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Ezetimibe alone or in combination with pitavastatin prevents kidney dysfunction in 5/6 nephrectomized rats fed high-cholesterol

*Yusaku Mori, Tsutomu Hirano**

Department of Diabetes, Metabolism, and Endocrinology, Showa University School of Medicine, Tokyo, Japan

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ABSTRACT

We attempted to elucidate the relationship between cholesterol absorption and kidney damage by investigating the renoprotective effect of ezetimibe, a cholesterol absorption inhibitor, in 5/6 nephrectomized rats (Nx). The Nx or sham-operated rats (Sham) were fed 1% high-cholesterol diet (HC) containing ezetimibe (10 mg/[kg d]), pitavastatin (3 mg/[kg d]), or both for 8 weeks. Pathological changes, endothelial nitric oxide synthase (eNOS) messenger RNA (mRNA), and oxidative stress were assessed in the kidney. The Sham fed HC exhibited hypercholesterolemia and glomerulosclerosis with macrophage infiltration in the kidney, and ezetimibe attenuated these changes. The Nx exhibited hypercholesterolemia, increased urinary 8-hydroxy-2′-deoxyguanosine (8-OHdG), glomerulosclerosis with macrophage infiltration and interstitial fibrosis, and downregulation of eNOS mRNA. The HC increased cholesterol further and worsened the kidney damage with increased 8-OHdG. Ezetimibe attenuated the hypercholesterolemia, kidney dysfunction, and pathological changes. The beneficial effects of ezetimibe were significantly associated with reduced 8-OHdG ($P < .01$). Pitavastatin did not reduce cholesterol or 8-OHdG, but it did significantly suppress the kidney damage with upregulated eNOS mRNA by 2.5-fold ($P < .02$). The combination of ezetimibe and pitavastatin synergistically ameliorated the kidney damage. The kidney dysfunction and pathological changes were *significantly* associated with cholesterol, markers of cholesterol absorption (campesterol and cholestanol), and 8-OHdG ($P < .001$-$.05$). Multiple regression analysis revealed that the markers of cholesterol absorption were independently associated with the kidney damage. Ezetimibe confers renoprotective effects by inhibiting cholesterol absorption, which in turn reduces oxidative stress; and pitavastatin additively ameliorates kidney damage by increasing NO production via mechanisms independent of cholesterol reduction.

1. Introduction

It remains uncertain whether the secondary dyslipidemia caused by chronic kidney disease (CKD) worsens primary kidney damage [1]. The Atherosclerosis Risk in Communities study [2] prospectively demonstrated that elevated serum creatinine is associated with the baseline levels of plasma cholesterol. A recent meta-analysis revealed that treatment

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* *Corresponding author*. Tel.: +81 3 3784 8947; fax: +81 3 3784 8948.
E-mail address: hirano@med.showa-u.ac.jp (T. Hirano).

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with statin, an inhibitor of cholesterol biosynthesis, can reduce urinary albumin exertion and prevent the decline of the estimated glomerular filtration rate (eGFR) in hypercholesterolemic subjects [3,4]. In the Treating to New Targets study subanalysis, a high dose of atorvastatin, one of the strong statins in common use, went so far as to increase eGFR in CKD populations [5]. These results suggest that cholesterol lowering ameliorates kidney damage in CKD. The statins have well-known anti-inflammatory and immunomodulating properties beyond their cholesterol-lowering effects. It remains to be determined whether the renoprotective effects of statins are mainly attributable to their cholesterol-lowering effects or pleiotropic effects. Several experimental studies performed to demonstrate the renoprotective effects of statins [6,7] have revealed that statins exert powerful effects of improving kidney dysfunction and preventing pathological changes of the kidney in animal models of CKD [8,9]. Unlike humans, rodents treated with statins do not manifest a cholesterol-lowering response [10,11]. As such, the renoprotective effects of statins in rodents can be attributed solely to the pleiotropic action.

