Effect of Three Statins on Glucose Uptake of Cardiomyocytes and its Mechanism

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Background: The aim of this study was to investigate the effects of different statins on glucose uptake and to confirm its mechanism in primary cultured rat cardiomyocytes after administration of atorvastatin, pravastatin, and rosuvastatin.

Material/Methods: Primary cultured rat cardiomyocytes were randomly assigned to 5 groups: normal control group (OB), insulin group (S1), statin 1-μM (S2), 5-μM (S3), and 10-μM (S4) groups for 3 different statins. The 2-[^3]H]-DG uptake of each group was determined and the mRNA and protein expression levels of glucose transporter type 4 (GLUT4), insulin receptor substrate (IRs), and RhoA were assessed.

Results: After treatment with different concentrations of statins and insulin, the 2-[^3]H]-DG uptake showed a significant negative correlation with the concentration of atorvastatin (P<0.05), and no significant correlation with pravastatin and rosuvastatin. The mRNA and protein expression levels of GLUT4 and IRs-1 in primary cultured cardiomyocytes were both significantly reduced by atorvastatin treatment (P<0.05). Pravastatin and rosuvastatin showed no significant effects on GLUT4 and IRs-1 expression. The mRNA and protein expression levels of RhoA both showed no significant difference when treated with the 3 statins.

Conclusions: These results confirm that atorvastatin can inhibit insulin-induced glucose uptake in primary cultured rat cardiomyocytes by regulating the PI3K/Akt insulin signal transduction pathway.

MeSH Keywords: Glucose • Glucose Transporter Type 4 • Hydroxymethylglutaryl-CoA Reductase Inhibitors • Insulin • Myocytes, Smooth Muscle

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Background

Cardiovascular diseases (CVD) are the major cause of mortality in subjects with type 2 diabetes (T2D) [1,2]. Dyslipidemia is a key factor contributing to cardiovascular disease in T2D patients [3]. Previous clinical studies have shown that impairment of lipid metabolism significantly increases the risk of CVD events [3,4]. Statins, 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase inhibitor, can control both hypercholesterolemia and hypertriglyceridemia. The early statin trials were first reported in the 1990s, and several studies of the effects of statins showing the benefits on CVD have been published [5,6]. More recent evidence has demonstrated that statins are beneficial in reducing the risk of CVD events in people without prior evidence of CVD [7]. The effects of statins in primary and secondary prevention of CVD, especially in patients with T2D, is well established [8].

Statins can control serum glucose and lipids by regulating insulin-mediated glucose uptake. Previous studies have demonstrated that statins can reduce insulin-stimulated glucose uptake by inhibiting translocation of glucose transporter type 4 protein (GLUT4) to the membrane [9,10]. GLUT4 vesicles can translocate to the plasma membrane upon insulin stimulation via a multi-step process. In the normal status, GLUT4 is mainly located at intracellular vesicles, whereas relatively little GLUT4 resides at the plasma membrane. When stimulated by insulin, these vesicles can move to the plasma membrane, where they dock and fuse, thereby increasing the number of GLUT4 molecules on the cell surface [11]. Insulin receptor substrate (IRS) proteins play critical roles in regulation of the insulin signaling pathway [12]. Many studies have focused on the effect of statins on glucose uptake in adipocytes rather than myocytes [13]. However, evidence from a number of randomized clinical trials over the last 2 decades show a potential association between statin therapy and increased risk of development of diabetes [14], although this risk is low when compared with the benefits of reduction in coronary events.

In this study, the glucose uptake of cardiomyocytes was determined after administration of atorvastatin, pravastatin, and rosvastatin to investigate the effects of various statins on the insulin-induced glucose signaling pathway and to confirm its mechanism in cardiomyocytes.

Material and Methods

Material

Neonatal Sprague-Dawley (SD) rats (age 24 h, male and female) were provided by the Experimental Animal Center of China Medical University. Atorvastatin, pravastatin, and rosuvastatin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The insulin purchased from the Sigma-Aldrich (St Louis, MO, USA). Rabbit anti-GLUT4, anti-pIRS-1, anti-RhoA, anti-β-actin, and goat anti-rabbit IgG antibodies were purchased from Sigma-Aldrich.

Cell culture

Primary cultured neonatal rat cardiomyocytes were prepared as previously described with minor modifications [15]. The heart was obtained from each neonatal SD rat with ophthalmic scissors under sterile conditions and cut into tissue blocks about 1 mm³. After complete digestion, the mixture was centrifuged at 1000 rpm for 8 min and the supernatant was discarded. The deposit was suspended in Dulbecco’s modified Eagle’s medium (DMEM) with 10% fetal calf serum and cultured in a CO₂ incubator for a differential plating period of 90 min to allow attachment of the non-myocardial cells. The cardiomyocytes were obtained from the deposit after centrifugation at 1000 rpm for 8 min and plated in 10-cm culture dishes (Falcon) at a density of 1×10⁵ cells/ml. Following incubation for 24 h, the cultures were washed 2 times with phosphate-buffered saline (PBS) to remove the dead and non-adherent cells. The medium was changed every 3 days during incubation and the treatment factors were administered on the fifth day.

