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

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ABSTRACT

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 suppresses 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 [2–5]. 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 [7–9]. 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

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.

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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 10 mg/ml of streptomycin). All the cells were grown under an atmosphere of 5% CO_2 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, 10 μM or DMSO as control group for 0 h, 24 h, 48 h, 72 h.

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 490 nm in a microplate reader BIO-RAD Model 680.

2.3. Cell viability tests

Myotubes were treated with 0–10 μM 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 3 h at 37 °CThen 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 min 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 myotudes stimulated with either insulin or ddH_2O.

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 18000*g* for 1 min, 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 h, 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 20 min. Washed with PBS and incubated with anti-mouse GLUT4 (cat no ab65267, Abcam, USA) or isotype control mouse IgG2a (ICIGG2A) for 30 min at room temperature in 50 μl PBS. The secondary antibody was used Dylight 488 goat anti-mouse IgG (H + L) (1:500 dilution, Abcam, USA) for 30 min at room