Accumulating evidence suggests that a high-cholesterol diet (HC) exacerbates kidney damage in animal models of kidney disease [12,13]. However, it remains largely unknown how dietary cholesterol affects pathological and physiological changes in the kidney. Ezetimibe is a new, widely used cholesterol-lowering agent that works by inhibiting cholesterol absorption in the intestine through the antagonistic action of a cholesterol transporter, Niemann-Pick C1 like 1 protein [14-16]. Our group speculated that ezetimibe may be a useful tool to elucidate the mechanisms of dietary cholesterol–induced nephrotoxicity. The 5/6 nephrectomy (Nx) model is one of the most widely used models of CKD without drug- or immune-mediated nephritis. The Nx rats typically manifest tubulointerstitial cell injury with interstitial fibrosis, followed by glomerular injury with focal segmental glomerulosclerosis [17-20]. In this study, we investigated how ezetimibe-modulated cholesterol absorption is associated with kidney function and pathological changes of the kidney in normal and Nx rats fed HCs. We also studied the effects of pitavastatin on kidney function and histological changes in the remnant kidney of the cholesterol-fed Nx rats for comparison with the effects of ezetimibe on the kidney.

2. Materials and methods

2.1. Experimental protocol

Male Wistar rats (Sankyo Labo Service, Tokyo, Japan) weighing approximately 200 g were kept in individual cages on a rotating 12-hour light-dark cycle with free access to water and diet. The rats underwent a 2-step 5/6 nephrectomy beginning with a right nephrectomy under anesthesia with sodium pentobarbital followed by removal of the upper and lower poles of the left kidney [21]. The Sham rats underwent a laparotomy only. Both groups were given 2 weeks to recover after the operation. In experiment 1, the Sham rats were randomly assigned into 3 groups (n = 8, each): normal chow, HC, and HC plus ezetimibe (Merck, Whitehouse Station, NJ). In experiment 2, the Nx rats were randomly divided into 5 groups (n = 9, each): normal chow, HC, HC plus ezetimibe, HC plus pitavastatin (Kowa Pharmaceutical, Aichi, Japan), and HC plus the combination of ezetimibe and pitavastatin. The normal chow consisted of 54.7% vegetable starch, 3.9% fat (0.02% cholesterol), and 18.8% protein. The HC consisted of the normal chow plus 1% cholesterol with 0.5% colic acid (Oriental Yeast, Tokyo, Japan). The agents were administered in powdered chow at a dose of 10 mg/(kg d) in the ezetimibe-treated groups [22,23] and 3 mg/(kg d) in the pitavastatin-treated groups [24,25] based on estimations of the average food intake per day. The urine volume was measured, and urine specimens were collected from rats in individual metabolic cages over 3 consecutive 24-hour periods from day 49 to day 52 after the start of treatment. The systolic blood pressure (SBP) and heart rate were measured by the tail-cuff method with a pulse transducer (Model MK-2000ST; Muromachi Kikai, Tokyo, Japan), and the animals were killed under pentobarbital anesthesia after an 8-hour fast on day 56. Trunk blood was collected, and the remnant kidney (or the left kidney in the Sham group) was removed. Half of each kidney was fixed in 10% phosphate-buffered formalin for microscopic evaluation of renal pathology. The rest was soaked in ISOGEN reagent (Nippon Gene, Tokyo, Japan) and stored at –80°C for evaluation of messenger RNA (mRNA) expression. All animal procedures were conducted according to the *Guide for the Care and Use of Laboratory Animals* of Showa University and approved by the Committee for the Care and Use of Animals of the same institution.

2.2. Measurement blood and urine samples

Serum obtained from centrifuged blood samples was used for measurement of total cholesterol, high-density lipoprotein cholesterol (HDL-C), triglyceride, total protein, creatinine (Cr), and creatine kinase with standard commercial kits (Wako Pure Chemical Industries, Osaka, Japan). Urinary total protein and Cr were measured in 3 consecutive days and shown as the average values. Creatinine clearance (CCr) was calculated as follows: {[urine volume (milliliters per minute) × urine Cr]/serum Cr}/body weight (kilograms). Non–high-density lipoprotein cholesterol was determined by subtracting HDL-C from total cholesterol. Markers of cholesterol absorption (campesterol and cholestanol) were measured in serum by high-performance liquid chromatography procedure (GC-2010; Shimazu, Kyoto, Japan). 8-Hydroxy-2′-deoxyguanosine (8-OHdG) was measured in the urine by the enzyme-linked immunosorbent assay kit (NIKKEN SEIL, Tokyo, Japan) [26].

