Original Article

Renoprotective Effect of Pravastatin in Salt-Loaded Dahl Salt-Sensitive Rats

Makiko KIDO, Katsuyuki ANDO, Shigeyoshi OBA, and Toshiro FUJITA

The pathophysiological features of nephrosclerosis may be analogous to those of atherosclerosis, which is intimately related to lipid metabolism. Thus, we examined whether a lipid-lowering agent, pravastatin, would ameliorate renal damage in hypertensive model animals. Salt-loaded Dahl salt-sensitive (S) rats were given pravastatin (2 mg/ml in drinking water) for 5 weeks. Pravastatin decreased systolic blood pressure. Although pravastatin did not influence the serum total, high-density, or low-density lipoprotein cholesterol, serum triglycerides were decreased. Pravastatin decreased urinary protein excretion and ameliorated histopathological damage in salt-loaded Dahl S rats. Increased urinary excretion of 8-*iso*-prostagaldin $F_{2\alpha}$ and 8-hydroxy-2′-deoxyguanosine and renal superoxide overproduction and decreased reduced glutathione in the renal parenchyma were ameliorated with pravastatin in Dahl S rats fed a high salt diet. Therefore, pravastatin inhibited the progression of renal injury in salt-loaded Dahl S rats, through its antioxidant as well as its depressor effects. (*Hypertens Res* 2005; 28: 1009–1015)

***Key Words*: 3-hydroxy-3-methyl coenzyme A reductase inhibitor, urinary protein, oxidative stress, pleiotropic effect, salt-sensitive hypertension**

Introduction

Low-density lipoprotein (LDL) and oxidized LDL cholesterol, monocytes/macrophages infiltration, and overexpression of cytokines, growth factors, and adhesion molecules have each been proposed to play significant roles in the pathophysiology of both atherosclerosis and nephrosclerosis (*1*). Indeed, lipid abnormality is commonly observed in various renal diseases. For example, renal damage was shown to be exacerbated with high cholesterol in an animal model of chronic renal failure (*2*). Also, renal dysfunction has been ameliorated with lipid-lowering agent 3-hydroxy-3-methyl coenzyme A (HMG-CoA) reductase inhibitors in several animal models (*3–9*). In addition, pravastatin was shown to have a renoprotective effect in patients with moderate renal insufficiency and cardiovascular disease in the Cholesterol and Recurrent Events (CARE) trial (*10*), which also demonstrated that pravastatin was effective for secondary prevention of cardiovascular events (*11*). In animal studies (*3–9*), however, the renoprotective effects of statins were not always associated with amelioration of lipid metabolism. Thus, the protective effect of statins on the kidneys may be attributable to mechanism(s) other than their lipid-lowering action.

Recently, HMG-CoA reductase inhibitors have been postulated to have pleiotropic effects, such as restoration of endothelial function, stabilization of atherosclerotic plaques, reduction of oxidative stress, anti-inflammatory actions, inhibition of thrombosis, suppression of smooth muscle cell proliferation, improvement of insulin sensitivity, and enhancement of vasculogenesis (*12*). Previously, we reported that an antioxidant retarded the progression of renal injury in Dahl salt-sensitive (S) rats (*13*). Similarly, supplementation with vitamins C and E blunted renal damage in experimental renovascular disease (*14*). Indeed, oxidative stress has been proposed to play an important role in the development of

