Simvastatin inhibits glucose uptake activity and GLUT4 translocation through suppression of the IR/IRS-1/Akt signaling in C2C12 myotubes

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Abbreviations: ML, mevalonolactone; HMG-CoA, 3-hydroxy-3-methyl-glutaryl-CoA; IR insulin receptor; IRS, insulin receptor substrate; GLUT4, glucose transporter member 4; GGPP, geranylgeranyl pyrophosphate; FPP, farnesyl pyrophosphate

Astract

Simvastatin, a 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) reductase inhibitor, is clinically used in the prevention and treatment of cardiovascular diseases. Numerous studies demonstrate that statins increase the risk of new-onset diabetes in long-term therapy, but mechanisms underpinning this effect are still unclear. Here, we investigated whether simvastatin inhibited the glucose uptake activity and the underlying mechanisms in C2C12 myotubes. Our studies showed that simvastatin significantly inhibited glucose uptake activity and GLUT4 translocation, whereas the effect was reversible with mevalonolactone (ML), which acts as an intermediate of cholesterol synthesis pathway. Mechanistically, the inhibition of glucose uptake and GLUT4 translocation elicited by simvastatin were associated with the suppression of the insulin receptor (IR)/IR substrate (IRS)/Akt signaling cascade. Simvastatin suppressed the phosphorylation of IR, IRS-1 and Akt, and total expression of IR or IRS-1, but did not affect Akt. Furthermore, simvastatin decreased Rac1 GTP binding. In conclusion, our findings indicate that simvastatin suppress glucose uptake activity and GLUT4 translocation via IR-dependent IRS-1/PI3K/Akt pathway. These results provide an important new insight into the mechanism of statins on insulin sensitivity which may be associated with new-onset diabetes.

1. Introduction

Statins, defined as 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors, are widely used cholesterol-lowering drugs and provide a tremendous advantage in reducing the morbidity and mortality of cardiovascular and cerebrovascular diseases in various at-risk populations [1]. Recently, so-called pleiotropic functions, such as glucose metabolism, anti-inflammation, anti-fibrosis and immunomodulation, are widely explored [2,3,4,5]. However, clinical trials and meta-analysis highlight that statins increase the risk of new-onset diabetes among patients receiving statins [2,6]. Although mechanisms for raised this risk following statins are not yet fully understood, some explanations about this effect are available. Statins may potentially reduce insulin secretion by suppressing adenosine triphosphate (ATP)-dependent potassium channels, impair glucose transport by inhibiting isoprenoids synthesis, delay ATP production, and induce cell apoptosis of islet β -cell [7,8,9]. Our previous study suggested that simvastatin could inhibit insulin synthesis and secretion in MIN6 cells through a reduction in saccharometabolism. [10]. Other attentions were focused on the effects of statins on glucose metabolism in peripheral target organ such as liver, skeletal muscles and adipocytes. However, mechanisms underlying the pro-diabetic effect of statins remain perplexing.

IRS-1/PI3K/Akt signaling pathways are the classical pathway in the regulation of glucose uptake and metabolism [11]. The process of insulin-stimulated glucose uptake in skeletal muscle cells is as following: the insulin binds to insulin receptor (IR), then leads to tyrosine phosphorylation of the insulin receptor substrate (IRS) proteins. Phosphorylated IRS proteins ultimately result in the translocation of GLUT4 from the intracellular storage compartment to the plasma membrane through downstream signaling cascades [12]. The decreased expression of GLUT4 in the plasma membrane contributes to insulin resistance and type 2 diabetes [13]. However, the effect of statins on IRS-1/PI3K/Akt signaling pathways remains unclear in skeletal muscle cells. Statins not only inhibit rate-limiting enzyme of the cholesterol biosynthetic, but also reduce the production of mevalonate (such as isoprenoid intermediates) which is the precursor of cholesterol. Its derivatives serve as a variety of important lipid ligands for the post-transcriptional modification of proteins [14].

