Short-term use of atorvastatin affects glucose homeostasis and suppresses the expression of LDL receptors in the pancreas of mice

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Abstract. Low-density lipoprotein receptors (LDLRs) may serve a role in the diabetogenic effect of statins; however, the effects of statins on LDLR expression and its regulation in the pancreas and islets have yet to be determined. To exclude the long-term effects of treatment with atorvastatin, which allows mice to adapt, male C57BL/j and apolipoprotein E-deficient mice were acutely treated with oral atorvastatin for 6 weeks, and glucose homeostasis and LDLR expression in the pancreas and islets were examined. In the present study, it was observed that the short-term use of atorvastatin affected insulin sensitivity in normal mice and glucose tolerance in hyperlipidemic mice. Furthermore, it was identified that 6 weeks of treatment with atorvastatin suppressed LDLR expression in the pancreas and pancreatic islets in C57BL/j mice, and an increase in proprotein convertase subtilisin/kexin type 9 expression was additionally observed in the pancreas. However, 6 weeks of treatment with atorvastatin did not affect LDLR expression in the pancreas of hyperlipidemic mice. It may be concluded that the short-term use of atorvastatin disturbs glucose homeostasis and suppresses LDLR expression in the pancreas and pancreatic islets in C57BL/j mice, suggesting that the role of LDLR in the diabetogenic effect of statins requires further investigation.

Introduction

Hyperlipidemia is strongly associated with the prevalence of type 2 diabetes mellitus (T2DM), and statins are the first line of drugs for treating hyperlipidemia. However, a previous study demonstrated that statins may increase the risk of new-onset T2DM, and the underlying mechanism requires determination (1). Notably, a series of studies on patients with familial hypercholesterolemia (FH) have investigated this (2,3). In these studies, a novel phenomenon demonstrated that patients with FH were protected against diabetes mellitus even when subjected to long-term statin therapy. Considering that the majority of the patients in these studies had a heterozygous mutation in the low-density lipoprotein receptor (LDLR) gene, these findings suggest a noteworthy hypothesis that LDLR-mediated cholesterol metabolism may be involved in the diabetogenic effect of statins (4). Indeed, statins may enhance cholesterol uptake in the liver and peripheral tissues via upregulation of LDLR and thereby reduce plasma levels of LDL-cholesterol (C) in the blood. Overaccumulation of cholesterol in peripheral tissues may cause lipotoxicity, which promotes a negative effect on the function of the pancreatic islets as previously described (5). Additionally, cholesterol impairs the function of pancreatic β cells and causes cellular apoptosis, which have been demonstrated in in vitro and in vivo studies (6).

Although LDLRs may provide a key association between statin therapy and the risk of new-onset T2DM, whether LDLR expression and its regulation in the pancreas and pancreatic islets are altered when patients receive statins is unknown (2). A previous study conducted by the present team demonstrated that 16 weeks of oral treatment with atorvastatin did not affect glucose metabolism in rabbits (7). Therefore, it was hypothesized that the short-term use of atorvastatin may disturb glucose homeostasis. Accordingly, it was proposed that the short-term use of statins may affect the function of pancreatic islets via regulation of LDLR expression. Therefore, the present study aimed to elucidate glucose homeostasis, LDLR expression and its possible regulation in the pancreas following the short-term administration of atorvastatin.

Materials and methods

Animals. The present study was conducted in accordance with the Guidelines for Animal Experimentation of Xi’an Medical University (Xi’an, China) and the Guide for the Care and Use of Laboratory Animals published by the US National
Institutes of Health (NIH; Bethesda, MD, USA; NIH publication no. 85-23, revised 2011). The experimental protocols were approved by the Laboratory Animal Administration Committee of Xi'an Medical University (Institutional Animal Care and Use Committee; permit no. XYJZS-1207011). Male C57BL/6j mice (12 weeks old; ~30 g) were obtained from the laboratory animal center at The Fourth Military Medical University (Xi'an, China), and male apolipoprotein E (ApoE)-deficient mice (12 weeks old; ~30 g) were obtained from the laboratory animal center at Xi'an Jiaotong University (Xi'an, China). A total of 20 C57BL/6j mice were randomly divided into two groups and received a normal chow diet. One group was treated with atorvastatin via intragastric administration (10 mg/kg, each day; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) for 6 weeks; the other group was given the same dose of double distilled water. A total of 16 ApoE-deficient mice were additionally divided into two groups and received a high-fat diet in addition to the treatment described above. As previously suggested by Capel et al. (8), a 10 mg dose of atorvastatin has been repeatedly demonstrated to be effective on lipid metabolism in mice, and the relevant literature is listed in Table 1 (8-17). All mice were individually housed in plastic cages (30x20x13 cm) throughout the extent of the present study and maintained on a 12/12 h light/dark cycle (light off at noon) at constant temperature (22°C) with 10-15 hly cycles of fresh air and relative humidity (60±10%). Food and water were available ad libitum.

