

Differential effect of statins on diabetic nephropathy in db/db mice

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Abstract. Recent studies suggest a potential benefit of the lipid-lowering medication in the treatment of chronic kidney disease (CKD) such as diabetic nephropathy. Although statins have been widely used to lower serum cholesterol levels, the effect of these drugs on diabetic nephropathy has not been fully elucidated. In the present study, therefore, we addressed the role of different kinds of statins on diabetic nephropathy in db/db mice. Mice were fed with a standard diet with 0.005% (w/w) of pitavastatin, rosuvastatin, and pravastatin for 8 weeks starting from 8 weeks of age. The treatment with statins did not affect the food intake, body weight gain, adiposity, or blood pressure in db/db mice. Treatment with statins also had no effect on plasma lipid levels. In terms of the effect on albuminuria, pitavastatin and rosuvastatin reduced the urinary excretion of albumin by 60 and 40%, respectively, but not pravastatin, suggesting the effect of these two drugs on diabetic nephropathy. Furthermore, pitavastatin and rosuvastatin improved glomerular hypertrophy. All statins treatment improved insulin resistance. In addition, rosuvastatin and pravastatin treatment reduced oxidative stress measured by urinary 8-OHdG level, whereas the statins had no effect on the inflammatory response in the kidney of db/db mice. These results are not consistent with the renoprotective effect of statins. In conclusion, our data suggest that pitavastatin and rosuvastatin can improve diabetic nephropathy through the suppression of glomerular hypertrophy, independent of lipid-lowering or anti-oxidative effects.

Introduction

Diabetic nephropathy is one of the most common forms of chronic kidney disease (CKD) and the most frequent cause

of mortality in patients with diabetes (1,2). The number of people affected by diabetic nephropathy or who need renal replacement is steadily increasing (3). Therefore, the establishment of therapeutic strategies for diabetic nephropathy is needed. Diabetic nephropathy results from complex interactions between genetic, metabolic, and hemodynamic factors, and can be characterized by mesangial expansion followed by glomerulosclerosis and a decline in renal function. The development of glomerulosclerosis in diabetes mellitus is always preceded by persistent albuminuria and glomerular hypertrophy (2). Therefore, these two manifestations could be promising therapeutic targets for the treatment of diabetic nephropathy.

3-Hydroxy-3-methylglutaryl (HMG)-coenzyme A (CoA) reductase inhibitors (statins) are widely used for diabetic patients to reduce their cardiovascular risk (4). Statins also have renoprotective actions and have been shown to reduce albuminuria in both experimental and clinical diabetic renal disease (5-8). Some of these benefits may be due to lipid lowering, since lipid levels are strongly associated with the development and progression of diabetic kidney disease (9,10). On the other hand, statins have a range of lipid-independent actions on cell proliferation, inflammation, and oxidative stress (11,12), which may impact the development and progression of renal damage in diabetes. These pleiotropic effects have been suggested to contribute to the renoprotective effect of statins. However, the precise mechanisms of the renoprotective effects are not fully understood. In addition, whether different statins have the same effect on diabetic nephropathy is not well known.

In this study, we addressed the role of various statins, such as pitavastatin, rosuvastatin, and pravastatin on the development of diabetic nephropathy in db/db mice.

Materials and methods

Materials. Pravastatin and rosuvastatin were provided by Daiichi Sankyo Co., Ltd. and pitavastatin was provided by Kowa Pharmaceutical Co., Ltd.

Animal procedure and experimental design. Male db/db mice (n=24) and their lean control db/m (n=6) mice were obtained from Charles River at 6 weeks of age. The mice were fed with normal chow without additional supplementation (non-treated group) or with chow supplemented with 0.005% (w/w) pravastatin, pitavastatin or rosuvastatin for 8 weeks starting from 8

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Table I. Characteristics of db/m and db/db mice treated with or without statins.

	db/m	db/db			
		Con	Pra	Pit	Ros
Body weight (g)	32.5±0.40	53.1±3.90	51.2±5.1	50.3±3.80	51.3±5.70
Liver weight (g)	1.15±0.29	2.99±0.41	2.78±0.54	2.55±0.36	3.19±0.85
eWAT weight (g)	0.37±0.05	3.23±0.25	3.08±0.59	3.01±0.41	3.10±0.62
Kidney weight (g)	0.31±0.07	0.50±0.02	0.51±0.06	0.42±0.01 ^a	0.41±0.03 ^a
Food intake (g/day)	3.82±0.33	7.45±2.43	7.15±0.72	7.70±1.65	7.04±1.58
SBP (mmHg)	NA	113.2±11.6	113.5±3.00	109.5±9.50	114.6±5.60