The prenylation of small GTP-binding proteins (or GTPases) is dependent on prenylation enzyme substrates, such as FPP (farnesyl pyrophosphate) and GGPP (geranylgeranyl pyrophosphate), which are the precursors of cholesterol. Modified by prenylation, the proteins are covalently added to members for regulating membrane targeting, trafficking and signaling[15]. Rac1, an important member of Rho GTPases family, regulates insulin-stimulated glucose uptake by induced GLUT4 translocation in mature skeletal mouse muscle [16]. Notably, the impairment of Rac1 signaling pathway was observed both in skeletal muscle of insulin resistant human subjects and in ob/ob mice [17]. Based on these findings, we hypothesis that statins inhibit glucose uptake and GLUT4 translocation through suppression of IRS-1/PI3K/Akt signaling pathways, which may be associated with statins-induced new-onset diabetes.

2. Materials and methods

2.1.C2C12 cell culture

The mouse muscle cell line C2C12 was purchased from the Chinese Academy of Sciences (Shanghai, China). The cells were cultured, proliferated and differentiated as described elsewhere [18]. Briefly, the cells were cultured as monolayer in Dulbecco's Modified Eagle's Medium (DMEM, Thermo scientific, USA) containing 10% (v/v) fetal bovine serum (FBS, Gibco, USA) and antibiotics (100 00IU/ml penicillin and 10mg/ml of streptomycin). All the cells were grown under an atmosphere of 5% CO₂ in air at 37°C until they were reached 90% confluence. Then this medium was substituted with differentiation medium DMEM containing 2% (v/v) horse serum and antibiotics, incubated for 6 days to induce myotube formation. Above medium changed every day. After myotube formation, cells were co-cultured with simvastatin at 2, 5, 10μM or DMSO as control group for 0h, 24h, 48h, 72h.

2.2. Uptake of glucose

Simvastatin (EMD Chemicals, USA) was prepared as 20 mM stock solutions in DMSO. Mevalonate was purchased as mevalonolactone (ML) from Wako Pure Chemical Industries (Osaka, Japan), prepared as a 0.5 M stock solutions in water, and utilized to the medium at a final concentration of 100 μM. The medium of treated cells were collected to measure the glucose level by Glucose Kit (Biosino Bio-technology and Science Inc, China). Absorbance was measured at 490nm in a microplate reader BIO-RAD Model 680.

2.3. Cell viability tests

Myotubes were treated with 0–10μM simvastatin with or without 100 μM ML for 72 h (changed medium every day) before cell viability was analyzed. Cell viability assays were determined by the manufacturer's protocol of Cell Counting Kit-8 (Dojindo, Japan). Briefly, 10 μl of WST-8 were added to each treated cell and incubated for 3h at 37°C Then measured absorbance at 450 nm.

2.4. Western blot analysis

The treated cells were washed twice with ice-cold PBS after treated with simvastatin and mevalonolactone (100 μM) or simvastatin alone at 72 h and at the last 30 min stimulated with insulin (100 nM), and harvested in lysis buffer with the presence of protease inhibitor cocktail, phosphatase inhibitor cocktail. Protein concentrations were measured by BCA protein assay kit (Thermo scientific, USA). An equal volume of sample (100 μg) were separated on 10% SDS-PAGE and transferred to polyvinylidene difluoride membrane. Non-specific sites were blocked with Tris-buffered saline (pH 7.4) and 0.05% Tween 20 (TBST) supplemented with 5% bovine serum albumin. And membranes were then incubated overnight at 4°C with individual respective antibody at respective dilution: rabbit anti-mouse GAPDH (1:1000 dilution; catalog no.5174, Cell Signaling Technology), mouse anti-mouse IR (1:750 dilution; catalog no.ab69508, Abcam), rabbit anti-mouse IRS-1 (1:1000 dilution; catalog no.2390, Cell Signaling Technology), rabbit anti-mouse AKT (1:1000 dilution; catalog no.4685, Cell Signaling Technology), rabbit anti-mouse phospho-IR on Tyr1361 (1:500 dilution; catalog no.ab60946, R&D System), rabbit anti-mouse phospho-IRS-1 on Tyr1222 (1:500 dilution; catalog no.3066, Cell Signaling Technology), rabbit anti-mouse phospho-AKT on Thr308 (1:500 dilution; catalog no.9275, Cell Signaling Technology), rabbit anti-mouse phospho-AKT on Ser473 (1:500 dilution; catalog no.4051, Cell Signaling Technology). After extensive washing with TBST, the membranes were incubated for 60 minutes with anti-rabbit/mouse HRP-coupled secondary antibodies at 1:10,000 dilution. And the signal was detected via a chemiluminescent detection system according to the manufacturer's instructions (Pierce, USA). Immunoreactive proteins were visualized by enhanced chemiluminescence and quantified by densitometry using ImageJ Software. Before above process, to determine whether insulin effect was acting at 30 min, we examined the time-dependent activation of Akt in myotubes stimulated with either insulin or ddH₂O.