From the Department of Nephrology and Endocrinology, Faculty of Medicine, University of Tokyo, Tokyo, Japan.
Address for Reprints: Katsuyuki Ando, M.D., Department of Nephrology and Endocrinology, Faculty of Medicine, University of Tokyo, 7−3−1, Hongo, Bunkyo-ku, Tokyo 113−8655, Japan. E-mail: katsua-tky@umin.ac.jp
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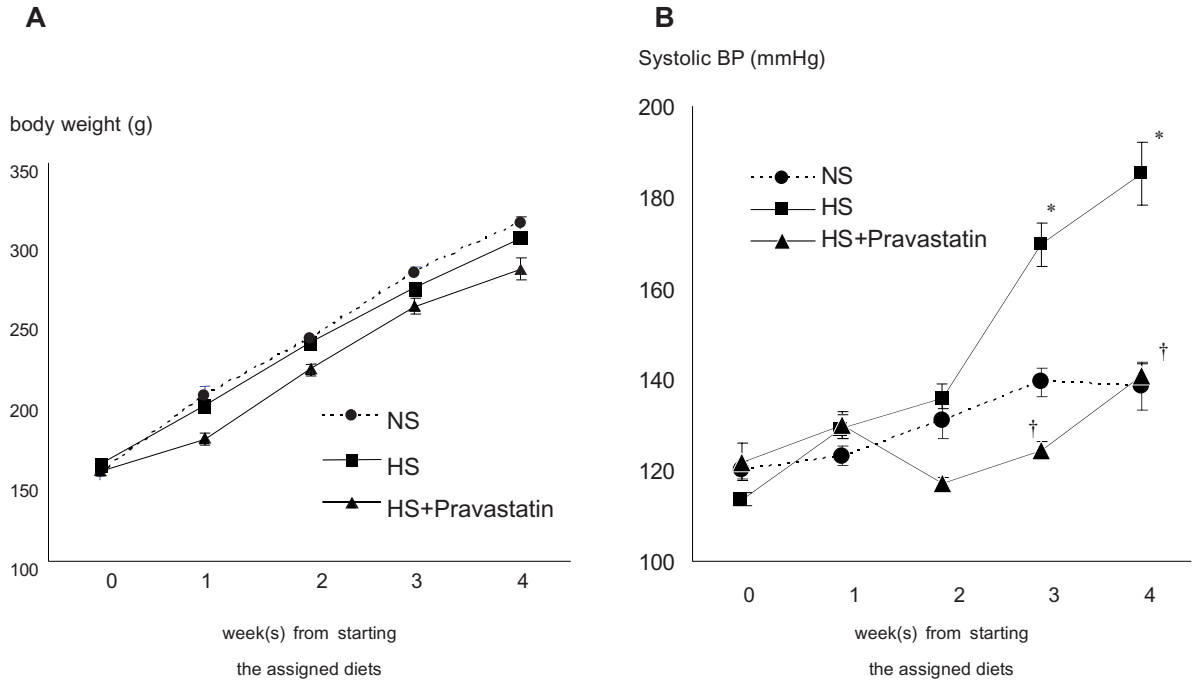

Fig. 1. *Changes in A: body weight and B: systolic blood pressure (BP). NS: normal salt diet; HS: high salt diet; HS+Pravastatin: high salt diet plus pravastatin treatment. Values are expressed as the means±SEM for 5 animals in each group.* *$p < 0.05$ vs. *NS,* †$p < 0.05$ vs. *HS.*

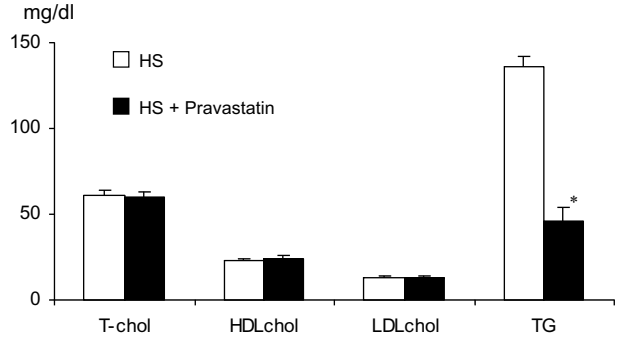

Fig. 2. *Serum total (T-), high-density lipoprotein (HDL), and low-density lipoprotein (LDL) cholesterol, triglycerides (TG). Abbreviations: see legend in Fig. 1.* *$p < 0.05$ vs. *HS.*

renal damage (*15–17*). Therefore, we hypothesized that statins ameliorate the development of hypertensive renal injury through their antioxidant effect. To clarify this viewpoint, salt-loaded Dahl S rats were given pravastatin and their renal function was examined. In addition, we measured the parameters of oxidative stress to evaluate the role of reactive oxygen species (ROS).

Methods

Animal Preparation

Four-week-old male Dahl S rats were purchased from SLC (Shizuoka, Japan). The rats were fed either a high salt diet containing 8% NaCl or a standard laboratory chow containing 0.66% NaCl (Oriental Yeast Co., Tokyo, Japan) for 5 weeks. Some Dahl S rats on a high salt diet were given drinking water containing 2 mg/ml of pravastatin. Food and water were freely available throughout the study. The rats were housed in a room maintained at a constant humidity (60±5%), temperature (23±1°C), and light cycle (0700–1900 h).

