Effects of Atorvastatin on Glucose Metabolism and Insulin Resistance in KK/Ay Mice

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Insulin resistance plays an important role not only in the development and progression of diabetes mellitus but also in the establishment of metabolic syndrome. Improvement of insulin resistance is thus of great importance both in improving glucose metabolism and preventing atherosclerosis. Although HMG-CoA reductase inhibitors appear to favorably affect glucose metabolism, as indicated by the results of a sub-analysis in the West of Scotland Coronary Prevention Study (WOSCOPS), their effects on glucose metabolism and insulin resistance have not been thoroughly investigated in animal models. In this study, the effects of atorvastatin on the glucose metabolism and insulin resistance of KK/Ay mice, an animal model of type II diabetes, were investigated. Atorvastatin significantly decreased the non-HDL-cholesterol level in the oral glucose tolerance test, inhibited increase in the 30-min glucose level, decreased plasma insulin levels before and 30 and 60 minutes after glucose loading, and decreased the insulin resistance index, compared with corresponding values in controls, indicating that atorvastatin appeared to improve glucose metabolism by improving insulin resistance. Northern blot analysis revealed decreases in levels of mRNA of sterol regulatory element binding protein-1 (SREBP-1) and glucose-6-phosphatase (G6Pase), and it may play a role in the improvement of glucose metabolism and insulin resistance. J Atheroscler Thromb, 2005; 12: 77–84.

Key words: Animal model, Oral-glucose tolerance test (OGTT), Diabetes, Obesity

Introduction

The report of the Multiple Risk Factor Intervention Trial (MRFIT) suggested that the prevalence of atherosclerosis in patients with diabetes mellitus is two or three times that in individuals without diabetes (1), and a Finnish study has reported that the risk of ischemic heart disease is extremely high in patients with type II diabetes mellitus (2). In order to prevent the development of ischemic heart disease in patients with diabetes mellitus, the risks of development of hyperlipidemia, hypertension and other relevant diseases should be carefully controlled. In the Third Report of the National Cholesterol Education Program Expert Panel on Detection, Evaluation, and Treatment of High Blood Cholesterol in Adults (ATP III), it is stated that the risk of development of ischemic heart disease in patients with diabetes should be considered equal to that in individuals with a history of ischemic heart disease, and the target level of LDL-cholesterol in patients with diabetes is set at < 100 mg/dl (3). It has been demonstrated that the development of ischemic heart disease in diabetic patients can be prevented by lowering LDL cholesterol level by treatment with HMG-CoA reductase inhibitors (4, 5).

Insulin resistance not only plays an important role in the development and progression of diabetes but also is
Suzuki et al. considered the pathological change principally responsible for metabolic syndrome. Insulin resistance promotes the development of atherosclerosis in association with a decrease in HDL-C, an increase in triglyceride level, hypertension and a decrease in nitric oxide (NO) production, among other changes (6). Since thiazolidine improved insulin resistance and prevented the development of atherosclerosis in animal models (7), improvement of insulin resistance is also expected to inhibit the development of atherosclerosis in humans. There are several hypotheses concerning how insulin resistance develops. Free fatty acids and triglycerides have been reported to induce insulin resistance in skeletal muscle in the presence of physiologically active substances released from fat tissues such as adiponectin, leptin and TNF-α (8, 9). Deposition of fat results in the development of insulin resistance not only in skeletal muscle but also in the liver (10), and biguanides improve the insulin resistance associated with fat deposition (11).

Recently, a sub-analysis in the West of Scotland Coronary Prevention Study (WOSCOPS) revealed that pravastatin significantly inhibited new onset of diabetes mellitus (12), suggesting that HMG-CoA reductase inhibitors may improve glucose metabolism. It has been reported that atorvastatin demonstrated no significant effects on glycemic control (13, 14). Although HMG-CoA reductase inhibitors are known to exert pleiotropic effects in inhibiting inflammation and enhancing restoration of endothelial function, the effects of statins on glucose metabolism and insulin resistance remain to be clarified. In the present study, we investigated the effects of atorvastatin on glucose metabolism and insulin resistance in KK/Ay mice, an animal model of insulin resistance and type 2 diabetes mellitus (15, 16).