2.5 Rac1 activity in C2C12 cell

The C2C12 cells were treated and collected as above. Rac1 activities were measured using a Rac1 activation assay kit (STA401, Cell Biolabs Inc.USA). In short, after centrifugation at 18000g for 1min, the protein concentration in the lysates was determined by BCA protein assay kit (Thermo scientific, USA). The equal amount of protein in supernatants was added to p-21-binding domain (PBD) domain of PAK1 beads, which only binds activated Rac1 in its GTP-bound form. Following incubation with gentle agitation at 4 °C for 1 hour, beads were washed twice with pull-down assay buffer. The amount of GTP-bound Rac1 was detected by immunoblot analysis using an antibody against Rac1.

2.6. Flow cytometry

For flow cytometry studies, the above treated cells were digested with trypsin EDTA-free and washed twice with phosphate-buffered saline (PBS). Next fixed with 4% paraformaldehyde with 20min. Washed with PBS and incubated with anti-mouse GLUT4 (cat no ab65267, Abcam, USA) or isotype control mouse IgG2a (ICIGG2A) for 30 minutes at room temperature in 50 μ l PBS. The secondary antibody was used Dylight 488 goat anti-mouse IgG (H+L) (1:500 dilution, Abcam, USA) for 30 min at room temperature in 100 μ l PBS. Before analysis, cells were washed twice with PBS and resuspended in 500 μ l PBS. The cells were analyzed on FACS Scan flow cytometer (BD Biosciences, San Diego, CA) and mean fluorescence intensity (MFI) determined using Flowjo Software Version 7.6 (Tree Star, Ashland, OR).

2.7. Statistical analysis

Data were collected from several independent experiments, with three replicates per experiment. All data were analyzed with One-Way ANOVA or student t-test in SPSS 13.0 and $P < 0.05$ was considered statistically significant. Bars in the graph represent standard deviation (S.D.)

3. Results

3.1 Simvastatin decreased glucose uptake in C2C12 myotubes

The cytotoxicity of simvastatin and mevalonolactone (ML) on C2C12 myotubes was examined after simvastatin with or without ML for 72 h. As shown in Fig.1A, simvastatin and ML were not cytotoxic to cells following these concentrations. The ability of glucose uptake in C2C12 myotubes was indirectly indicated via measuring the concentration of glucose in medium. The initial glucose concentration in DMEM is 4.5g/l. We study the overall glucose consumption of the control cells is 2.5 ± 0.05 g/l for 72 hours. With the time exposure to 5 μ M simvastatin, the inhibited effect on glucose uptake tended to be more obvious and was significant at 72 hours compared to DMSO (Fig.1B). Simvastatin significantly inhibits glucose uptake in a dose-dependent manner in mouse muscle cells without insulin (Fig.1C). To study whether the inhibition of glucose uptake induced by simvastatin is associated with the deficient of isoprenoid intermediates, we used ML which is an intermediate of the mevalonate pathway. Interestingly, the effect was reversed by co-administration of simvastatin and 100 μ M ML (Fig.1D).

