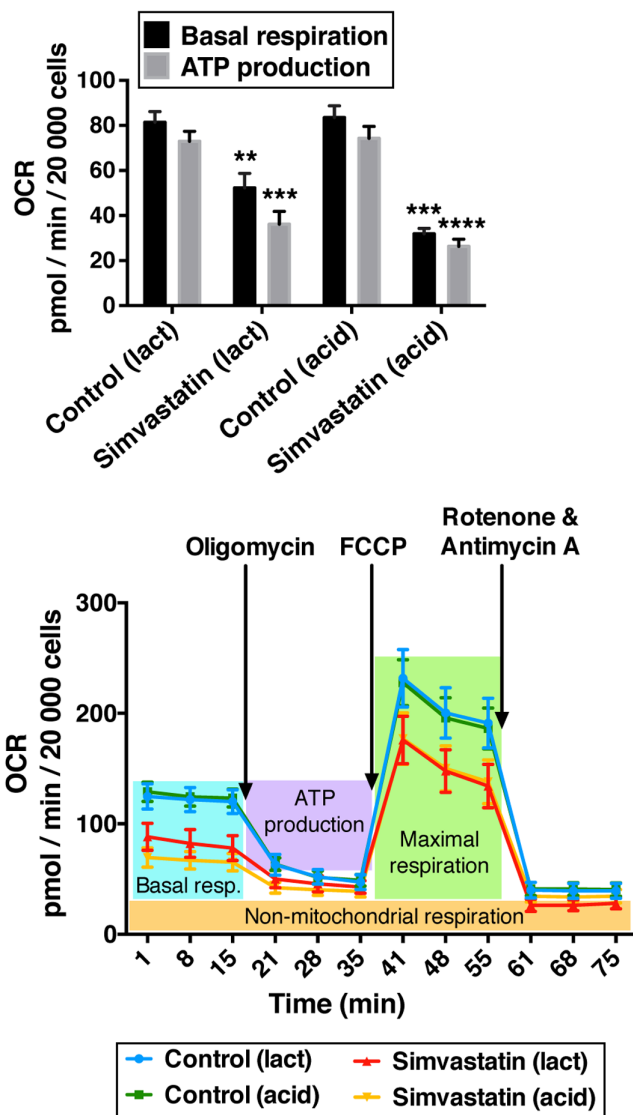


Figure 4 Mitochondrial respiration and ATP production in response to pre-exposure to lactone- and acid-form simvastatin were measured by detecting oxygen consumption rate (OCR) in primary human myoblasts with Seahorse analyzer. Data (in pmol/min/20,000 cells) are expressed as mean \pm s.e.m. from four men. $**P < 0.01$, $***P < 0.001$ and $****P < 0.0001$ vs respective control, one-way ANOVA with repeated measurements, Sidak's *post hoc* test.

insulin-stimulated glucose uptake were reduced by lactone-form simvastatin (20, 21). While there may be differences in metabolic responses between rodent-derived and human muscle cells, simvastatin also reduced basal glucose uptake in human myotubes (33). The reason for these apparently contradictory results is not obvious. However, we observed that insulin-stimulated glycogen synthesis was also increased in response to lactone-form simvastatin, with a tendency for an increase also in basal glycogen synthesis. Thus, these data are consistent with

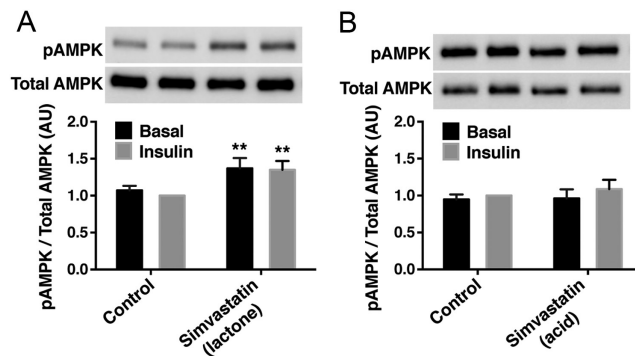


Figure 5 Activation of AMP-activated protein kinase (AMPK). Western blot analysis showing the effect of lactone- (A) and acid-form (B) simvastatin in primary human myotubes. The representative blots and quantification of phosphorylated AMPK-Thr¹⁷² and total AMPK. Data are expressed as mean \pm s.e.m. from nine (lactone) and five (acid) men, $**P < 0.01$ vs respective control, two-way ANOVA with repeated measurements, Sidak's *post hoc* test, AU, arbitrary units.

the observed activation of glucose uptake in response to lactone-form simvastatin. Exposure of human muscle cells to acid-form simvastatin has previously been reported to reduce basal glucose uptake (32), whereas in our study acid-form simvastatin did not affect glucose uptake. This discrepancy may be related to methodological differences to assay glucose uptake, which was detected by glucose analogue 6-NBDG by Nowis *et al.* (32) whereas we assayed glucose uptake with radiolabelled 2-deoxy-glucose. However, we observed an inhibition of both basal and insulin-stimulated glycogen synthesis by acid-form simvastatin. Taken together, our data suggest different metabolic effects for acid- and lactone-forms of simvastatin during 48 h exposure. An inhibition of basal and insulin-stimulated glycogen synthesis by active acid-form simvastatin may contribute to a reduction in insulin sensitivity observed with simvastatin treatment (6).