Con, control; Pra, pravastatin; Pit, pitavastatin; Ros, rosuvastatin; eWAT, epididymal white adipose tissue; SBP, systolic blood pressure. Results are expressed as mean ± SD (n=6 in each group). ^aP<0.05 vs. Con.

weeks of age. Animals had access to food and water *ad libitum* and were maintained on a 12-h light/dark cycle. All animal experiments were conducted according to the Guidelines for Animal Experiments at Kyoto University.

Analysis of metabolic parameter. Plasma glucose concentration was measured with a Glutest Ace (Sanwa Kagaku Kenkyusho Co., Ltd.). Plasma insulin concentration was measured with an insulin assay kit (Morinaga Institute of Biological Science). Plasma cholesterol and triglyceride levels were respectively measured with the Cholesterol E and Triglyceride E tests (Wako Pure Chemical Industries, Ltd.).

Measurement of urinary albumin and creatinine. Urinary albumin and creatinine were measured at 16 weeks of age from 24-h collection samples from mice housed in individual metabolic cages. During the urine collection, the mice were allowed free access to food and water. Albumin concentration in the urine was measured by Albuwell (Exocell). Urinary creatinine was measured with a Hitachi Mode 736 analyzer (Hitachi). The urinary albumin concentration was adjusted by the urinary creatinine concentration.

Measurement of urinary oxidative stress. Urinary 8-OHdG concentrations were measured at 16 weeks of age using a competitive enzyme-linked immunosorbent assay kit (8-OHdG Check, Japan Institute for the Control of Aging). Urinary 8-OHdG excretion was expressed as the total amount excreted in 24 h.

Quantitative real-time PCR. Total-RNA was extracted from frozen kidney tissue (50 mg) at 16 weeks of age using an RNeasy mini kit (Qiagen). The cDNA was synthesized from total-RNA using SuperScript III (Invitrogen). Real-time PCR was performed on an ABI PRISM 7900 using the SYBR-Green PCR Master Mix (Applied Biosystems). Primer sets were as follows: tumor necrosis factor (TNF)- α forward, 5'-CCCAGACCTCACACTCAGATC-3' and reverse, 5'-GCCACTCCAGCTGCTCCTC-3'; β -actin forward, 5'-TACCACAGGCATTGTGATGG-3' and reverse, 5'-TTTGATGTACGCACGATTT-3'. The mRNA levels were normalized relative to the amount of β -actin mRNA and expressed in arbitrary units.

Measurement of glomerular size. The mice were euthanized at 16 weeks of age. The kidneys were rapidly fixed in 10% formaldehyde, and embedded in paraffin. Paraffin sections were cut at 3 μ m. For measurement of the glomerular size, paraffin sections were stained with hematoxylin and eosin. The size of the glomerular surface area was measured using the Image-Pro Plus software version 3.0.1 (Media Cybernetics, Inc.).

Statistical analysis. Data are expressed as the mean ± SD. Multiple comparisons among the groups were conducted by one-way analysis of variance with Fisher's PLSD test for post hoc analysis. P-values of <0.05 were considered significant.

Results

Effect of statin treatment on body weight, adiposity and systolic blood pressure. In db/db mice fed with a standard diet for 8 weeks starting at 8 weeks of age, body weight, epididymal white adipose tissue (eWAT) weight, liver weight were increased compared to those of db/m mice. Treatment with statins had no effect on body weight, food intake, liver weight and eWAT weight in db/db mice (Table I). In addition, there was no difference in systolic blood pressure between statin-treated and non-treated db/db mice.

Effect of statin treatment on renal function in db/db mice. Because albuminuria reflects renal function (13), we measured the urinary excretion of albumin in normal chow-fed db/db mice at 16 weeks of age. Urinary excretion of albumin was markedly increased in db/db mice compared with db/m mice (Fig. 1). Pitavastatin, rosuvastatin, but not pravastatin improved albuminuria in db/db mice. Kidney weights in pitavastatin- and rosuvastatin-treated db/db mice were reduced compared with non-treated db/db mice (Table I). These data suggest that pitavastatin and rosuvastatin treatment improves renal function in db/db mice.