Methods

Animals
Male 8-week-old KK/Ay mice were obtained from Clea Japan, Inc. (Osaka, Japan). KK/Ay mice are produced by mating female black KK mice with impaired glucose tolerance and male yellow obese Ay mice, and are known to be a good model of obesity-associated type II diabetes mellitus with insulin resistance. The mice were housed individually in cages located in a room with controlled temperature, humidity and lighting (illuminated from 7:30 to 20:30), and were allowed water and rodent chow (CMF, Oriental Yeast, Co., Ltd., Tokyo, Japan) ad libitum. Blood was taken from animals with food available ad libitum to select animals with a triglyceride level of ≥ 280 mg/dl. The eligible animals were then allocated into four groups, i.e., (A) the oral glucose tolerance test (OGTT)-control (N = 12), (B) OGTT-atorvastatin (N = 12), (C) insulin tolerance test (ITT)-control (N = 8), and (D) ITT-atorvastatin groups (N = 8), to equalize the mean values of plasma glucose level, plasma triglyceride level, FFA level and body weight of animals among groups (Table 1). Atorvastatin suspended in water containing 0.5% carboxymethylcellulose was administered orally at a dose of 30 mg/kg once daily in the morning during the study period. Animals in the control groups were orally administered an aqueous 0.5% carboxymethylcellulose suspension. Mice were anesthetized with diethyl ether, and livers were collected immediately. The experiments were performed in accordance with the regulations of the Animal Ethical Committee of University of Tsukuba following “Principles of Laboratory Animal Care” (NIH publication no. 85–23, revised 1985).

Determination of plasma parameters
Levels of cholesterol, HDL-cholesterol, non-esterified fatty acid, triglyceride and glucose in plasma were determined using enzyme methods. ELISA kits were used to measure plasma levels of adiponectin (Otsuka Pharmaceutical Co., Ltd, Tokyo, Japan) and leptin (Life Science Laboratory, Morinaga Milk Industry Co., Ltd., Kanagawa, Japan).

RNA isolation and northern blotting
Total RNA from liver was isolated with Trisol reagent (Invitrogen, Carlsbad, CA, USA), and 10-µg RNA samples equally pooled from three mice each of the control or

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**Table 1.** Blood chemistry parameters and body weight at baseline.

<table>
<thead>
<tr>
<th></th>
<th>OGTT groups</th>
<th>ITT groups</th>
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<tr>
<td></td>
<td>(A) Control (N = 12)</td>
<td>(B) Atorvastatin (N = 12)</td>
</tr>
<tr>
<td>TG (mg/dl)</td>
<td>454 ± 35</td>
<td>473 ± 28</td>
</tr>
<tr>
<td>Glucose (mg/dl)</td>
<td>550 ± 19</td>
<td>530 ± 22</td>
</tr>
<tr>
<td>FFA (mEq/l)</td>
<td>1.07 ± 0.05</td>
<td>1.04 ± 0.04</td>
</tr>
<tr>
<td>B.W. (g)</td>
<td>38.4 ± 0.5</td>
<td>38.1 ± 0.4</td>
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Blood samples were collected under ad libitum feeding conditions. Values are means ± SEM.

OGTT: oral glucose tolerance test, ITT: insulin tolerance test, TG: triglyceride, FFA: free fatty acid, B.W.: body weight
atorvastatin-treated group were run on a 1% agarose gel containing formaldehyde, and then transferred to a nylon membrane. The cDNA probes used were cloned as previously described (17, 18). The probes were labeled with $[\alpha-^{32}\text{P}]d\text{CTP}$ using a Rediprime II random prime labeling system kit (Amersham Biosciences, Piscataway, NJ, USA). The membranes were hybridized with the radiolabeled probe in Rapid-hyb Buffer (Amersham Biosciences) at 65°C and washed in 0.1 × SSC, 0.1% SDS at 65°C. Blots were analyzed by digital imaging (Fujix BAS2500; Fujifilm, Tokyo, Japan) and quantified using ImageQuaNT software (Amersham Biosciences, Carlsbad, CA, USA). The results of quantification were normalized to the signal generated from 36B4 (acidic ribosomal phosphoprotein P0) mRNA.