2.3. Renal histological and immunohistochemical examination

Coronal sections of the kidney were cut at thicknesses of 3 to 4 mm and embedded in paraffin. Sections of 2 to 3 μm in thickness were stained with periodic acid Schiff for light microscopic evaluation of glomerulosclerosis; stained with Masson trichrome for evaluation of interstitial fibrosis; and immunostained for CD68, a macrophage marker, for

Table 1 – Measurements of sham-operated rats fed normal chow, HC, or HC with ezetimibe (EZE)

	Sham	Sham + HC	Sham + HC + EZE
Number	8	8	8
BW (g)	304 ± 13	320 ± 9a	290 ± 13b
Diet intake (g/d)	20.6 ± 1.1	22.6 ± 2.7	22.3 ± 3.4
HR (/min)	413 ± 22	440 ± 25	432 ± 32
SBP (mm Hg)	120 ± 11	117 ± 9	122 ± 13
TP (g/dL)	6.6 ± 0.1	7.5 ± 0.2a	7.3 ± 0.3a
CK (IU/L)	587 ± 176	363 ± 103a	283 ± 77a

Values are means ± SD. Significance ($P < .05$): a, vs sham; b, vs sham + HC. EZE indicates ezetimibe; BW, body weight; HR, heart rate; TP, total protein; CK, creatine kinase.

evaluation of macrophage infiltration into glomeruli. Glomerulosclerosis was quantified by the glomerulosclerosis index (GSI) [27]. One hundred glomeruli per animal was graded as 0 (absent), 1 (<25%), 2 (25%-50%), 3 (51%-75%), or 4 (>75%). The GSI was calculated as follows: (N1 × 1 + N2 × 2 + N3 × 3 + N4 × 4)/n, where N1, N2, N3, and N4 represented the numbers of glomeruli assessed as grades 1, 2, 3, and 4, respectively, and n was the total number of glomeruli assessed. Interstitial fibrosis was quantified by the interstitial fibrosis score (IFS) [28]. Five contiguous fields at the corticomedullary junction were digitally photographed at ×4 magnification. The area of fibrosis, including the interstitial cell infiltrate and atrophic tubules, was measured as a percentage of the total area using Image J software (National Institutes of Health, Bethesda, MD) and expressed as the average of 5 fields per animal.

The immunostaining was performed as follows: Sections deparaffinized in xylene and rehydrated in 100% ethanol were blocked with 3% H_2O_2 in phosphate-buffered saline for 20 minutes and 3% bovine serum albumin in phosphate-buffered saline for 30 minutes. After the blocking, the sections were incubated with a mouse anti-rat CD68 monoclonal antibody (Millipore, Billerica, MA) and treated with horseradish peroxidase–conjugated goat anti-mouse IgG (Sigma-Aldrich, Tokyo, Japan) as the secondary antibody. The immune complex was detected with 3,3′-diaminobenzidine tetrahydrochloride (Sigma-Aldrich). Macrophage infiltration was quantified by counting the numbers of CD68-positive cells in the glomeruli and expressed as the average of 50 glomeruli per animal. All of the analyses were performed twice on the same sections by 2 investigators with no knowledge of the groups, and the averages from each analysis were used.

2.4. *RNA extraction and quantitative real-time reverse transcription polymerase chain reaction analysis*

Total RNA was extracted from the kidney homogenates using ISOGEN (Nippon Gene, Tokyo, Japan) reagent according to the manufacturer's protocol. First-strand complementary DNA was synthesized with a TaqMan High Capacity cDNA Reverse Transcription Kit. Gene expression was determined by real-time reverse transcription polymerase chain reaction using TaqMan Gene Expression Assays (Life Technologies, Carlsbad, CA) and an ABI PRISM 7000 Sequence Detection System (Life Technologies) on endothelial nitric oxide synthase (eNOS; assays ID, Rn02132634_s1), transforming growth factor–β

