Original Article

Distinct Effects of Pravastatin, Atorvastatin, and Simvastatin on Insulin Secretion from a β-cell Line, MIN6 Cells

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In addition to the prevention of cardiovascular diseases by lowering plasma LDL cholesterol, recent studies suggest that statins could have some impact on insulin action. To estimate the direct effects of statins on insulin secretion from pancreatic β-cells, MIN6 cells were treated with pravastatin, simvastatin, or atorvastatin. Basal insulin secretion at low glucose concentration was unexpectedly increased at very high doses of simvastatin or atorvastatin after 24- and 48-hour incubation. Insulin secretion at high glucose was not significantly changed, and thus, net glucose-stimulated insulin secretion was apparently decreased by these lipophilic statins. The changes in insulin secretion were highly associated with increased endogenous SREBP activities in response to HMG-CoA inhibition as estimated by SRE-luciferase assays, and finally after 48-hour incubation, accompanied by impaired cell viability as estimated by MTT assays. In contrast, these changes were much less prominent by the addition of pravastatin. Meanwhile, glucose-stimulated insulin secretion of islets isolated from C57BL/6 mice was not significantly changed by any of the statins. Overall, taken up by β-cells, statins can affect insulin secretion through either HMG-CoA inhibition or cytotoxicity, as observed by the addition of extraordinary high doses of lipophilic statins, but not hydrophilic statins, to the medium.


Key words; SREBP, Cholesterol, HMG-CoA reductase, Lipotoxicity

Introduction

Statins are specific and potent competitive inhibitors of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase and are widely used as plasma cholesterol-lowering drugs to prevent the development and progression of atherosclerosis. Evidence for reduction of the incidence of cardiovascular diseases by statins has now been fully established1-6. Diabetes is a major risk for cardiovascular diseases, and statins are also used for patients with diabetes7, 8; however, the effects of statins on glucose insulin metabolism have been implicated, but not fully understood. Statins could have some beneficial impact on peripheral insulin resistance, which can potentially contribute to a potential protective role of statins against the development of diabetes9. We recently found that atorvastatin ameliorates insulin resistance in experimental animals10; however, it has been reported that statins might have some inhibitory effects on glucose-stimulated insulin secretion11, 12. These diverse effects of statins to potentially modify the pathology of type 2 diabetes warrant studies on the effects of various statins on β-cell functions, especially insulin secretion in various conditions. In this study, we investigate effects of pravastatin, atorvastatin, and simvastatin on insulin secretion from a β-cell line, MIN6 cells.
Materials and Methods

Reagents
Simvasatin, atorvastatin, and pravastatin were purchased from Wako (Osaka, Japan).
Dimethyl sulfoxide (DMSO) was purchased from Sigma (St. Louis, MO, USA).

Cell Culture and Treatment
MIN6 cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) containing 25 mmol/L glucose supplemented with 15% fetal bovine serum (FBS), β-mercaptoethanol (5 μL/L), penicillin (100 units/mL), and streptomycin (100 μg/mL) at 37°C in an atmosphere of 95% air/5% CO₂. The cells were seeded onto 24-well plates at a density of 3 × 10⁵ cells/mL and grown overnight to 80% confluency. The cells were washed with phosphate-buffered saline (PBS) and incubated for 24 hours or 48 hours at 37°C in 0.5 mL of medium containing the indicated concentrations (×IC50 for HepG2) of pravastatin, atorvastatin, or simvastatin. The IC50 of pravastatin, atorvastatin, and simvastatin estimated by HMG-CoA reductase inhibition in HepG2 cells was 9.4 nM, 1.9 nM, and 2.7 nM, respectively.
DMSO was used as a solvent of the agents, and the final concentrations did not exceed 0.1% (v/v).

Preparation of Murine Pancreatic Islets
All the animal husbandry and animal experiments were consistent with the University of Tsukuba’s Regulations for Animal Experiments, and permitted by the Animal Experiment Committee, University of Tsukuba. All procedures of animal experiments were performed in accordance with Tsukuba University’s Animal Studies Committee. Isolation of islets from C57BL/6 mice (Charles River Laboratories) was carried according to the Ficoll-Conray protocol. After isolation, the islets were incubated for 2 hours at 37°C in an atmosphere of 95% air/5% CO₂ in RPMI-1640 medium supplemented with 10% FBS, 100 units/mL penicillin and 100 μg/mL streptomycin.