Plasma lipid, glucose and insulin metabolism. Following overnight fasting, blood samples were collected via the retro-orbital sinus in tubes containing EDTA as an anticoagulant. Blood samples were centrifuged (1,500 x g; 15 min; 4°C) to collect plasma. Plasma total cholesterol (TC), total triglycerides (TG) and high-density lipoprotein (HDL-C) were measured using a Varioskan Flash plate reader (Thermo Fisher Scientific, Inc., Waltham, MA, USA) with assay kits (BioSino Bio-Technology and Science Inc., Beijing, China) during the final week of the treatment as previously described (18). Intravenous glucose tolerance tests (IGTT) and intravenous insulin tolerance tests (IITT) were performed as previously described (7). The mice were injected with an intravenous glucose solution following 12 h of fasting, and the blood samples were subsequently drawn at 0, 15, 30, 60, 90, 120 and 150 min (1.5 g/kg body weight). For IITT, subsequent to the intravenous injection of insulin (1 U/kg body weight; Wanbang Biopharmaceuticals Co., Ltd., Xuzhou, China), blood samples were collected to examine blood sugar levels at 0, 15, 30, 60, 90, 120 and 150 min.

RNA extraction and reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA of the pancreas and liver were extracted using RNAlater Plus (Takara Bio, Inc., Otsu, Japan). RT-qPCR was performed and quantified using the 2 ΔΔ Ct method as previously described (18-20). The sequences of the primers are listed as follows: LDLR, 5'-TGACCTTTCATCCGAGGCTT-3' and 5'-GGCATGCGGGGTATCCTC-3'; propionate convertase subtilisin/kexin type 9 (PCSK9), 5'-TATCCACGATGGCA CCAGA-3' and 5'-ATGGTGACCCCTCTCAGA-3'; sterol regulatory element-binding protein 2 (SREBP-2), 5'-CCCTTG ACTTCCTTGTCGCA-3' and 5'-GGTCTGAGTGTGTGGCC AATC-3'; E3 ubiquitin-protein ligase MYLIP (IDOL), 5'-AGG AGATCAACTCCACTTCTCAG-3' and 5'-ATCTGCGAGACCG GACAGG-3'; GAPDH, 5'-ACTGGAGACCAGTGGTGC-3' and 5'-TGCTGTAGCCTATTCATTG-3'.

Protein extraction and western blot analysis. Total protein was extracted from the pancreas and liver as previously described (19). The primary antibodies were against LDLR (1:500; cat. no. ab52818; Abcam, Cambridge, UK), ATP-binding cassette transporter A1 (ABCA1; 1:1,000; cat. no. sc-58219), SREBP-2 (1:1,000; cat. no. sc-271616; both Santa Cruz Biotechnology, Inc., Dallas, TX, USA), PCSK9 (1:1,000; cat. no. ab181142; Abcam), IDOL (1:2,000; cat. no. SAB4501317; Sigma-Aldrich; Merck KGaA) and GAPDH (1:2,000; cat. no. sc-32233; Santa Cruz Biotechnology, Inc.) overnight at 4°C. Membranes were subsequently incubated with horseradish peroxidase-conjugated secondary antibodies (1:2,500; cat. nos. A0216 and A0208; Beyotime Institute of Biotechnology, Haimen, China) for 3 h at room temperature. A western blot analysis was performed as previously described, and relative protein expression was quantified by ImageJ (bundled with Java 1.8.0_172; NIH) (19).

Islet isolation and culture. Pancreatic islets in each mouse were isolated as previously described (21). Pancreatic islets were isolated from fasting mice with collagenase type V (0.8 mg/ml; Sigma-Aldrich; Merck KGaA) digestion and, subsequently, islets were purified using a continuous Histopaque (Sigma-Aldrich; Merck KGaA) gradient. Purified islet fractions were collected, and all islets (including islets with normal architecture and islets more or less affected by fibrosis) were selected under a stereomicroscope (magnification, ×10). Mouse islets were cultured in RPMI 1640 medium (Invitrogen; Thermo Fisher Scientific, Inc.) and seeded on a 24-well plate (20 islets/well) coated with rat-tail collagen (Sigma-Aldrich; Merck KGaA). Islets were left in wells for 24 h to adhere and were subsequently treated with different concentrations of atorvastatin (0, 1, 10 and 100 nM; Sigma-Aldrich; Merck KGaA) for 48 h, according to previously described protocols (22).