Effect of statin treatment on plasma lipid level in db/db mice. To clarify the mechanism by which statins ameliorated renal function, we first examined the effect of statin treatment on lipid metabolism in db/db mice. Plasma triglyceride and total cholesterol level were increased in non-treated db/db mice compared with db/m mice (Fig. 2A and B). On the other hand,

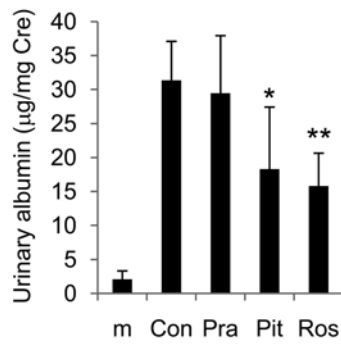


Figure 1. Effect of statins on renal function in db/db mice. The graph shows the urinary excretion of albumin in db/m mice (m), non-treated (Con), pravastatin-treated (Pra), pitavastatin-treated (Pit) and rosuvastatin-treated (Ros) db/db mice. Results are expressed as mean \pm SD. * P <0.05, ** P <0.01 vs. non-treated db/db mice (n=6 in each group).

statin treatment had no effect on plasma lipid levels in db/db mice (Fig. 2A and B), suggesting that the renoprotective effect of statins is independent of their lipid-lowering action.

Effect of statin treatment on insulin resistance in db/db mice. It has been reported that the development of insulin resistance contributes to renal dysfunction (14). Therefore, we next examined the effect of statin treatment on glucose metabolism in db/db mice. Blood glucose level, plasma insulin level, and HOMA-IR were markedly increased in db/db mice compared with db/m mice, indicating an increase in insulin resistance (Fig. 2C-E). Although statin treatment had no effect on plasma glucose, all statins reduced plasma insulin levels, resulting in a decrease in HOMA-IR (Fig. 2C-E). The data suggest that statin treatment improves insulin resistance.

Because hypoadiponectinemia is associated with the development of insulin resistance and kidney disease (15), we examined the effect of statin treatment on plasma adiponectin levels in db/db mice. In non-treated db/db mice, plasma adiponectin levels were decreased compared with db/m mice. Meanwhile, statin treatment had no effect on plasma adiponectin level in db/db mice (Fig. 2F).

Effect of statin treatment on the renal inflammation in db/db mice. Accumulating evidence now indicates that inflammatory mechanisms play a significant role in the development and progression of diabetic nephropathy. Especially, TNF- α is a pleiotropic inflammatory cytokine and has been shown to cause enhanced albumin permeability (16). Therefore, we next examined the effect of statin treatment on inflammation in the kidney of db/db mice. The expression of TNF- α mRNA was increased in the kidney of db/db mice compared with that of db/m mice, whereas statin treatment had no effect on its expression in db/db mice (Fig. 3A). These data suggest that statins had no effect on the inflammatory response in the kidneys of db/db mice.

Effect of statin treatment on the oxidative stress in db/db mice. To examine the effect of statin treatment on oxidative stress, we measured urinary 8-OHdG concentrations in db/db mice. Urinary 8-OHdG levels in non-treated db/db mice were significantly higher than those in db/m mice. Pravastatin and rosuvastatin reduced urinary 8-OHdG levels in db/db mice, whereas pitavastatin had no effect on oxidative stress despite detecting the amelioration of albuminuria (Fig. 3B).

Effect of statin treatment on glomerular hypertrophy in db/db mice. Glomerular hypertrophy is a hallmark in diabetic