Metabolic actions were accompanied by changes in insulin signalling cascade, as insulin-stimulated phosphorylation of AS160 and GSK3 β were upregulated with lactone-, but not with acid-form simvastatin. Interestingly, the activation of both Akt sites at Ser⁴⁷³ or Thr³⁰⁸ were inhibited with lactone-form simvastatin, suggesting an Akt-independent mechanism for phosphorylation of AS160 and GSK3 β with lactone-form simvastatin.

In order to get more detailed insight into the glucose metabolism in terms of energy production, we determined the rate of glycolysis and mitochondrial respiration, both of which produce ATP. After 48 h exposure of myoblasts to both forms of simvastatin, the rate of glycolysis and mitochondrial respiration were measured in real-time

with the Seahorse analyzer. Either form of simvastatin led to a profound reduction in both glycolysis and glycolytic capacity, indicating that simvastatin-treated cells had diminished capability to catabolize glucose. These findings are consistent with a recent study where simvastatin reduced glycolysis and glycolytic capacity in neuroblastoma cells (34). Statins have been implicated with impaired mitochondrial function in several studies (16, 17, 35). Lactone-form statins, including lactone-form simvastatin, have especially been shown to be more potent in inducing cytotoxicity and reducing mitochondrial respiration and ATP production rate, when compared to their corresponding acid-forms (22). Acid-form simvastatin also reduces ATP content in C2C12 myotubes (17). Our findings are in agreement with the idea of overall negative impact of statins on mitochondrial function, as exposure to both lactone and acid-form of simvastatin led to a decrease in mitochondrial oxygen consumption and ATP production.

The observed increase in glucose uptake in response to lactone-form simvastatin may be a consequence of increased cellular stress and AMPK activation. Reduced mitochondrial ATP production leads to an increase in cellular AMP to ATP ratio. This activates AMPK, a sensor of cellular energy status (31). Indeed, we observed that exposure of primary human myotubes to lactone-form simvastatin led to a significant increase in AMPK-Thr¹⁷² phosphorylation. This was not observed with acid-form simvastatin, which may be related to the more profound inhibition of mitochondrial respiration and ATP production with lactone-form simvastatin (22). Even though both forms of simvastatin reduced mitochondrial respiration and ATP production in our study, it is possible that differences in their inhibitory potency may not have been revealed by our experimental set-up. AMPK directly phosphorylates AS160 (36, 37) and increases glucose uptake in an insulin-independent fashion (38). AS160 phosphorylation was increased in myotubes exposed to lactone-form simvastatin even if the Akt phosphorylation was blunted. These data suggest an Akt-independent mechanism for increased glucose uptake in response to lactone-form simvastatin, which may be AMPK-mediated. As glucose uptake was increased and glucose oxidation markedly diminished, glucose fluxes were directed towards non-oxidative glucose disposal in muscle cells treated with lactone-form simvastatin. The increase in glucose incorporation into glycogen may have been facilitated by increased phosphorylation of GSK3 β in response to lactone-form simvastatin. As the activation of Akt was reduced, these data suggest an Akt-independent

phosphorylation for GSK3 β . One possible mechanism may be related to activation of protein kinase C (PKC) (39), especially its α , β 1 and γ isoforms (40), which are known to phosphorylate GSK3 β . A schematic presentation of the effects of lactone-form simvastatin on glucose metabolism and signalling events in primary human skeletal muscle cells is given in Fig. 6.

The limitation of the current study is that we investigated in detail the effects of only one of the statins used as cholesterol-lowering drug. Inclusion of other lipophilic statins, such as lovastatin or atorvastatin (10), could have improved the interpretations of the effects observed with simvastatin. However, in some experiments we included hydrophilic and clinically far less diabetogenic pravastatin, which did not affect glucose uptake or insulin-stimulated glycogen synthesis. This strengthens our observations regarding simvastatin. The concentration of the simvastatin used in this study was 6 μ g/mL and the concentration of pravastatin was 13 μ g/mL, which are in line with those used in other *in vitro* studies (19, 20, 22, 33). Plasma concentrations for simvastatin and pravastatin after oral intake are in the scale of nanograms per milliliter (18, 41). Thus, the concentrations used usually in *in vitro* experiments are substantially higher than those reached *in vivo*, and are at supraphysiological concentrations to provide a maximal stimulus during a short-term exposure. The time and pH-dependent conversion of the lactone-form simvastatin to the corresponding hydroxy acid in cell culture conditions at 37°C has been observed to be substantial after 24–48 h incubation (42). However, the effect of lactone-form simvastatin exposure for 48 h on