Immunofluorescence. Following 48 h of incubation, islets were fixed for 1 h with 4% formaldehyde at room temperature and were blocked with 5% bovine serum albumin (cat. no. P0007; Beyotime Institute of Biotechnology) in PBS for 1 h at room temperature. To avoid the detection of internalized LDLR in the intracellular lysosomes, islets were not permeabilized with Triton X-100 prior to incubation. Pancreatic islets were incubated overnight at 4°C with primary antibodies (1:500; cat. no. ab52818; Abcam) against LDLR, and the islets were incubated with Alexa Fluor 488 (1:2,000; cat no. Z25302; Thermo Fisher Scientific, Inc.) secondary antibodies for 3 h at room temperature. Pictures were taken on the fluorescent Nikon TE2000 Inverted Microscope (magnification, x4; TE2000S; Nikon Corporation, Tokyo, Japan) and analyzed with the ImageJ software as previously described (23).

ELISA. Plasma PCSK9 and insulin levels were measured using commercial ELISA kits (cat. no. MPC900; Mouse Quantikine
Statistical analysis. All data are expressed as the mean ± standard error. Comparisons between two groups were performed using the Student’s t-test. Comparisons between multiple groups were conducted using one-way analysis of variance with the Bonferroni test. P<0.05 was considered to indicate a statistically significant difference. The experiments were repeated three times and statistical calculations were analyzed using the SPSS 19.0 software (IBM Corp., Armonk, NY, USA).

Results

Short-term use of atorvastatin affects glucose homeostasis in mice with hyperlipidemia. To study the effect of acute atorvastatin administration on glucose metabolism, IGTT and IITT were performed following 3 and 6 weeks of treatment with atorvastatin in C57BL/6J mice. As demonstrated in Fig. 1A, a 3-week treatment with atorvastatin did not affect glucose tolerance, and only the glucose levels at time 0 (fasting state) were decreased in atorvastatin-treated mice. However, treatment with atorvastatin for 3 weeks significantly affected insulin tolerance following 50 min (P<0.05) and significantly increased the AUC (P<0.05), indicating that atorvastatin decreased insulin sensitivity (Fig. 1A). However, when the AUCs were compared, it was observed that treatment with atorvastatin for 6 weeks did not affect glucose tolerance or insulin sensitivity (Fig. 1B). Additionally, it was identified that treatment with atorvastatin for 6 weeks decreased insulin levels following 12 h of fasting (Fig. 1B).

As it is known that hyperlipidemia increases the risk of cardiovascular disease (CVD) and T2DM, it was hypothesized that the short-term use of atorvastatin may affect glucose metabolism in mice with hyperlipidemia (24). It was observed that treatment with atorvastatin for 6 weeks increased glucose tolerance in mice with hyperlipidemia (Fig. 2A); whereas, there was no marked effect on insulin sensitivity (Fig. 2B).

Short-term use of atorvastatin affects blood lipid levels in normal mice. The lipid profile was examined in the two types of mice following 6 weeks of treatment with atorvastatin. The present results demonstrated that treatment with atorvastatin significantly decreased the TC, TG and insulin levels in the C57BL/j mice compared with the control plasma lipid levels (P<0.05); however, there was no significant difference in plasma LDL-C, HDL-C or glucose (Fig. 3A). In hyperlipidemic mice, treatment with atorvastatin did not affect plasma TC, LDL-C, HDL-C, TG or glucose (Fig. 3B). This result is consistent with a previous study demonstrating that atorvastatin does not markedly reduce the blood lipid levels in ApoE-deficient mice (25). Furthermore, it was identified that treatment with atorvastatin for 6 weeks did not affect insulin levels following 12 h of fasting (Fig. 3B).
Treatment with atorvastatin suppresses LDLR expression in the pancreas of C57BL/6j mice. To study whether treatment with atorvastatin may affect LDLR expression in the pancreas and liver, the protein and mRNA levels of LDLR were measured by western blotting and PCR. It was observed that 6 weeks of treatment with atorvastatin significantly reduced LDLR protein in the pancreas (P<0.05), and this result was additionally confirmed with PCR, which demonstrated that LDLR mRNA expression was altered significantly (Fig. 4A; P<0.05). As it is known that ABCA1 is a key receptor that mediates cellular cholesterol efflux, ABCA1 expression was additionally examined; however, no alterations in ABCA1 expression were observed in the pancreas with acute atorvastatin administration (Fig. 4A) (26). Contrary to the findings in the pancreas, it was identified that 6 weeks of treatment with atorvastatin did not affect expression of LDLR and ABCA1 at the protein and mRNA levels in the liver (Fig. 4B).