Systolic blood pressure (BP) was measured by the tail-cuff method (P-98A; Softron, Tokyo, Japan). Urine was collected for 24 h at the last day of the treatment, and indomethacin (10 mg/ml) was added to the urine samples to inhibit degradation of 8-*iso*-prostaglandin $F_{2\alpha}$ (8-isoprostane). The rats were then anesthetized with sodium pentobarbital (100 mg/kg intraperitoneally) and euthanized after blood samples were obtained from the abdominal vein. The kidneys were excised for histological evaluation and measurement of reduced glutathione. All animal procedures conformed to the guiding principles for animal experimentation as enunciated by the Ethics Committees on Animal Research of the Faculty of Medicine, University of Tokyo, which complies with the guidelines of the National Institute of Health (NIH).

Table 1. Urinary Protein, Serum Creatinine, Urinary 8-Hydroxy-2′-Deoxyguanosine (8OHdG), and Urinary 8-*iso*-Prostaglandin $F_{2\alpha}$ (8-Isoprostane) of Dahl Salt-Sensitive Rats on Normal Salt Diet (NS), High Salt Diet (HS), and High Salt Diet plus Pravastatin Treatment (HS+Pravastatin)

	Urinary protein (mg/day)	Serum creatinin (mg/dl)	8-Isoprostane (ng/day)	8OHdG (ng/day)
NS	2.85±0.14	0.27±0.005	4.46±0.41	255.7±23.0
HS	14.2±0.85*	0.21±0.007	13.5±0.51*	425.0±21.1*
HS+Pravastatin	3.59±0.35†	0.21±0.015	10.4±0.39†	365.8±5.35†

*$p<0.05$ *vs*. NS, †$p<0.05$ *vs*. HS.

Analysis of Serum and Urine

Serum was used for the measurement of total, LDL, and high-density lipoprotein (HDL) cholesterol and triglycerides (enzymatic methods). Urinary protein was evaluated using the Bradford method. Serum and urinary creatinine were measured by Jaffe color reaction. Enzyme immunoassay procedures were used to measure 8-isoprostane following the methods supplied in the kit provided by Assay Designees Inc. (Ann Arbor, USA). Urinary 8-hydroxy-2′-deoxyguanosine (8OHdG) was extracted, purified and measured using an enzyme-linked immunosorbent assay kit (8OHdG Check; Nikken Food Co., Shizuoka, Japan).

Measurement of ROS Production in the Kidneys

The superoxide (O_2^-) production was measured by the lucigenin (bis-*N*-methylacridinium nitrate)-enhanced chemiluminescence method as described previously (*18*, *19*) with some modifications. Briefly, for lucigenin chemiluminescence, the kidney was excised and freed of surrounding tissues. The whole kidney specimen was weighed, minced on dry ice, and homogenized in lysis buffer (PBS containing: 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS, 1 mmol/l PMSF, 10 U/ml aprotinin, and 10 mmol/l sodium orthovanadate), and the mixture was centrifuged. The supernatant was aspirated and kept on ice for free radical measurement. After 5 min of dark adaptation, a kidney sample was placed in a scintillation counter (LB9507; Berthold Technologies, Bad Wildbad, Germany) and measured. Repeated measurements of the specimen were interpreted every 30 s, and the average value was reported over a 5-min period. The final readings were expressed as counts per min per 100 mg kidney weight.

Measurement of Renal Reduced Glutathione

To measure levels of total glutathione, 100 mg of whole kidney tissues were mixed with 2 ml of cold 0.1 mol/l phosphate buffer (pH 7.0) and homogenized for about 1 min. Then, 1 ml of mixture was kept on ice for 10 min after adding 1 ml of 0.6 Eq/l $HClO_4$–1 mmol/l EDTA. The sample was centrifuged and the supernatant was kept frozen at −80°C until assayed. Levels of total glutathione were measured by the enzymatic cycling method using 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) (*20*). To obtain the desired amount of the oxidized form of glutathione (GSSG), whole kidney tissue was homogenized with 20 mmol/l *N*-ethylmaleimide (NEM)–0.1 mol/l phosphate buffer (pH 7). The sample was kept on ice for 10 min, and then 0.6 Eq/l $HClO_4$–1 mmol/l EDTA was added and the mixture was centrifuged. Quantities of GSSG were analyzed as stated above (*20*). Reduced glutathione was gained from subtraction of GSSG from total glutathione and the levels were expressed in μg/ml/100 mg of whole kidney tissue.