3.2 Simvastatin inhibited insulin-induced glucose uptake and the translocation of GLUT4 in C2C12 myotubes

To study the effect of simvastatin on insulin action, the C2C12 myotubes were treated with or without insulin. The results showed that insulin obviously promoted the glucose uptake of the muscle cells without simvastatin. However, simvastatin impaired the insulin action. Interestingly, ML could reverse the phenomenon (Fig.2A). GLUT4 is a predominant glucose transporter in mouse myotubes and has a direct influence on glucose uptake [19]. The action of insulin is known to result in the translocation of GLUT4 from an intracellular storage site to the plasma membrane (PM), where it binds with glucose and uptakes glucose from extracellular milieu into the cell [20]. Simvastatin at the concentration of 2 μ M, 5 μ M and 10 μ M significantly inhibited the expression of GLUT4 in PM. However, 100 μ M ML rescued the diminishment of GLUT4 translocation (Fig.2B and 2C). These results suggested that insulin effectively enhanced the muscles's ability to soak up glucose, but the insulin action was suppressed by simvastatin via reducing the translocation of GLUT4.

3.3 Simvastatin inhibited canonical insulin signaling through the IR/IRS-1/Akt signaling pathways

Because the activations of several proteins, such as IR, IRS, PI3K, and Akt, are involved in insulin-induced translocation of GLUT4, we investigate whether simvastatin inhibits the insulin signaling pathway in C2C12 myotubes. As shown in Fig.3A, simvastatin significantly inhibited the phosphorylation of insulin-stimulated IR Tyr 1361, IRS-1 Tyr 1222 and AKT Thr308, which play a critical role in promoting GLUT4 translocation after insulin action. The suppressing effect of simvastatin on insulin-induced IR, IRS-1, Akt activation was reverted by co-administration of ML. The total expressions of IR and IRS-1 were obviously down-regulated with simvastatin, which were consistent with the tendency of phosphorylation of these proteins. ML also prevented the inhibitory effect. Intriguing, simvastatin had no effect on the expression of AKT (Fig.3B) and PI3K (data not shown).

3.4 Simvastatin inhibited Rac1 activity in C2C12 myotubes

Previous study suggested that Rac1 regulated insulin-stimulated glucose uptake by induced GLUT4 translocation in mature skeletal mouse muscle. To test the effect of simvastatin on Rac1 activity, we measured Rac1-GTP binding in C2C12 myotubes treated with simvastatin for 72 hours in response to insulin. Simvastatin decreased Rac1-GTP binding, which is the active state of Rac1 to control downstream signaling cascades (Fig.4). On the other hand, the Rac1 GTP binding was increased in S5+ML groups compared to S5. These findings suggest that the impairment of GLUT4 translocation induced by simvastatin may be associated with decrease in Rac1-GTP activity.

4. Discussion

Statins are inhibitors of hydroxy-methylglutaryl coenzyme A (HMG-CoA) reductase, which can inhibit the synthesis of endogenous cholesterol [1]. It has been demonstrated that statins are associated with the risk of new-onset diabetes in long-term therapy [2,6], but the underlying mechanism remains perplexing. Dysfunction of islet β cells and insulin resistance are the major mechanisms of Type-2 diabetes. Previous studies have been showed that statins can inhibit glucose transport, delay ATP production, suppress calcium channel-dependent insulin secretion and induce cell apoptosis in β -cell [7,21]. However, the effects of statins on peripheral target organ (liver, skeletal muscles and adipocytes) are still controversial. In our study, C2C12 myotubes were treated by simvastatin with or without insulin. The results showed that insulin increased the glucose uptake compared with ddH₂O control group. But simvastatin significantly inhibited glucose uptake and GLUT4 translocation in the presence of insulin, whereas this effect was reversible with mevalonate.

First, we measured the effect of simvastatin treatment on cell viability by Cell Counting Kit-8 method, which is broadly accepted to check the cell viability, proliferation or cytotoxicity. We find that simvastatin has no obvious cytotoxicity on mouse myotubes in these concentrations. However, some researchers have found that simvastatin reduced human or rat skeletal muscle cells cell viability [22,23]. Conversely, other studies demonstrated that simvastatin has no effect on cell viability in primary human myotubes from 0 μ M to 40 μ M with or without ML in medium [24]. We believe the contrary results may be caused by the differences of cell species and culture conditions.