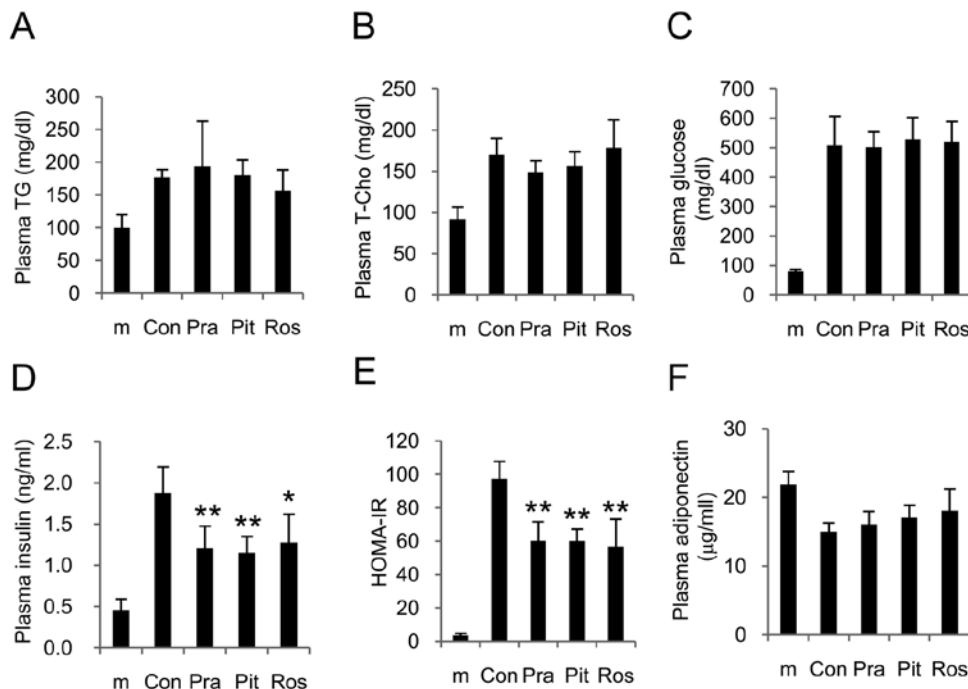


Figure 2. Effect of statins on lipid and glucose metabolism in db/db mice. (A) Plasma triglyceride (TG), (B) total cholesterol (T-Cho), (C) glucose, (D) insulin, (E) HOMA-IR and (F) adiponectin levels in db/m mice (m), non-treated (Con), pravastatin-treated (Pra), pitavastatin-treated (Pit) and rosuvastatin-treated (Ros) db/db mice. Results are expressed as mean \pm SD. * P <0.05, ** P <0.01 vs. non-treated db/db mice (n=6 in each group).

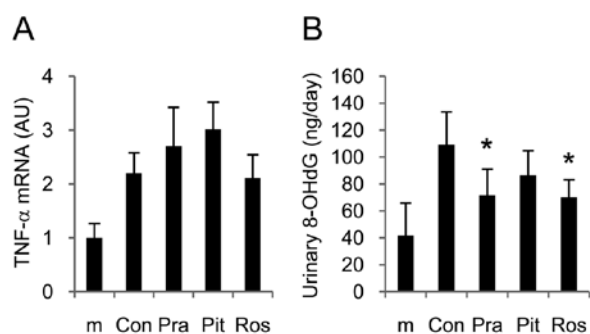


Figure 3. Effect of statins on renal inflammation and oxidative stress in db/db mice. (A) Expression of TNF- α mRNA in whole kidney and (B) urinary 8-OHdG levels in db/m mice (m), non-treated (Con), pravastatin-treated (Pra), pitavastatin-treated (Pit) and rosuvastatin-treated (Ros) db/db mice. Results are expressed as mean \pm SD. * P <0.05 vs. non-treated db/db mice (n =6 in each group).

nephropathy along with albuminuria. Therefore, we assessed the glomerular hypertrophy in db/db mice and the effect of statins by measuring the glomerular surface area. Mean glomerular surface area size in db/db mice was increased compared with db/m mice. Pitavastatin and rosuvastatin treatment, but not pravastatin treatment, suppressed the glomerular hypertrophy as well as urinary excretion of albumin in db/db mice (Fig. 4).

Discussion

In the present study, we showed that pitavastatin and rosuvastatin treatment improved albuminuria and suppressed glomerular hypertrophy, independent of its lipid-lowering and anti-oxidative effect in db/db mice.

In CKD patients, there is an increase in total cholesterol and LDL levels (17). The level of cholesterol is directly correlated with the degree of albuminuria (18), suggesting that hyperlipidemia is associated with the development of CKD such as diabetic nephropathy. In fact, lipid-lowering therapy by statin has been successful to the amelioration of renal function in patients with diabetic nephropathy (19,20). However, the present study and other animal studies showed that statin treatment significantly improved renal function without affecting the plasma lipid profile (5,8,21). Therefore, the renoprotective

effect of statins may be mainly caused by its pleiotropic action rather than their lipid-lowering action.

Insulin resistance is associated with the development of renal dysfunction in type 2 diabetes. It has been shown that insulin resistance correlates with the onset of microalbuminuria in patients with type 2 diabetes as well as in nondiabetic subjects (14). Several studies showed that amelioration of insulin resistance resulted in a restoration of renal function (22-24). Statin also has an ability to ameliorate insulin resistance. Takagi *et al* (25) reported that pravastatin treatment improved insulin resistance through the increase in plasma adiponectin levels in db/db mice. In the present study, we also observed that all statin treatment improved insulin resistance detected by the reduction of HOMA-IR, while adiponectin was not altered by statin treatment in db/db mice. However, this amelioration was not consistent with the renoprotective effects of statins in db/db mice.