Oral glucose tolerance test
Animals in Groups A and B were subjected to oral glucose tolerance test (OGTT) on Day 28 of treatment after a 16-hour fasting period. The mice were orally administered a 20% glucose solution at a dose of 2 g/kg, and blood samples were obtained immediately before and 30, 60 and 120 minutes after glucose loading. Insulin resistance index was calculated from plasma insulin and glucose levels after oral glucose load (19).

Insulin tolerance test
Animals in Groups C and D were subjected to insulin tolerance test (ITT) on Day 35 of treatment after a 16-hour fasting period. The mice underwent intraperitoneal administration of human insulin (Noborin R; Novo Nordisk, Bagsvaerd, Denmark) at a dose of 1 U/kg, and blood samples were obtained immediately before and 20, 40, 60 and 80 minutes after the insulin loading.

Determination of plasma levels of adiponectin and leptin
The mice in Groups A and B were separated into the following subgroups: the A-fast ($N = 6$), A-fed ($N = 6$), B-fast ($N = 6$) and B-fed ($N = 6$) groups. Blood samples of animals in the A-fast and B-fast groups were obtained on Day 38 of treatment after a 16-hour fasting period, and those in the A-fed and B-fed groups were obtained on day 35 of treatment without restriction of food intake.

Statistical analysis
All results were analyzed by the two-tailed Student’s $t$-test using Statistical Analysis System version 6.11 (SAS Institute, NC, USA). All values are expressed as mean ± SEM.

Results
Levels of glucose, insulin and lipids in plasma in animals subjected to oral glucose tolerance test
KK/Ay mice receiving atorvastatin at 30 mg·kg$^{-1}$·day$^{-1}$
for 4 weeks were subjected to OGTT to evaluate the effect of atorvastatin on glucose tolerance. The effect of the drug on plasma lipid levels after a 16-hour fasting period and immediately before glucose loading was also examined. Plasma glucose level during OGTT was significantly lower at 30 minutes after glucose loading in animals receiving atorvastatin than in those receiving the vehicle. Glucose levels at 60 and 120 minutes after glucose loading were also lower in the animals receiving atorvastatin than in control animals (Fig. 1A). Plasma insulin levels were significantly lower in animals receiving atorvastatin than in control animals immediately before and 30 and 60 minutes after glucose loading (Fig. 1B). Insulin response and insulin sensitivity after glucose loading were evaluated by Σ insulin and insulin resistance index, respectively. Σ insulin after glucose loading in atorvastatin group was significantly less than that in control group (131.2 ± 10.6 vs 189.6 ± 13.9, p < 0.005). Insulin resistance index was significantly reduced after atorvastatin treatment (Fig. 2). Plasma levels of total cholesterol, HDL cholesterol, non-HDL-cholesterol, triglyceride and free fatty acid after a 16-hour fasting period and immediately before glucose loading were lower in the animals receiving atorvastatin than those in the control animals by 8%, 3%, 21%, 12% and 15%, respectively. Non-HDL-cholesterol level was decreased significantly in the animals receiving atorvastatin (Table 2). Body weight did not change significantly during the study.

**Change in plasma glucose level during insulin tolerance test**

Plasma glucose levels during the insulin tolerance test were lower in the animals receiving atorvastatin than in the control animals at all time points before and after glucose loading. At 20 minutes after glucose loading, plasma glucose level tended to decrease (22%, p = 0.08), but the decrease in plasma glucose level was significant at none of the time points evaluated (Fig. 3).