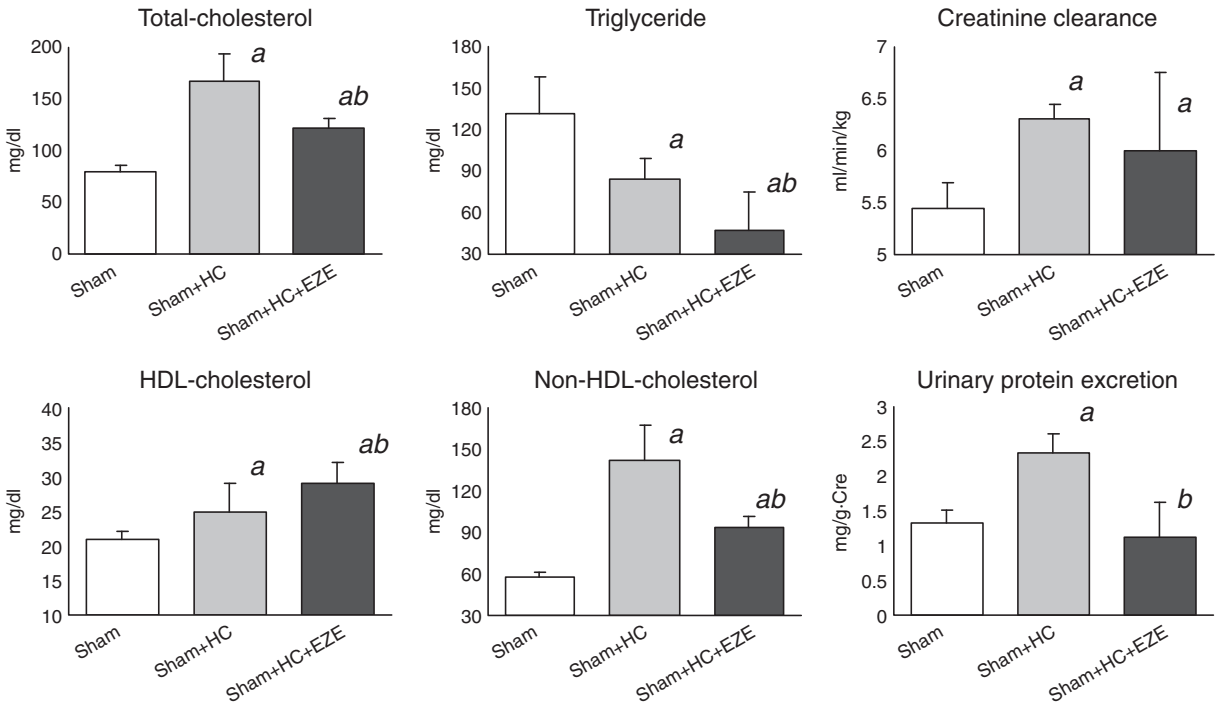

Fig. 1 – Serum lipids and kidney functions in Sham fed normal chow or an HC (1% cholesterol with 0.5% colic acid) alone or with ezetimibe (10 mg/[kg d]). Kidney functions were assessed by CCr and urinary protein excretion. Significance ($P < .05$): a, vs sham; b, vs sham + HC. Each group consisted of 8 animals. EZE indicates ezetimibe.