Oxidative stress and inflammation are also far more prevalent in CKD patients than in normal subjects (26). In the present study, we also observed the elevation of oxidative stress and inflammation in the kidneys of db/db mice compared with that of lean control mice. Renal disease is associated with a graded increase in oxidative stress markers even in early CKD (27). This oxidative stress can accelerate renal injury progression. In addition, inflammatory markers such as C reactive protein and cytokines increase with renal function deterioration suggesting that CKD is a low-grade inflammatory process (28). Therefore, the agents which have anti-oxidative and anti-inflammatory action have been attracted as a therapeutic strategy for renal dysfunction (29). Anti-oxidative and anti-inflammatory actions are also major pleiotropic effects of statins (12). Several reports have shown that these actions of statins contribute to their renoprotective effects (5,30,31). In the present study, we also observed that pravastatin and rosuvastatin suppressed oxidative stress in db/db mice as well as these reports, whereas we could not detect the anti-inflammatory effect of statins in the kidneys of db/db mice. Pitavastatin had no effect on oxidative stress, despite the presence of the restored renal function in db/db mice. This result suggests that the anti-oxidant action of statins is not primarily responsible for their renoprotective effect.

In the present study, we observed a correlation between the renoprotective effects of statins and their suppressive effect

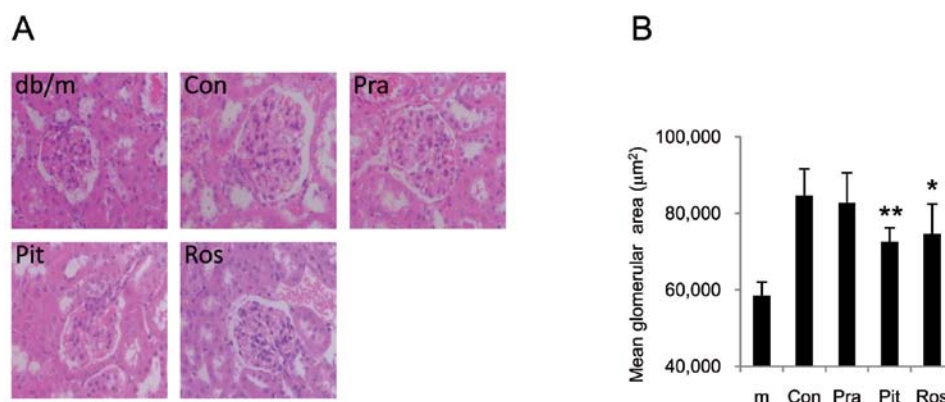


Figure 4. Effects of statins on the glomerular hypertrophy in db/db mice. (A) H&E staining of glomeruli (magnification, x200) and (B) mean glomerular surface area of db/m mice (m), non-treated (Con), pravastatin-treated (Pra), pitavastatin-treated (Pit) and rosuvastatin-treated (Ros) db/db mice. The mean area of fifty glomeruli per mouse was analyzed. Results are expressed as mean \pm SD. * P <0.05, ** P <0.01 vs. non-treated db/db mice (n =6 in each group).

of glomerular hypertrophy in db/db mice. The glomerular morphological changes in diabetic nephropathy are characterized primarily by mesangial expansion and glomerular based membrane (GBM) thickening. It has been reported that the dysregulated cell cycle by the increased inhibitor of cyclin dependent kinase (such as p21 and p27) contributes to these morphological changes and renal dysfunction (32,33). Pleiotropic effects of statins on the cell cycle are well known (12). Furthermore, Danesh *et al* (34) reported that statin treatment normalized the cell cycle through the suppression of p21 expression in high glucose-stimulated mesangial cells. In the present study, pleiotropic effects of statin on the cell cycle thus might improve glomerular hypertrophy and albuminuria. However, further study is required to clarify the effect of statins in glomerular hypertrophy and renal dysfunction.

In conclusion, we have shown the effects of various statins on diabetic nephropathy in db/db mice. Our study suggests that its renoprotective effect is mainly dependent on suppressing the glomerular hypertrophy, independent of its lipid-lowering or anti-oxidative effects, and there may be differences in the renoprotective ability between various statins.

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