2-[³H]-DG uptake determination

Primary cultured cardiomyocytes were randomly divided into 5 groups: normal control group, insulin group, and statin 1-μM, 5-μM, and 10-μM group, for each of the 3 different statins. The cardiomyocytes were pretreated with 0, 1, 5, and 10-μM of pravastatin, rosuvastatin, and atorvastatin for 72 h and then treated with 0.1-μM insulin for 30 min. Cells were washed twice with preheated PBS and the medium was then replaced by serum-free DMEM medium containing 2-[³H]-DG at a concentration of 1 μCi/ml. [U-¹⁴C] mannitol (0.1 μCi/ml) was also used as osmolality control [16]. The uptake of 2-[³H]-DG was determined after the cells were further incubated at 37°C and 5% CO₂ in an incubator for 10 min. The medium was then replaced by pre-cooled PBS and the reaction was terminated by the addition of 10 mM phloretin. The cells were rapidly washed twice in pre-cooled PBS to remove phloretin and were dissolved in 1% SDS/0.1 M NaOH lysis buffer overnight at room temperature. The lysis solution (300 μl) was assayed for radioactivity by use of a liquid scintillation counter (Triathler 425-034, Hidex Oy) to determine the uptake of 2-[³H]-DG in each group [11, 17].

Protein extraction and western blotting

Primary cultured cardiomyocytes were randomly divided into 5 groups: normal control group, insulin group, statin 1-μM, 5-μM,
The expression levels of GLUT4, IRs-1, and RhoA were semi-quantified with Western blotting. The gel was transferred to a polyvinylidene fluoride (PVDF) membrane (Solvay Chemicals, Belgium) and blocked for 1 h. The rabbit anti-GLUT4, anti-IRs-1, and anti-RhoA antibody were used as primary antibody at a dilution of 1:1000. The secondary antibody was goat anti-rabbit IgG antibody at a dilution of 1:10 000. Signals were visualized with the ECL kit (Amersham International, Amersham, UK). Image J software (NIH, Bethesda, MD, USA) was used to compare the gray values between the proteins of interest and the internal control protein, as well as between the phosphorylated protein and the total protein.

RT-PCR analysis

The expression levels of GLUT4, IRs-1, and RhoA in each group were assessed by RT-PCR. Total RNA was extracted from cells using the Trizol method [22], after which cDNA was synthesized from the RNA by reverse transcription. Using specific primers, PCR amplification was performed to allow for fluorescence-based quantitation of the gene expression. PCR reaction volumes were 10 μl and composed of cDNA (1 μl), primers (0.2 μl each), 2× Premix Ex Taq (5 μl), and H2O (3.6 μl). The primer sequences used are listed in Table 1.

Table 1. The primer sequences used in the RT-PCR.

| Gene   | Upstream primer          | Downstream primer          |
|--------|--------------------------|----------------------------|
| β-actin| CGTGCGTGACATTAAAGAG      | TTGCCGATAGTGATGACCT        |
| GLUT4  | CTTCATCATTGCGATGGGGTT    | AGGACCGCAAATAAGAGAAGAAGA  |
| IRs-1  | CACCCACTCTATCCCG         | CCCTACTCCGTTTGTCCAT       |
| RhoA   | CTGGTGATTGTGGGTAGG       | GCGATCATAATCTTCCTGCC      |

and 10-μM group for each of the 3 different statins. The cardiomyocytes were pretreated with corresponding concentrations of statin for 72 h and then treated with 0.1-μM insulin for 30 min. The cells were collected after being washed 2 times with PBS. The membrane protein, cytoplasm protein, and total cell proteins were extracted using a cell fractionation kit (Biovision, California, USA) according to the method of previous studies [11,18–20]. The protein samples were then separated with 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) [21].