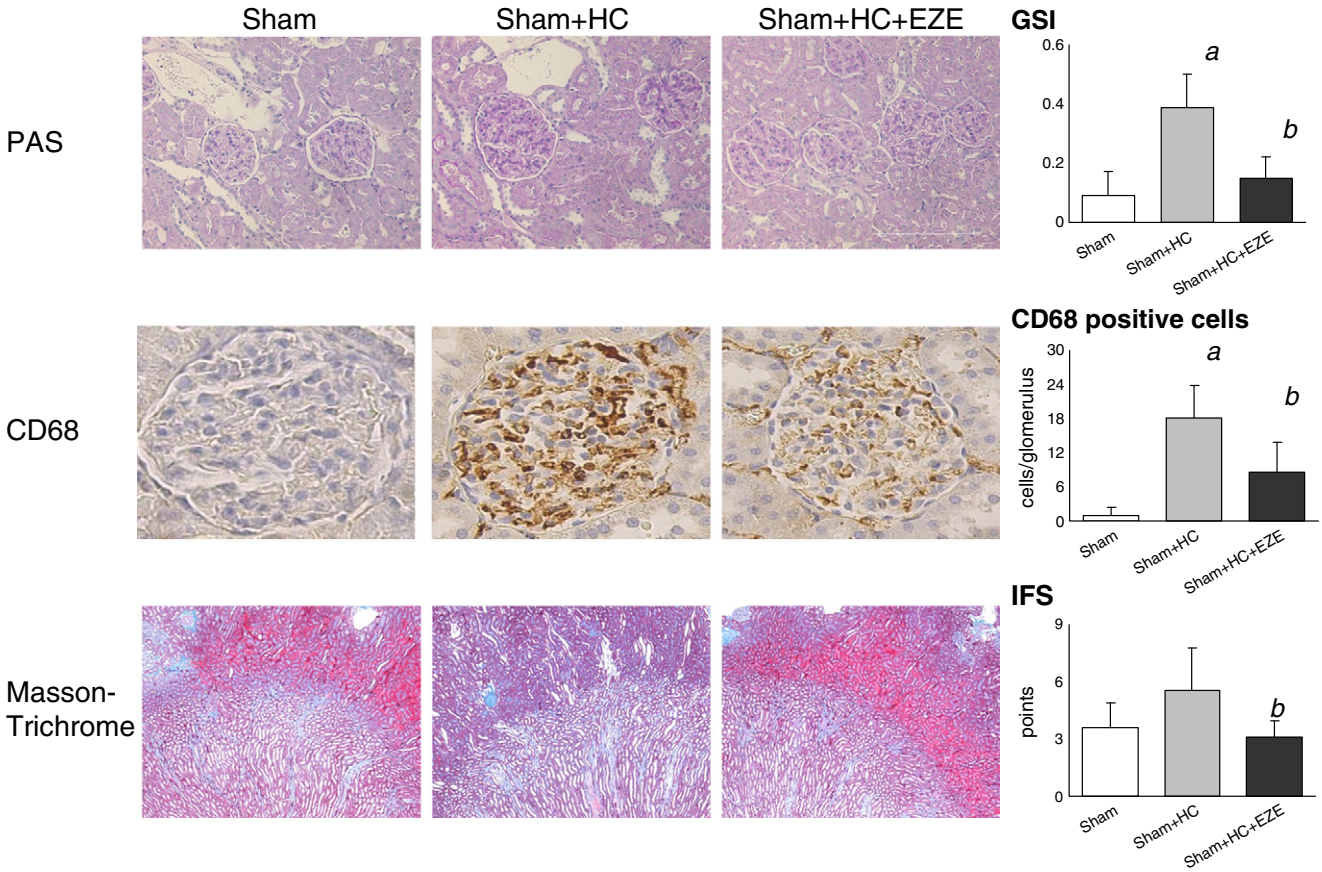

Fig. 2 – Renal histology in Sham fed normal chow or HC without or with EZE. Periodic acid Schiff staining of glomerulus (magnification, ×20), CD68-positive cells of glomerulus (magnification, ×40), Masson trichrome staining of corticomedullary junction (magnification, ×4). Significance ($P < .05$): a, vs sham; b, vs sham + HC. The number of each group is 8.

(TGF-β; assays ID, Rn00821748_g1), and connective tissue growth factor (CTGF; assays ID, Rn01537278_g1) according to the procedure described previously [29,30].

2.5. *Analysis of data*

Analysis of variance, followed by post hoc Tukey-Kramer multiple comparison, was used to test the significance of differences between groups. Relationships between 2 variables were tested by Pearson correlation coefficient, and multiple regression analyses were performed to determine independent variables for kidney damage. Statistical significance was accepted at $P < .05$. The procedures were carried out using SPSS software (release 15.0; SPSS, Chicago, IL). Results are given as means ± standard deviation (SD).