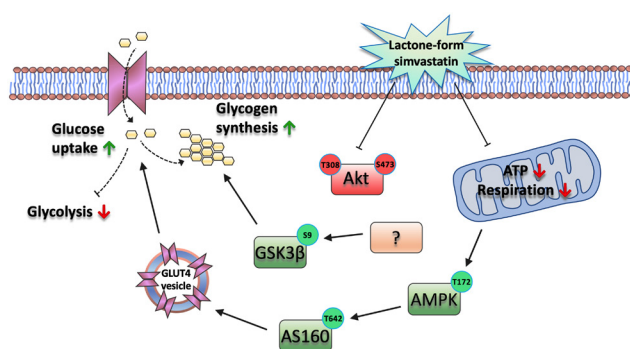


Figure 6

Schematic presentation of the effects of lactone-form simvastatin on glucose metabolism and signalling events in primary human skeletal muscle cells. Down- and upregulated metabolic and signalling events are indicated in red and green color, respectively. Dashed line indicates the metabolic action. Akt, protein kinase B; AMPK, adenosine monophosphate (AMP)-activated protein kinase; AS160, Akt-substrate 160 kDa; ATP, adenosine triphosphate; GLUT4, glucose transporter 4; GSK3 β , glycogen synthase kinase 3 β .

glucose uptake and glycogen synthesis was completely different to acid-form in our studies, suggesting that even lower amount of lactone-form simvastatin is sufficient to elicit different responses on glucose uptake and glycogen synthesis as compared to acid-form. Muscle cell cultures had been established only from men, which is also a limitation. As, for example, estrogens promote insulin sensitivity (43), it would be interesting to investigate in the future if gender would affect the responses of muscle cells to statins. Another limitation of the study is that glycolysis and mitochondrial respiration were studied in myoblasts, and in other experiments differentiated myotubes were used. While myoblasts and myotubes may exhibit differences in their metabolic responses (26), our observation of reduced mitochondrial respiration in simvastatin-treated human myoblasts is in good agreement with Kwak *et al.* (35) who observed reduced mitochondrial respiration in simvastatin-treated human myotubes. Thus, simvastatin inhibits mitochondrial respiration both in myoblasts and myotubes. The overall strength of our study is the use of biological replicates for the comprehensive and complementary approaches to investigate glucose metabolism, as methods included determination of glucose uptake, glycogen synthesis and glycolysis.

In conclusion, our data indicate that lactone- and acid-forms of simvastatin exhibit differential effects on non-oxidative glucose metabolism as lactone-form increases and acid-form impairs glucose storage into glycogen, suggesting impaired insulin sensitivity in response to acid-form simvastatin. Both forms of simvastatin demonstrate similar inhibitory effect on oxidative glucose metabolism and energy production in human skeletal muscle cells. Taken together, these effects may contribute to increased risk of myotoxicity and type 2 diabetes that has been observed with simvastatin use (6, 12).

Supplementary materials

This is linked to the online version of the paper at <https://doi.org/10.1530/EC-20-0444>.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Funding

This study has been supported by grants to H A K from Academy of Finland (grant no. 258753), Diabetes Wellness Sverige (grant no 598-174), Finnish Diabetes Research Foundation, Finska Läkaresällskapet, Finnish Medical Foundation, Helsinki University Central Hospital (funding

from hospital administration and governmental subsidy for research), Jalmari and Rauha Ahokas Foundation, Laboratoriolääketeen edistämissäätiö sr, Liv och Hälsa Foundation, Novo Nordisk Foundation, and Sigrid Juselius Foundation. S M has received support from Doctoral School of Health Sciences (Doctoral Programme in Clinical Research) of University of Helsinki, Finnish Diabetes Research Foundation, and Finska Läkaresällskapet.

Ethics approval and consent to participate

The study was approved by the ethical committee of Helsinki University Central Hospital (261/13/03/01/2008) and was conducted according to the standards of the Declaration of Helsinki as revised in 2008. Written informed consent was obtained from the subjects before participation.

Author contribution statement

S M, N D, Y H N, M L and H A K designed the study. S M, N D, Y H N, P K and H A K acquired the data. S M, N D and H A K analyzed and interpreted the data. S M and H A K drafted the article, which was reviewed and edited by N D, Y H N, P K and M L. H A K supervised the study and acquired the funding.

Acknowledgement

The authors thank all the volunteers who participated in the study.

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Received in final form 14 October 2020

Accepted 29 October 2020

Accepted Manuscript published online 29 October 2020