As a polarity molecule, glucose transporting should be mainly mediated by glucose transporter which localized in the plasma membrane. In insulin-sensitive cells, such as muscle and fats cells, the glucose uptake is mediated primarily by the facilitative glucose transporter isoform GLUT4 expression on PM which is dependent on insulin-signaling process. Insulin leads to the translocation of GLUT4 from an intracellular storage site to PM [20]. Although previous studies revealed that the atorvastatin and lovastatin can inhibited GLUT4 protein expression in differentiated 3T3-L1 cells [25,26], whether the effect exists in myotubes and the underlying mechanisms remain unclear. Here, we demonstrated that simvastatin also decreased the amount of GLUT4 in PM by inhibiting its translocation.

There are three signaling pathways involved in the translocation of GLUT4 in response to insulin in muscle: IRS signaling through the PI3K-dependent pathway, APS/Cbl/CAP signaling through the PI3K-dependent pathway, and Munc18c signaling to the SNARE complexes [27]. IRS-1 and Akt are the critical components in the translocation of GLUT4 from intracellular storage site to PM. Recent study revealed that perilipin 2 inhibited insulin-induced glucose uptake activity through decreasing IRS-1 expression in C2C12 myotubes [28]. Besides, Pollen Typhae, an active ingredient from Chinese herbal medicine, improved insulin-stimulated glucose uptake via increased the phosphorylation of Akt at Thr308 in an insulin-dependent manner [29]. So we focused on IRS-1/PI3K/Akt pathways. Our study demonstrated that simvastatin could significantly inhibit the phosphorylation of IR, IRS-1 and Akt in mouse myotubes, which was coincident with the results that atorvastatin could decrease the expression of phosphorylation of Akt in adipocytes [25]. These results suggested that simvastatin impaired glucose metabolism through inhibiting IRS-1/PI3K/Akt signaling pathways. We further found that simvastatin could suppress the total expression of IR and IRS-1 rather than PI3K and Akt. In fact, about 90% of the insulin receptor on cell membrane is in a state of "standby", only 10% - 20% of the IR in PM interacting with insulin could reach to maximum effect [30]. The down-regulation of total IR plays a tiny role in insulin action, but its phosphorylation is crucial.

Statins can competitively bind to the portion of HMG-CoA and disturb the mevalonic acid pathway which inhibit the synthesis of sterol isoprenoids and nonsterol isoprenoids [31]. To explore whether

statins impair glucose metabolism by inhibiting the mevalonic acid pathway, we treated cells with mevalonolactones and simvastatin. We found that simvastatin significantly inhibited glucose uptake and impaired insulin pathway. Interestingly, the effect was reversible with mevalonolactone, which probably represented that the impairment of isoprenoids acid pathways led to inhibit glucose uptake. The prenylation enzyme substrates, such as geranylgeranyl pyrophosphate (GGPP) and farnesyl pyrophosphate (FPP), are the critical ligands for post-transcriptional prenylation of multiple proteins, including the Rho GTPases which was dependent on a post-translational modification and are involved in the membrane-associated protein trafficking[32]. Without prenylation, the small GTPases are unable to anchor to the cell membrane [33]. Recent studies revealed that Rac1, belonging to the family of Rho GTPases, governs the glucose uptake through regulation of GLUT4 translocation both in response to insulin or to exercise in vivo [34,35]. Our studies indicate that the impairment of glucose uptake in response to insulin after treatment with simvastatin may be associated with the lack of Rac1 active form.

Taken together, simvastatin at clinical doses inhibits glucose uptake, decreases the GLUT4 translocation in the plasma membrane and impairs insulin sensitivity in mouse muscle cells, which can be reversed by mevalonic acid. These effects were mediated by the impairment of the IRS-1/PI3K/Akt pathways. Therefore, it should be taken into consideration of the side effects of statins on glucose metabolism in myotubes, which may be associated with *de novo* type 2 diabetes mellitus.

Disclosure

The authors declare no conflict of interest concerning this article.