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| IRs-1  | CACCCACTCTATCCCG         | CCCTACTCCGTTTGTCCAT       |
| RhoA   | CTGGTGATTGTGGGTAGG       | GCGATCATAATCTTCCTGCC      |

Results

The effects of statins on 2-[3H]-DG uptake

We detected the 2-[3H]-DG uptake status in primary cultured cardiomyocytes after being treated by insulin and different statins. The 2-[3H]-DG uptake showed a significant negative correlation with the concentration of atorvastatin, but no significant correlation with pravastatin or rosuvastatin. Control – the primary cultured cardiomyocytes; Atorvastatin – the cardiomyocytes treated with insulin and atorvastatin; Pravastatin – the cardiomyocytes treated with insulin and pravastatin; Rosuvastatin – the cardiomyocytes treated with insulin and rosuvastatin. * P<0.05 compared with pravastatin and rosuvastatin groups; # P<0.05 compared with atorvastatin 1-μM group.
2-[14C]-DG uptake in atorvastatin groups were significantly lower than that in pravastatin and rosuvastatin groups (P<0.05).

**The expression of GLUT4 protein and mRNA**

The expression of GLUT4 protein on the membrane was determined by Western blot and shown in Figure 2A. The protein expression level of GLUT4 was increased when treated by insulin (S1 group) compared with the control group (OB). The bands in Western blotting showed that the protein expression of GLUT4 was significantly reduced by atorvastatin compared with the S1 group and decreased significantly with the increase of atorvastatin concentration, showing a significant negative correlation. In pravastatin and rosuvastatin 1-, 5-, and 10-μM groups, no significant difference was observed between the groups compared with the insulin groups (Figure 2A). The mRNA expression level of GLUT4 in primary cultured cardiomyocytes treated with insulin and atorvastatin was consistent with the expression of GLUT4 protein. The GLUT4 mRNA expression in atorvastatin 1-, 5-, and 10-μM groups was all significantly lower than in the insulin group (P<0.05), and the GLUT4 mRNA expression in the atorvastatin 10-μM group was significantly lower than in the 1-μM group (P<0.05) (Figure 2B). Pravastatin and rosuvastatin showed no significant effects on the GLUT4 mRNA expression (data not shown).

**The expression of IRS-1 protein and mRNA**

The mRNA and protein expression levels of IRS-1 in primary cultured cardiomyocytes were both significantly reduced by atorvastatin treatment, which was the same with the expression of GLUT4. Pravastatin and rosuvastatin showed no significant effects on the IRS-1 expression (Figure 3).

**The expression of RhoA protein and mRNA**

The mRNA and protein expression levels of RhoA both showed no significant difference when treated with the 3 different statins (Figure 4).
substances into the cell [23]. Therefore, lipophilic atorvastatin can enter the cardiomyocytes and inhibit the insulin-mediated glucose uptake more easily than hydrophilic pravastatin and rosuvastatin.

To investigate the mechanism by which atorvastatin inhibits glucose uptake, we detected the expression of GLUT4, IRS-1, and RhoA. Glucose mainly depends on the glucose transporters (GLUTs) to enter into cardiomyocytes. In cardiomyocytes, the 2 most highly expressed glucose transporters are GLUT1 and GLUT4, and GLUT4 is the most abundant. GLUT1 mediates basal glucose transport, whereas GLUT4 is mainly responsible for insulin- or contraction-mediated glucose transport. In quiescent myocytes, the majority of GLUT4 protein resides in a specialized vesicle population in an intracellular compartment. Upon insulin stimulation, GLUT4 vesicles are translocated to the plasma membrane (PM) via a multiple-step process by which GLUT4 storage vesicles (GSVs) move to the PM, tether, dock, and ultimately fuse with the PM to expose GLUT4 proteins on the cell surface [9]. IRS-1 is the insulin receptor substrate protein and mainly located in the hepatic and adipose tissue, which is sensitive to insulin [27]. IRS-1 can associate with insulin receptor (IR) as the key intermediate in the insulin phosphatidylinositol 3-kinase/protein kinase B (PI3K/Akt) signaling pathway [9]. The blocking of phosphorylation of IRS-1 can reduce IRS-1–associated PI3K and Akt activity and ultimately decrease insulin-stimulated glucose transport activity [28]. In our study, the protein level of IRS-1 on the PM and the translocation of GLUT4 to the membrane in cardiomyocytes was reduced by atorvastatin. It is confirmed that atorvastatin can inhibit the uptake of cardiomyocytes by blocking the PI3K/Akt insulin signaling pathway. RhoA, a member of the Rho family of small GTPases, is a monomeric G protein that regulates a number of cell functions, including cytoskeletal reorganization, cell motility, and gene expression [29]. There have been several reports of RhoA in the diabetic mesangial cells regulating glucose metabolism [30,31], but no effect was observed in cardiomyocytes after treatment with atorvastatin in our study.

Conclusions

In primary cultured cardiomyocytes, lipophilic atorvastatin can inhibit insulin-mediated glucose uptake by blocking the PI3K/Akt insulin signaling pathway, whereas hydrophilic pravastatin and rosuvastatin showed no effects on glucose uptake.

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