Histological Evaluation of the Kidney

Kidneys were excised, fixed in PBS containing 4% paraformaldehyde for 4 to 6 h at room temperature, and then paraffin-embedded. The sections of kidney specimens were stained with hematoxylin and eosin and evaluated microscopically. Histological evaluation was done blindly by one of the authors (S.O.). The degree of tubular injury in terms of tubular dilation and cast formation was graded with an arbitrary score of 0 to 3: grade 0, normal; grade 1, mild; grade 2, moderate; grade 3, severe (*21*, *22*) (there was no tubular epithelial injury or debris accumulation).

Data Analysis

All values are expressed as the mean±SEM from at least 5 independently performed experiments. Data were analyzed by a one-way ANOVA followed by the post hoc test. A value of $p<0.05$ was considered significant.

Results

Body weight gain was not different among non-salt-loaded Dahl S rats, salt-loaded rats without pravastatin, and salt-loaded rats with pravastatin (Fig. 1A). The salt-induced rise in systolic BP was significantly decreased with pravastatin (Fig. 1B). Pravastatin did not affect serum total, HDL, or LDL cholesterol in salt-loaded rats, but decreased the serum triglyceride level (Fig. 2).

Salt loading increased daily urinary protein in Dahl S rats (Table 1). However, pravastatin decreased urinary protein

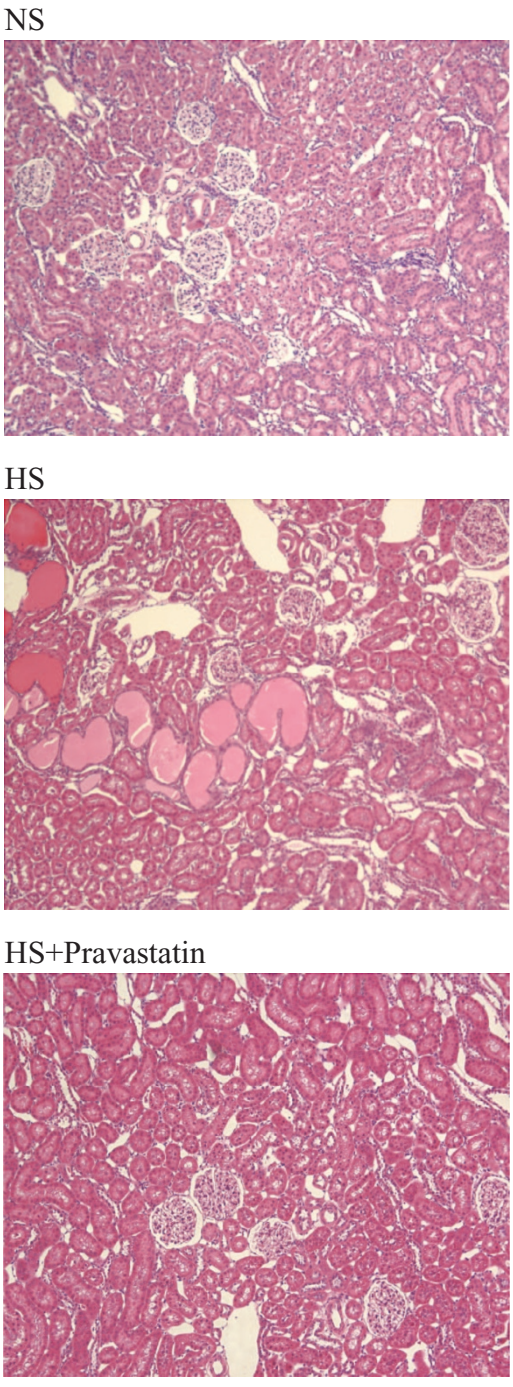

Fig. 3. *Renal histological photographs stained with hematoxylin-eosin (×100). Abbreviations: see legend in Fig. 1.*