**Levels of adiponectin and leptin in plasma, and levels of expression of genes related to lipid/glucose metabolism in the liver**

Animals subjected to the oral glucose tolerance test after treatment with atorvastatin or vehicle were separated into two sub-groups and received the respective treatment for an additional 1 week before autopsy and blood sampling after (1) a 16-hour fasting period or (2) intake of food ad libitum. Plasma levels of adiponectin and leptin as well as levels of mRNA of factors involved in lipid or glucose metabolism were determined. Atorvastatin did not significantly affect plasma levels of adiponectin and leptin compared with those in control animals (adiponectin 13.2 ± 1.1 vs 13.3 ± 0.7, p = 0.98 (fasting) and 10.2 ± 0.7 vs 9.2 ± 0.3, p = 0.25 (ad libitum); leptin 42 ± 2 vs 49 ± 4, p = 0.16 (fasting), 39 ± 2 vs 45 ± 2, p = 0.06 (ad libitum)). Atorvastatin affected body weight significantly in neither those animals tested after the fasting period nor those tested with food available ad libitum (41.0 ± 0.6 vs 42.8 ± 1.3, p = 0.24 (fasting), 43.9 ± 0.4 vs 44.1 ± 0.4, p = 0.65 (ad libitum)).

Expressions of mRNA of factors involved in lipid metabolism, glucose metabolism or insulin signal-transduc-

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**Table 2. Effects of four-week atorvastatin treatment on body weight and plasma parameters in KK/Ay mice**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control (mg/dl)</th>
<th>Atorvastatin (mg/dl)</th>
<th>P-value</th>
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<tbody>
<tr>
<td>TC (mg/dl)</td>
<td>151 ± 9</td>
<td>139 ± 5</td>
<td>0.26</td>
</tr>
<tr>
<td>HDL-C (mg/dl)</td>
<td>108 ± 9</td>
<td>105 ± 5</td>
<td>0.78</td>
</tr>
<tr>
<td>non-HDL-C (mg/dl)</td>
<td>43 ± 3</td>
<td>34 ± 3</td>
<td>0.04</td>
</tr>
<tr>
<td>TG (mg/dl)</td>
<td>186 ± 17</td>
<td>164 ± 15</td>
<td>0.35</td>
</tr>
<tr>
<td>FFA (mEq/l)</td>
<td>1.04 ± 0.08</td>
<td>0.89 ± 0.06</td>
<td>0.13</td>
</tr>
<tr>
<td>B.W. (g)</td>
<td>40.2 ± 0.7</td>
<td>39.2 ± 0.3</td>
<td>0.20</td>
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</table>

Blood samples were collected after 16 h fasting following 4-week treatment with atorvastatin at 30 mg/kg/day. Values are means ± SEM, N = 12 in each group. Numbers in parentheses represent percent change compared to vehicle treatment. P-values were calculated by Student’s t-test.
Atorvastatin on Glucose Metabolism

Fig. 3. Effect of atorvastatin on insulin resistance in KK/Ay mice. ITT was performed after administration of atorvastatin at 30 mg/kg/day for 5 weeks. Blood samples were collected sequentially and plasma glucose were measured. ○: control, ■: atorvastatin. Values are means ± SEM, N = 8 in each group. P-values were calculated by Student’s t-test.

Fig. 4. Effects of atorvastatin on mRNA expression of various genes in liver from vehicle- or atorvastatin-treated KK/Ay mice. Liver samples were collected on day 38 after 16 h fasting (fast), and on day 35 under ad libitum feeding conditions (fed). Northern blot analysis of various genes in liver is shown. Total RNA (10 µg) in samples pooled equally from three mice was subjected to Northern blotting, followed by hybridization with the indicated cDNA probes.

Discussion

Insulin resistance is a major pathological change observed in patients with coronary heart disease (6). Improvement of insulin resistance may play an important role in preventing atherosclerosis in patients with hyperlipidemia complicated by diabetes mellitus. Clinical studies have reported that statins affect glucose metabolism and insulin resistance (21), but there have been no reports of investigations of their mechanism of action in animal models. In the present study, we investigated the effects of atorvastatin on glucose metabolism and insulin resistance and their mechanisms of action in KK/Ay mice, a model of type II diabetes mellitus in which obesity, hyperinsulinemia, insulin resistance, hyperlipidemia and hyperglycemia develop.