To test whether the effect of atorvastatin on LDLR expression may be affected by hyperlipidemia, the LDLR expression was measured in the pancreas and liver of ApoE-deficient mice. It was observed that 6 weeks of treatment with atorvastatin did not affect the protein and mRNA expression levels of LDLR in the pancreas (Fig. 5A). Furthermore, it was identified that treatment with atorvastatin did not affect the protein and mRNA expression of LDLR in the liver (Fig. 5B).

Treatment with atorvastatin suppresses LDLR expression in pancreatic islets. To test whether atorvastatin may affect LDLR expression in pancreatic islets, increasing concentrations of atorvastatin were used to treat adhered islets from C57BL/6j mice for 48 h. It was identified that incubation with atorvastatin affected LDLR expression in islets in a concentration-dependent manner (Fig. 6). Subsequent to analyzing the fluorescence intensity by using ImageJ software, it was observed that islets incubated with a high dose of atorvastatin (100 nM) exhibited a significantly decreased fluorescence intensity compared with the 0 nm group (Fig. 6B; P<0.05).
Short-term use of atorvastatin induces an increase in PCSK9 in the pancreas. To investigate the possible regulation of LDLR by atorvastatin, the protein expression of SREBP-2, PCSK9 and IDOL was measured in the pancreas of C57BL/6j mice. It was observed that 6 weeks of treatment with atorvastatin did not affect SREBP-2 or IDOL expression; whereas, treatment with atorvastatin significantly increased PCSK9 expression in the pancreas compared with the control (Fig. 7A; P<0.05). To confirm these results, RT-qPCR was used to measure the mRNA expression of SREBP-2, PCSK9 and IDOL in the pancreas. It was observed that the mRNA expression of PCSK9 was upregulated by treatment with atorvastatin; however, SREBP-2 and IDOL were not affected (Fig. 7B). Furthermore, given that plasma PCSK9 may affect LDLR expression in peripheral tissues, a commercial ELISA kit was used to detect plasma PCSK9 expression levels. It was identified that 6 weeks of treatment with atorvastatin did not affect plasma PCSK9 expression levels (Fig. 7C). A schematic diagram represents the regulation of hepatic LDLR by statins: Statins upregulate LDLR via SREBP-2; SREBP-2 additionally increases transcription of PCSK9 to degrade LDLR; and cellular cholesterol...
accumulation may activate LXR, which upregulates IDOL to degrade LDLR (Fig. 7D).

Discussion

Statins are 3-hydroxy-3-methyl-glutaryl coenzyme-A reductase inhibitors, which are used to decrease blood LDL-C levels by inhibiting cellular cholesterol synthesis and upregulating LDLR expression levels in the liver and peripheral tissues (27). Although previous research suggests that statins increase the risk of new-onset T2DM, it is noteworthy that statins are highly effective for the prevention of CVD in individuals with or without DM (28). A recent study additionally concluded that the net absolute benefit observed with statin therapy in such individuals is >50 times greater compared with any putative effect on DM (29). As statins have an important role in the primary and secondary prevention of CVD, a mechanistic understanding of the diabetogenic effect of statins requires investigation. However, there are inconsistent findings on the diabetogenic effect of statins in animal experiments (28). A previous study conducted by the present team demonstrated that long-term treatment with atorvastatin does not affect glucose homeostasis in rabbits with normal blood lipid levels (7). Given that adaptation may occur in islets when experimental animals are subjected to long-term treatment with atorvastatin, it was hypothesized that acute administration of atorvastatin may affect glucose homeostasis. Notably, the present study supports the hypothesis that short-term administration of atorvastatin may slightly affect glucose homeostasis in normal and hyperlipidemic mice. In clinical studies, treatment with statins has been associated with the risk of new-onset T2DM; there is additional evidence to suggest that statins may induce the onset of T2DM (29,30). Although diabetic rodent models are useful for the majority of diabetes studies, these animals usually have impaired pancreatic islets and present dysfunction of β cells, which may not be suitable for observation of early changes in
the diabetogenic process or a slight effect of statins on normal islets (31). It was hypothesized that the diabetogenic effect of statins may contribute to LDLR-mediated LDL-C uptake in β cells, and hyperlipidemia may be involved in the diabetogenic effect of statins. Therefore, normal C57BL/J and hyperlipidemic ApoE-deficient mice were examined in the present study.