Measurement of Insulin Secretion
Prior to stimulation by glucose, MIN6 cells or ten pancreatic isolated islets were preincubated for 30 min in 2.8 mM glucose in 0.5% BSA KRBH buffer (129.4 mM NaCl, 5.2 mM KCl, 2.7 mM CaCl₂, 1.3 mM KH₂PO₄, 1.3 mM MgSO₄, 24.8 mM NaHCO₃, and 10 mM HEPES at pH 7.4). The cells were incubated with either 2.8 mM (low) or 20 mM (high) glucose in KRBH buffer for 1 hour. The insulin content of the medium was measured using a mouse insulin ELISA kit (Shibayagi Co., Ltd., Gunma, Japan).

MTT (3-(4,5-Dimethyl-2-thiazoly)-2,5-diphenylte trazolium Bromide) Assays
As another set of the statin experiments, MIN6 cells were supplemented with MTT and incubated for 4 hours. The medium was switched to 0.5 mL of 0.04 N HCl/isopropanol for extraction by shaking for 5 min. MTT values were measured by absorbance.

Transfection and Luciferase Assays
MIN6 cells were seeded onto 24-well plates and cultured overnight. The cells were transfected with a SRE-luciferase reporter plasmid (500 ng) and a pRL-SV40 plasmid (50 ng, Promega) using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA) according to the manufacturer’s instructions as previously described. After 4-hour incubation, the cells were subjected to statin treatment as described above. After 24- or 48-hour incubation, the amount of firefly luciferase activity in transfectants was measured and normalized to the amount of renilla luciferase activity.

Statistical Analysis
Data are presented as the mean ± s.e.m. Statistical significance was tested with an unpaired two-tailed Student’s t-test.

Results
MIN6 cells are a well-established murine β-cell line and maintain glucose-stimulated insulin secretion as a physiologic feature of pancreatic β-cells (control data of Fig. 1 and 2). The cells were treated with pravastatin, simvastatin, or atorvastatin. Preliminary data demonstrated that the addition of these statins at the respective IC50 doses reported to inhibit HMG-CoA reductase activity in HepG2 cells did not significantly change insulin secretions at low glucose (2.8 mM, basal) or high glucose (20 mM) concentrations. Therefore, we used ×100, ×1000, and ×10000 concentrations of the IC50 for each statin. When cells were treated with these high doses for 24 and 48 hours, simvastatin and atorvastatin increased basal insulin secretion dose-dependently starting at ×100 and at ×1000, respectively (Figs. 1 and 2). Pravastatin exhibited this effect only at the highest dose (×10000). In contrast, insulin secretion at high glucose exhibited a trend to increase with each statin in a pattern similar to basal insulin secretion, but was not significant. Thus, glucose stimulation as calculated by the fold-change of high glucose vs. low glucose was reduced at the high doses of simvastatin and atorvastatin, respectively, but not
pravastatin. This apparent effect was enhanced when the incubation was extended to 48 hours (Fig. 2). Collectively, the time-course and dose-dependent impairment of insulin induction by high glucose was observed in simvastatin and atorvastatin, but not pravastatin.

The primary action of statin is the inhibition of HMG-CoA reductase that catalyzes a rate-limiting step for cholesterol biosynthesis. This inhibition is eventually compensated by adaptive up-regulation of the endogenous pathway at the transcription level through the activation of SREBPs, primarily by SREBP-2 16). For the estimation of HMG-CoA inhibition states in statin-treated MIN6 cells, we measured endogenous SREBP activities by SRE-luciferase reporter gene assays 17). Atorvastatin and simvastatin dose-dependently induced SREBP activities at 24-hour and 48-hour incubation (Fig. 3). The dose-dependency of these statins to cause SREBP activation was roughly similar to that of the effect on insulin secretion. In contrast, pravastatin treatment showed a trend, but did not significantly activate SREBPs. These data indicated that only high dose of lipophilic statins added to the medium exhibited HMG-CoA inhibition in MIN6 cells.

MTT assays as a marker for cell viability and
growth demonstrated no effects by any statins after 24-hour incubation; however, extension of the statin treatment to 48 hours significantly affected cell viability at high doses of simvastatin, atorvastatin, and pravastatin in a pattern similar to the effects of these agents on insulin secretion (Fig. 4).

Finally, pancreatic islets were isolated from C57BL/6 mice and subjected to treatment with statins. As shown in Fig. 5, the islets exhibited significant glucose-stimulated insulin secretion. However, the administration of statins to islets at the concentrations used for MIN6 cells did not evoke significant changes in glucose-stimulated insulin secretion in pancreatic islets.

**Fig. 2.** Effect of incubation with pravastatin (A), atorvastatin (B), or simvasatin (C) for 48 hours on insulin secretion measured at low (black column, 2.8 mM) and high (white column, 20 mM) glucose concentrations in MIN 6 cells. Fold change of insulin secretion at high glucose level vs. low glucose level was indicated (D). Experimental procedures were described in the legend under Fig. 1 except 48-hour incubation with statins.