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References

- [1] D.R. Illingworth, D.W. Erkelens, U. Keller, G.R. Thompson, M.J. Tikkanen, Defined daily doses in relation to hypolipidaemic efficacy of lovastatin, pravastatin, and simvastatin, *Lancet* 343 (1994) 1554-1555.
- [2] N. Sattar, D. Preiss, H.M. Murray, P. Welsh, B.M. Buckley, A.J. de Craen, S.R. Seshasai, J.J. McMurray, D.J. Freeman, J.W. Jukema, P.W. Macfarlane, C.J. Packard, D.J. Stott, R.G. Westendorp, J. Shepherd, B.R. Davis, S.L. Pressel, R. Marchioli, R.M. Marfisi, A.P. Maggioni, L. Tavazzi, G. Tognoni, J. Kjekshus, T.R. Pedersen, T.J. Cook, A.M. Gotto, M.B. Clearfield, J.R. Downs, H. Nakamura, Y. Ohashi, K. Mizuno, K.K. Ray, I. Ford, Statins and risk of incident diabetes: a collaborative meta-analysis of randomised statin trials, *Lancet* 375 (2010) 735-742.
- [3] D.D. Waters, J.E. Ho, D.A. DeMicco, A. Breazna, B.J. Arsenault, C.C. Wun, J.J. Kastelein, H. Colhoun, P. Barter, Predictors of new-onset diabetes in patients treated with atorvastatin: results from 3 large randomized clinical trials, *J Am Coll Cardiol* 57 (2011) 1535-1545.
- [4] B. Zhu, A.Q. Ma, L. Yang, X.M. Dang, Atorvastatin attenuates bleomycin-induced pulmonary fibrosis via suppressing iNOS expression and the CTGF (CCN2)/ERK signaling pathway, *Int J Mol Sci* 14 (2013) 24476-24491.
- [5] F. Kong, B. Ye, L. Lin, X. Cai, W. Huang, Z. Huang, Atorvastatin suppresses NLRP3 inflammasome activation via TLR4/MyD88/NF- κ B signaling in PMA-stimulated THP-1 monocytes, *Biomedicine & Pharmacotherapy* 82 (2016) 167-172.
- [6] K.K. Koh, M.J. Quon, S.H. Han, Y. Lee, S.J. Kim, J.B. Park, E.K. Shin, Differential metabolic effects of pravastatin and simvastatin in hypercholesterolemic patients, *Atherosclerosis* 204 (2009) 483-490.
- [7] U.K. Sampson, M.F. Linton, S. Fazio, Are statins diabetogenic?, *Curr Opin Cardiol* 26 (2011) 342-347.
- [8] M. Ruscica, C. Macchi, B. Morlotti, C.R. Sirtori, P. Magni, Statin therapy and related risk of new-onset type 2 diabetes mellitus, *Eur J Intern Med* 25 (2014) 401-406.