On the basis of previous studies, statins may increase the transcription of LDLRs in the liver by upregulating SREBP-2. As a transcription factor, SREBP-2 additionally upregulates PCSK9 to induce the degradation of LDLR (27). Statins may promote an increase in IDOL, which serves as a ubiquitin ligase to degrade LDLR and may reduce LDLR expression (32). As statins may dually upregulate and downregulate LDLR, the abundance of the LDLR protein is not markedly increased in the liver (33). In contrast to the liver, the pancreas and islets may not be fully subjected to such dual regulation. In the present study, it was observed that atorvastatin suppressed LDLR expression in the pancreas and islets, whereas this phenomenon was not observed in the liver. Furthermore, it was not observed that atorvastatin affected LDLR expression in the pancreas and liver in hyperlipidemic mice. As mentioned previously, LDLR expression is primarily regulated by SREBP-2, PCSK9 and IDOL. Therefore, these genes were investigated in the pancreas. Interestingly, compared with no difference in plasma PCSK9, treatment with atorvastatin may promote an increase in PCSK9 in the pancreas, indicating that decreased LDLR may be attributed to upregulated PCSK9 in situ.

As previously mentioned, upregulation of LDLR may enhance LDLR-mediated LDL-C (or modified LDL-C) uptake and causes dysfunction or cytotoxicity in these cells (2). LDLR therefore provides an association between treatment with statins and new-onset T2DM. Although the present data demonstrated that atorvastatin decreases LDLR in the pancreas, the pathological role of LDLR in T2DM requires further investigation. Firstly, it is unclear whether treatment with atorvastatin may induce a similar effect on LDLR expression in the pancreas and islets of humans, and this effect may be enhanced or weakened in patients with hyperlipidemia or T2DM. Secondly, ApoE-deficient mice present hypercholesterolemia due to accumulation of remnant lipoproteins compared with LDLs, which is not completely consistent with the physiopathological process of patients with hyperlipidemia or FH. Therefore, a clinical study may provide novel insight into the pathological role of LDLR in the diabetogenic effect of statins. Notably, it was additionally identified that atorvastatin did not promote an increase in ABCA1 in the liver and pancreas. Given that ABCA1 is a pivotal receptor for mediating cholesterol efflux, whether atorvastatin may enhance the ABCA1-mediated cholesterol efflux in mice requires determination (34).

In conclusion, the present study presents novel findings on the effect of acute administration of atorvastatin on disturbing glucose homeostasis. It was additionally demonstrated that acute administration of atorvastatin downregulates LDLR expression in the pancreas of normal mice; however, this reduction in LDLR was not observed in hyperlipidemic mice. Furthermore, the present data demonstrated that LDLR expression in the pancreas is more affected by atorvastatin compared with in the liver of mice with normal blood lipid levels, suggesting that the role of LDLR in the diabetogenic effect of statins requires further investigation.

Figure 7. Expression of SREBP-2, PCSK9 and IDOL in the pancreas of C57BL/6j mice following 6 weeks of treatment with atorvastatin. (A) Protein expression of SREBP-2, PCSK9 and IDOL was quantified by western blotting. n=4/group. (B) mRNA expression of SREBP-2, PCSK9 and IDOL was assessed by reverse transcription-quantitative polymerase chain reaction. n=4/group. (C) Plasma PCSK9 was quantified using an ELISA kit. n=10/group. (D) A schematic diagram representing the regulation of hepatic LDLR by statins: Statins upregulate LDLR via SREBP-2; SREBP-2 additionally increases transcription of PCSK9 to degrade LDLR; and cellular cholesterol accumulation may activate LXR, which upregulates IDOL to degrade LDLR. Data are expressed as the mean ± standard error. *P<0.05 vs. respective control. PCSK9, proprotein convertase subtilisin/kexin type 9; SREBP-2, sterol regulatory element-binding protein 2; LDLR, low-density lipoprotein receptor; IDOL, E3 ubiquitin-protein ligase MYLIP; LXR, oxysterols receptor LXR-α.
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Availability of data and materials

All data generated or analyzed during the present study are included in this published article.

Authors’ contributions

QY, CX and SW contributed to the experimental design, discussion of result and critical revision of the manuscript. QY contributed to the drafting of the manuscript. QY, FW, XM, YG and YW conducted the experiments. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

The experimental protocols were approved by the Laboratory Animal Administration Committee of Xi’an Medical University (Institutional Animal Care and Use Committee; permit no. XYJZS-120701).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References