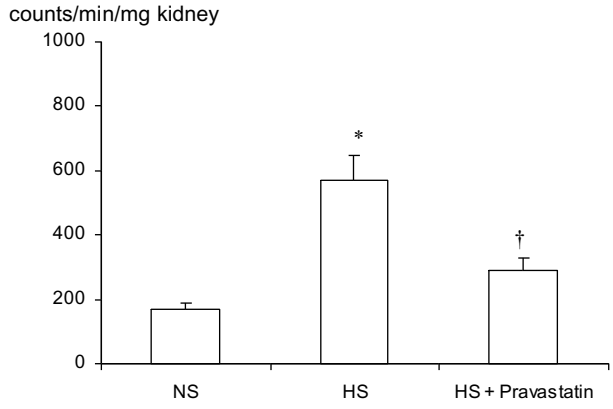

Fig. 4. *Superoxide production from the whole kidney measured by lucigenin chemiluminescence. Abbreviations: see legend in Fig. 1.* *$p < 0.05$ vs. *NS*, †$p < 0.05$ vs. *HS*.

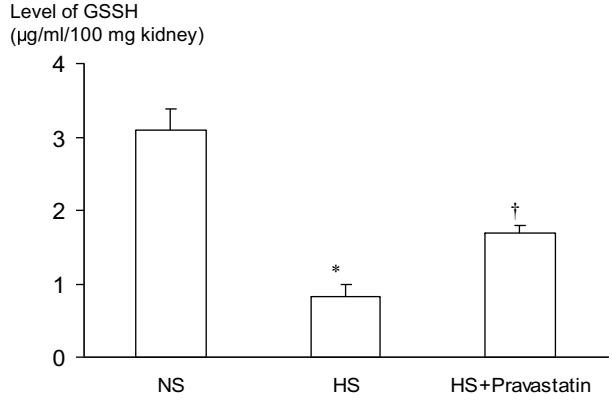

Fig. 5. *Reduced glutathione in the renal parenchyma. Abbreviations: see legend in Fig. 1.* *$p < 0.05$ vs. *NS*, †$p < 0.05$ vs. *HS*.

greatly. Salt loading caused tubular injury in Dahl S rats (morphological severity: 2.5±0.19 *vs*. 0.2±0.2, $p<0.01$) (Fig. 3). However, pravastatin reversed renal damage in salt-loaded Dahl S rats (1.4±0.69, $p<0.05$).

Urinary 8-isoprostane and 8OHdG were increased by salt loading, but pravastatin partially inhibited them (Table 1). Superoxide production from the kidney, as measured by lucigenin chemiluminescence, was also increased by a high salt diet in Dahl S rats, and this increase was reduced with pravastatin (Fig. 4). On the other hand, renal reduced glutathione was decreased with salt loading but normalized with pravastatin (Fig. 5).

Discussion

In the present study, pravastatin treatment normalized urinary protein excretion and tubulointerstitial injury in salt-loaded Dahl S rats. The present data are consistent with the previous findings; HMG-CoA reductase inhibitors including pravastatin have been reported to ameliorate renal injury in several model animals of renal impairment (*3–9*). In addition, pravastatin suppressed renal ROS generation, which was demonstrated by the data of urinary 8-isoprostane, 8OHdG, and superoxide production measured by lucigenin chemilumines-

cence. Thus, the present data suggest that an antioxidant effect of pravastatin plays an important role in renoprotection, because it has been demonstrated that antioxidant intervention prevented renal injury (*13*, *14*). In addition, pravastatin decreased systolic BP in salt-loaded Dahl S rats and, therefore, its antihypertensive effect may also contribute to the renoprotective effect of pravastatin. Because, in salt-loaded Dahl S rats, serum lipid levels were not decreased with pravastatin, except in the case of serum triglycerides, the lipid-ameliorating effect could play a minor role in its renoprotective action.