- [9] D. Cheng, Y. Wang, S. Gao, X. Wang, W. Sun, L. Bai, G. Cheng, Y. Chu, S. Zhao, E. Liu, Atorvastatin delays the glucose clearance rate in hypercholesterolemic rabbits, *Biomed Pharmacother* 72 (2015) 24-29.
- [10] J. Zhou, W. Li, Q. Xie, Y. Hou, S. Zhan, X. Yang, X. Xu, J. Cai, Z. Huang, Effects of simvastatin on glucose metabolism in mouse MIN6 cells, *J Diabetes Res* 2014 (2014) 376570.
- [11] T. Satoh, Molecular mechanisms for the regulation of insulin-stimulated glucose uptake by small guanosine triphosphatases in skeletal muscle and adipocytes, *Int J Mol Sci* 15 (2014) 18677-18692.
- [12] A.F. Rowland, D.J. Fazakerley, D.E. James, Mapping insulin/GLUT4 circuitry, *Traffic* 12 (2011) 672-681.
- [13] W.T. Garvey, L. Maianu, T.P. Huecksteadt, M.J. Birnbaum, J.M. Molina, T.P. Ciaraldi, Pretranslational suppression of a glucose transporter protein causes insulin resistance in adipocytes from patients with non-insulin-dependent diabetes mellitus and obesity, *J Clin Invest* 87 (1991) 1072-1081.
- [14] J.K. Liao, U. Laufs, Pleiotropic effects of statins, *Annu Rev Pharmacol Toxicol* 45 (2005) 89-118.
- [15] M.D. Resh, Trafficking and signaling by fatty-acylated and prenylated proteins, *Nat Chem Biol* 2 (2006) 584-590.
- [16] L. JeBailey, O. Wanono, W. Niu, J. Roessler, A. Rudich, A. Klip, Ceramide- and oxidant-induced insulin resistance involve loss of insulin-dependent Rac-activation and actin remodeling in muscle cells, *Diabetes* 56 (2007) 394-403.
- [17] L. Sylow, T.E. Jensen, M. Kleinert, K. Højlund, B. Kiens, J. Wojtaszewski, C. Prats, P. Schjerling, E.A. Richter, Rac1 signaling is required for insulin-stimulated glucose uptake and is dysregulated in insulin-resistant murine and human skeletal muscle *Diabetes* 62 (2013) 1865–1875.
- [18] J. Hanai, P. Cao, P. Tanksale, S. Imamura, E. Koshimizu, J. Zhao, S. Kishi, M. Yamashita, P.S. Phillips, V.P. Sukhatme, S.H. Lecker, The muscle-specific ubiquitin ligase atrogin-1/MAFbx mediates statin-induced muscle toxicity, *J Clin Invest* 117 (2007) 3940-3951.
- [19] C.B. Dugani, A. Klip, Glucose transporter 4: cycling, compartments and controversies, *EMBO Rep* 6 (2005) 1137-1142.
- [20] R. Govers, Cellular regulation of glucose uptake by glucose transporter GLUT4, *Adv Clin Chem* 66 (2014) 173-240.
- [21] N. Sattar, M.R. Taskinen, Statins are diabetogenic--myth or reality?, *Atheroscler Suppl* 13 (2012) 1-10.
- [22] H.B. Kwak, A. Thalacker-Mercer, E.J. Anderson, C.T. Lin, D.A. Kane, N.S. Lee, R.N. Cortright, M.M. Bamman, P.D. Neuffer, Simvastatin impairs ADP-stimulated respiration and increases mitochondrial oxidative stress in primary human skeletal myotubes, *Free Radic Biol Med* 52 (2012) 198-207.
- [23] M. Itagaki, A. Takaguri, S. Kano, S. Kaneta, K. Ichihara, K. Satoh, Possible mechanisms underlying statin-induced skeletal muscle toxicity in L6 fibroblasts and in rats, *J Pharmacol Sci* 109 (2009) 94-101.
- [24] R. Smith, R. Solberg, L.L. Jacobsen, A.L. Voreland, A.C. Rustan, G.H. Thoresen, H.T. Johansen, Simvastatin inhibits glucose metabolism and legumain activity in human myotubes, *PLoS One* 9 (2014) e85721.
- [25] M. Nakata, S. Nagasaka, I. Kusaka, H. Matsuoka, S. Ishibashi, T. Yada, Effects of statins on the adipocyte maturation and expression of glucose transporter 4 (SLC2A4): implications in glycaemic control, *Diabetologia* 49 (2006) 1881-1892.
- [26] L.H. Chamberlain, Inhibition of isoprenoid biosynthesis causes insulin resistance in 3T3-L1 adipocytes, *FEBS Lett* 507 (2001) 357-361.
- [27] L. Ramalingam, E. Oh, D.C. Thurmond, Novel roles for insulin receptor (IR) in adipocytes and skeletal muscle cells via new and unexpected substrates, *Cell Mol Life Sci* 70 (2013) 2815-2834.
- [28] K.A. Cho, P.B. Kang, PLIN2 inhibits insulin-induced glucose uptake in myoblasts through the activation of the NLRP3 inflammasome, *Int J Mol Med* 36 (2015) 839-844.