In this study, atorvastatin significantly decreased plasma non-HDL cholesterol level in KK/Ay mice: It was thus found to exert lipid-lowering effects in an animal model of insulin resistance. Atorvastatin also inhibited increase in the plasma glucose level, and decreased plasma insulin levels and the insulin resistance index significantly during the oral glucose tolerance test: These findings suggest that atorvastatin may improve insulin resistance.

It has been reported that free fatty acids and triglycerides induce insulin resistance in skeletal muscle. In clinical studies, patients with type II diabetes mellitus exhibited significant decreases in HbA1c level and HOMA index after treatment with atorvastatin, and a significant correlation was found between the decrease in HOMA index and the decrease in plasma triglyceride level (22). In the present study, plasma levels of free fatty acids and triglycerides were decreased in animals receiving atorvastatin (by 15% and 12%, respectively) but these decreases were not statistically significant: The improvement of insulin resistance by atorvastatin cannot be explained by decreases in free fatty acid and triglyceride levels alone.
In the present study, we investigated the effects of atorvastatin on plasma levels of various factors known to affect insulin resistance. Adiponectin is believed to improve insulin resistance, since it inhibits the expression of TNF-α (23) and decreases the content of triglycerides in tissues by enhancing oxidation of fatty acids in skeletal muscles (24). Leptin is believed to improve insulin resistance, since it decreases triglyceride content in tissues by promoting beta-oxidation of fatty acids (25). In this study, atorvastatin did not significantly affect the plasma adiponectin level, but did tend to decrease the plasma leptin level (KK/Ay mice exhibit hypoleptinemia and leptin resistance (26). Few reports are available on the effects of statins on these adipocytokines, and further studies of them are needed.

Analysis of mRNA expressions in the liver revealed that atorvastatin did not change the level of expression of PEPCK but did decrease the expression of G6Pase in fasting conditions: these findings suggest that atorvastatin inhibits gluconeogenesis. In addition, atorvastatin affected levels of expression of neither IR nor IRS-2 in fasting conditions, and thus appeared not to inhibit the insulin signaling system. The decrease in expression of SREBP-1 noted in this study may support the hypothesis that atorvastatin prevents fatty liver by inhibiting fatty acid synthesis. In the animals with food available ad libitum, atorvastatin decreased the levels of expression of SREBP-1 and FAS: these findings further suggest that atorvastatin may inhibit fat synthesis in the liver.

Although it has been reported that fatty liver induces insulin resistance in the liver (27), the mechanism of development of insulin resistance in fatty liver remains to be clarified. Metformin, a biguanide, is known to improve insulin resistance in the clinical setting (11), and inhibition of fatty liver is believed to play a role in this (28, 29). Polyunsaturated fatty acids have been reported to prevent fatty liver and to improve insulin resistance (30, 31) via several mechanisms, including inhibition of the expression of lipogenic enzymes (32) by hindering binding of LXR to LXRE (33) to inhibit transcription of SREBP-1, and decrease in the content of triglycerides in the liver by enhancement of beta-oxidation of fatty acids through activation of PPARα (34). The suppression of expression of SREBP-1 by atorvastatin observed in this study suggests the possibility that atorvastatin decreases fat synthesis by decreasing the activity of lipogenic enzymes including FAS. Inhibition of fatty liver by atorvastatin was also indicated in a study in rats loaded with sucrose in which atorvastatin inhibited increase in triglyceride level in liver and enhanced beta-oxidation of fatty acids in liver (35). Therefore, atorvastatin may improve insulin resistance in the liver in the same fashion as metformin and PUFA, by inhibiting development of fatty liver.

In conclusion, atorvastatin decreased lipid levels, improved glucose metabolism after glucose loading and improved insulin resistance in KK/Ay mice, an animal model of type II diabetes mellitus. Possible mechanisms of improvement of glucose metabolism by atorvastatin may include improvement of insulin resistance by inhibition of synthesis of fats through inhibition of the expression of SREBP-1 in the liver as well as inhibition of gluconeogenesis.

References


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