Our data suggest an antioxidant effect of pravastatin. Renal reduced glutathione, an ROS-eliminating substance, was also normalized by pravastatin in the present study. This change in renal reduced glutathione levels can be explained as a result of the reduced ROS generation by pravastatin. Indeed, pravastatin decreased plasma lipid peroxide in patients with hypercholesterolemia (*23*), and treatment with pravastatin as well as atorvastatin decreased NADPH oxidase activity in the right atrial myocardium in patients undergoing elective cardiac surgery for coronary artery bypass grafting (*24*). Because hypercholesterolemia has been demonstrated to increase endothelial ROS generation (*25*), the lipid-lowering effect of pravastatin may contribute to its antioxidant effect. However, the antioxidant effect of HMG-CoA reductase inhibitors has been shown to be independent of the triglyceride-lowering action (*26*) and, in Dahl S rats in the present study, LDL cholesterol was low and unaffected by pravastatin. *In vitro* as well as *in vivo* studies revealed that HMG-CoA reductase inhibitors directly inhibited ROS generation by reducing prenylation and translocation of rac1 (*24*, *27*), and by reducing the expression of NAD(P)H oxidase subunits and scavenging free radical molecules (*28*). However, further experiments will be needed to clarify the mechanism(s) of the antioxidant effect of pravastatin.

Pravastatin almost completely suppressed a rise in systolic BP in Dahl S rats with salt loading in the present study. Decreased BP may also contribute to the amelioration of renal damage in salt-loaded Dahl S rats, since BP control has been shown to play a critical role in renoprotection (*29*). However, BP reduction could not account for the entire renoprotective effect of pravastatin, since a lower dose of pravastatin than that used here was reported to decrease urinary protein and ameliorate renal injury in salt-loaded Dahl S rats without BP changes (*5*).

The depressor effect of pravastatin in the present study cannot be due to its toxicity, because body weight gain was not different between salt-loaded Dahl S rats with and those without pravastatin treatment. In a study by Wilson *et al*., pravastatin decreased mean BP in salt-loaded Dahl S rats when it was started concomitantly with salt loading, but not when it was started 2 weeks after the start of salt loading (*5*). Also, pravastatin was shown to decrease BP in spontaneously hypertensive rats (SHR) (*9*). The antihypertensive effect of HMG-CoA reductase inhibitors may be due to an improvement in endothelial function (*30*). Indeed, a higher dose of statin was shown to promote endothelial NO synthase protein expression (*31*). Moreover, statin inhibited the development of nitric oxide (NO)-deficient hypertension (*32*). On the other hand, an HMG-CoA reductase inhibitor was also reported to greatly suppress an angiotensin II-induced increase in BP to 67%, possibly by blunting the pressor effect of angiotensin II (*33*). Pravastatin-ameliorated insulin resistance (*34*) may also lead to a depressor effect of pravastatin, as proposed for the antihypertensive effect of fibrate (*35*). However, BP was not normalized by pravastatin in the previous studies. The greater reduction in BP in the present study may be due to the difference in the dose of pravastatin used: the dose was several times greater in the present study compared with Wilson's study (*5*). In addition, they started pravastatin administration at 8 weeks of age (*5*), while we started it at 4 weeks of age. The delay in starting the salt loading results in a less extreme rise in systolic BP and slower development of hypertension in Dahl S rats (*36*). In contrast, earlier treatment with a high dose of statin might cause a greater reduction in BP as in the present study, because NO deficiency (*37*), activation of the tissue renin-angiotensin system (*38*), and insulin resistance (*39*) are pathophysiological characteristics in salt-induced hypertension in Dahl S rats. However, further studies will be needed to confirm this hypothesis.

The lipid-lowering effect of pravastatin may play a minor role in renoprotection because pravastatin did not affect serum total, HDL, or LDL cholesterol. Moreover, the lower levels of triglycerides do not suggest a pathophysiological role of lipid metabolism in the development of renal injury with salt loading. It remains uncertain why pravastatin decreased only the serum triglyceride levels in salt-loaded Dahl S rats in the present study. In agreement with our data, a previous study reported that pravastatin decreased serum triglycerides but did not affect serum total or HDL cholesterol in SHR (*9*). The same influence of lipid metabolism has been reported in simvastatin (*9*) and cerivastatin (*40*). The different effect of HMG-CoA reductase inhibitors in lipids might be due to the relatively lower levels of serum total, HDL, and LDL cholesterol and the relatively higher level of triglycerides.

In conclusion, pravastatin prevented the development of renal injury in salt-loaded Dahl S rats, in association with an antioxidant effect in the kidney and BP reduction. Thus, HMG-CoA reductase inhibitors may have a beneficial effect on kidney function beyond their lipid-ameliorating action.

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