- [29] X.T. Feng, T.Z. Wang, Y. Chen, J.B. Liu, Y. Liu, W.J. Wang, Pollen Typhae total flavone improves insulin-induced glucose uptake through the beta-arrestin-2-mediated signaling in C2C12 myotubes, *Int J Mol Med* 30 (2012) 914-922.
- [30] J. Buren, H.X. Liu, J. Jensen, J.W. Eriksson, Dexamethasone impairs insulin signalling and glucose transport by depletion of insulin receptor substrate-1, phosphatidylinositol 3-kinase and protein kinase B in primary cultured rat adipocytes, *Eur J Endocrinol* 146 (2002) 419-429.
- [31] E.S. Istvan, J. Deisenhofer, Structural mechanism for statin inhibition of HMG-CoA reductase, *Science* 292 (2001) 1160-1164.
- [32] S.J. McTaggart, Isoprenylated proteins, *Cell Mol Life Sci* 63 (2006) 255-267.
- [33] G.L. Kucera, S.E. Rittenhouse, Human platelets form 3-phosphorylated phosphoinositides in response to alpha-thrombin, U46619, or GTP gamma S, *J Biol Chem* 265 (1990) 5345-5348.
- [34] L. Sylow, I.L. Nielsen, M. Kleinert, L.L. Moller, T. Ploug, P. Schjerling, P.J. Bilan, A. Klip, T.E. Jensen, E.A. Richter, Rac1 governs exercise-stimulated glucose uptake in skeletal muscle through regulation of GLUT4 translocation in mice, *J Physiol* (2016).
- [35] N. Takenaka, Y. Nihata, T. Satoh, Rac1 Activation Caused by Membrane Translocation of a Guanine Nucleotide Exchange Factor in Akt2-Mediated Insulin Signaling in Mouse Skeletal Muscle, *PLoS One* 11 (2016) e0155292.

Fig.1. Simvastatin inhibited glucose uptake in C2C12 myotubes. (A) C2C12 myotubes viability was assessed using the CCK8 assay. Cells incubated in a medium with simvastatin and DMSO were defined as control and were considered to have a 100% proliferation rate. (B) Time-response of simvastatin on consuming of medium glucose after incubation 5 μ M simvastatin for 24h, 48h, 72 h and compared to untreated 0 h (incubated with DMSO for 72 h) (n=4, student t-test, * $P<0.05$ vs. DMSO). (C) Dose-response of simvastatin on consuming of medium glucose after 72 h incubation (n = 3, student t-test, * $P<0.05$ vs. DMSO). (D) Effects of 5 μ M simvastatin with or without ML on consuming of medium glucose after 72 h incubation and 5 μ M simvastatin was defined as control (n = 3, student t-test, * $P<0.05$ vs. DMSO and # $P<0.05$ vs. S5).

Fig.2. Simvastatin inhibited insulin-induced glucose uptake and translocation of GLUT4 in myotubes. (A) Myotubes were incubated with 5 μ M simvastatin with or without 100 μ M ML for 72 h. The ability of glucose uptake was measured with or without 100 nM insulin to the medium above for 24 h. (n = 3, student t-test, * $P<0.05$ vs. ddH₂O). (B) Determine mean fluorescence intensities (MFI) for expression of GLUT4 in plasma membrane and the number of DMSO group was defined as control. (n = 3, student t-test, * $P<0.05$ vs. DMSO and # $P<0.05$ vs. S5) (C) One representative result was shown.

Fig.3. Simvastatin inhibited the phosphorylation level of IR, IRS-1, Akt and the total expression of IR, IRS-1. (A) Representative blots of protein were shown and band intensity analysis for phosphorylation of IR on Tyr1361, phosphorylation of IRS-1 on Tyr1222, phosphorylation of AKT on Thr308 were normalized to the control protein and expressed as % mean control values (n = 3, student t-test, * $P<0.05$ vs. untreated and # $P<0.05$ vs. S5). (B) Representative blots of proteins were shown and band intensity analysis for expression of total-IR, total-IRS-1, total-AKT were normalized to the control protein and expressed as % mean control values (n = 3, student t-test, * $P<0.05$ vs. untreated and # $P<0.05$ vs. S5).

Fig.4 Simvastatin decreased Rac1 GTP binding. Representative blots and band intensity analysis of Rac1 GTP binding was normalized to the DMSO group in C2C12 myotubes (n = 3, student t-test, * $P<0.05$ vs. untreated and # $P<0.05$ vs. S5).

A

B

C

D

A

B

C

A

B