All values are the means ± SE. *p<0.05, compared with the control with high glucose. *p<0.05 and **p<0.01, compared with the control with low glucose (A, B, and C). *p<0.05 and **p<0.01, compared with the control (D).

**Discussion**

Our data demonstrated that high doses of lipophilic statins such as simvastatin and atorvastatin significantly increased basal insulin secretion in MIN6 cells. Since insulin secretion at high glucose was not significantly increased by these statins, the net glucose-stimulation as estimated by the fold-change of insulin secretion at high glucose vs. low glucose was apparently decreased. The mechanism for these unexpected results is currently unknown. It is highly plausible that the cytotoxicity by high concentrations of lipophilic statins leads to leakage of insulin from insulin-con-
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Taining granules. Supportively, MTT assays indicate that cell growth and viability are impaired by lipophilic statins. Cytotoxicity could impair ATP production, leading to decreased insulin secretion in response to glucose. Meanwhile, before impaired cell viability was observed after 48-hour incubation, both increased endogenous SREBP activities and changes in insulin secretion were already observed after 24-hour incubation. Thus, HMG-CoA reductase inhibition could be directly involved in the effects on insulin secretion by lipophilic statins. Supportively, mevalonate rescue essentially abolished the statin effect on insulin secretion as well as SREBP activation (data not shown). It is well known that, depending upon its concentration, cellular cholesterol could be both indispensable and cytotoxic for the cell and it is conceivable to speculate that disturbed cholesterol metabolism could be a cause of impaired β-cell function as observed by the addition of oxidized LDL.18 It is also possible that the activation of SREBPs by statins directly impairs insulin secretion.

Fig. 3. Endogenous SREBP activities in MIN6 cells after incubation for 24 hours (A) or 48 hours (B) with pravastatin, atorvastatin, or simvasatin as estimated by transfection with an SRE-luciferase reporter gene in MIN6 cells. MIN6 cells were transfected with SRE-luciferase reporter gene and the SV40 expression vector of renilla luciferase as a reference, followed by 4-hour incubation. The cells were treated with statins as described in the legend to Fig. 1 for 24 hours. Finally, the cells were subjected to reporter assays for measurement of firefly luciferase activity normalized to the amount of renilla luciferase activity.

All values are the means ± SE. *p < 0.05 and **p < 0.01, compared with the control.

Fig. 4. Effect of incubation with pravastatin, atorvastatin, or simvasatin for 24 hours (A) or 48 hours (B) on MTT assays in MIN6 cells. Cell culture conditions were described in the legend to Fig. 1. After harvesting the cells, MTT assays were performed.

All values are means ± SE. *p < 0.05 and **p < 0.01, compared with the control.
secretion as reported in \( \beta \)-cell-specific SREBP-1c transgenic mice\(^ \text{19} \) and SREBP-2 transgenic mice (paper in preparation, Ishikawa M).

Whether the effects of lipophilic statins on insulin secretion of MIN6 cells are due to a cytotoxic effect, HMG-CoA reductase inhibition, or both, pravastatin, a hydrophilic statin, exhibits only a minimal trend to this effect. The distinction of SREBP activation among statins suggests that uptake of statins in the medium by MIN6 cells was much less efficient in hydrophilic statins than in lipophilic statins, which also explains the difference in the potential cytotoxicity indicated by MTT assays.

A previous work demonstrated that simvastatin, but not pravastatin, disturbed glucose-stimulated insulin secretion through blockage of an L-type Ca channel in rat isolated single \( \beta \)-cells\(^ \text{12} \). In our experimental settings, glucose-stimulated insulin secretion in mouse islets was not inhibited by statins. In contrast to our data on the complex statin effects on MIN6 cells, other statins clearly decreased insulin secretion in HIT cells, a hamster \( \beta \)-cell line, (unpublished data, Okajima F and Oikawa S). Thus, the actions of statins on pancreatic \( \beta \)-cells vary and should be carefully estimated by various cell lines in different conditions. Current data indicate that supra-physiological concentrations of statins are required to see the inhibition of insulin secretion, suggesting that regular doses of statins used for hypercholesteremic patients are unlikely to cause deteriorated effects on insulin action, especially in the case of pravastatin. Although isolated islets seem to exhibit less sensitivity to statins than MIN6 cells through the route of addition to the medium, there may be still another mechanism for the uptake of both lipophilic and hydrophilic statins by pancreatic \( \beta \)-cells \textit{in vivo}. Further \textit{in vivo} studies are needed to clarify the cytotoxicity of statins, and more importantly, the physiological roles of cellular cholesterol synthesis in insulin secretion.

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