3. Results

Table 1 lists various measurements in experiment 1. The final body weight was somewhat increased by the HC, whereas coadministration of ezetimibe rectified the excess body

Table 2 – Measurements of Nx fed normal chow or HC alone, or HC with pitavastatin (PTV), with EZE, or with both

	Nx	Nx + HC	Nx + HC + EZE	Nx + HC + PTV	Nx + HC + PTV + EZE
n	9	9	9	9	9
BW (g)	299 ± 15	279 ± 16	267 ± 22	293 ± 20	273 ± 17
Diet intake (g/d)	22.5 ± 3.5	21.6 ± 3.4	21.4 ± 3.2	23.9 ± 2.2	24.7 ± 4.9
HR (/min)	474 ± 15	460 ± 20	449 ± 33	458 ± 34	457 ± 38
SBP (mm Hg)	123 ± 9	166 ± 44a	140 ± 13	148 ± 16	128 ± 15b
TP (g/dL)	6.3 ± 0.1	6.8 ± 0.3a	6.7 ± 0.3a	6.5 ± 0.3b	6.3 ± 0.1bc
CK (IU/L)	416 ± 199	586 ± 150	579 ± 336	638 ± 385	669 ± 422

Values are means ± SD. Significance ($P < .05$): a, vs Nx; b, vs Nx + HC; c, vs Nx + HC + EZE; d, Nx + HC + PTV.

weight. Diet intake, pulse, and SBP were comparable between the Sham rats fed normal chow, HC, and HC plus ezetimibe. Cholesterol feeding increased serum total protein levels and decreased creatine kinase, whereas ezetimibe had no effect on these levels. Fig. 1 shows serum lipids and kidney functions in experiment 1. The HC increased the total cholesterol, HDL-C, and non–HDL-C levels, but decreased triglyceride levels. Ezetimibe ameliorated the hypercholesterolemia and further decreased the triglyceride levels. Cholesterol feeding had no deleterious effect on kidney function, but it increased the urinary protein excretion. Ezetimibe attenuated the dietary cholesterol–induced proteinuria.

Fig. 2 demonstrates pathological changes in the kidney in experiment 1. The HC led to glomerulosclerosis and macrophage infiltration in the Sham rats. Cholesterol feeding tended to promote interstitial fibrosis. Ezetimibe remarkably attenuated these pathological changes induced by cholesterol feeding.

Table 2 lists various measurements in experiment 2. The final body weights, food intake, and heart rates were comparable between the 5 Nx groups. The SBP was significantly increased by HC in the Nx rats, and the hypertension was attenuated by the combination of pitavastatin and ezetimibe. Creatine kinase levels were comparable between the 5 groups. Fig. 3 shows serum lipids and kidney functions in experiment 2. The non–HDL-C levels were twice as high in the Nx rats than in the Sham rats. The Nx rats exhibited significantly decreased CCr and proteinuria. The HC brought about severe hypercholesterolemia, moderate hypertriglyceridemia, deteriorated kidney function, and marked proteinuria in the Nx rats, whereas ezetimibe attenuated the hypercholesterolemia, hypertriglyceridemia, and kidney dysfunction. Pitavastatin slightly decreased total cholesterol and non–HDL-C levels, but significantly ameliorated kidney dysfunction and proteinuria in the cholesterol-fed Nx rats. The combination therapy group showed a lipid profile similar to the ezetimibe-alone group, but the combination therapy further increased CCr over each agent administered alone. Fig. 4 demonstrates pathological changes in the kidney in experiment 2. Glomerulosclerosis with macrophage infiltration was observed in the remnant kidney of the Nx group, and the HC doubled the GSI and increased CD68-positive cells in the glomeruli. Interstitial fibrosis was observed in the remnant kidney, and the HC exacerbated the IFS. Ezetimibe substantially decreased the GSI, CD68-positive area, and IFS. Pitavastatin also significantly suppressed the glomerulosclerosis, CD68-positive cell number, and interstitial fibrosis. The combination of ezetimibe and pitavastatin substantially attenuated the GSI and IFS to levels comparable to those observed in the chow-fed Nx group and nearly eliminated the CD68-positive area altogether.

Fig. 5 shows the levels of serum campesterol and cholestanol (markers of cholesterol absorption), urinary 8-OHdG (a marker of oxidative stress), and eNOS mRNA in the kidneys

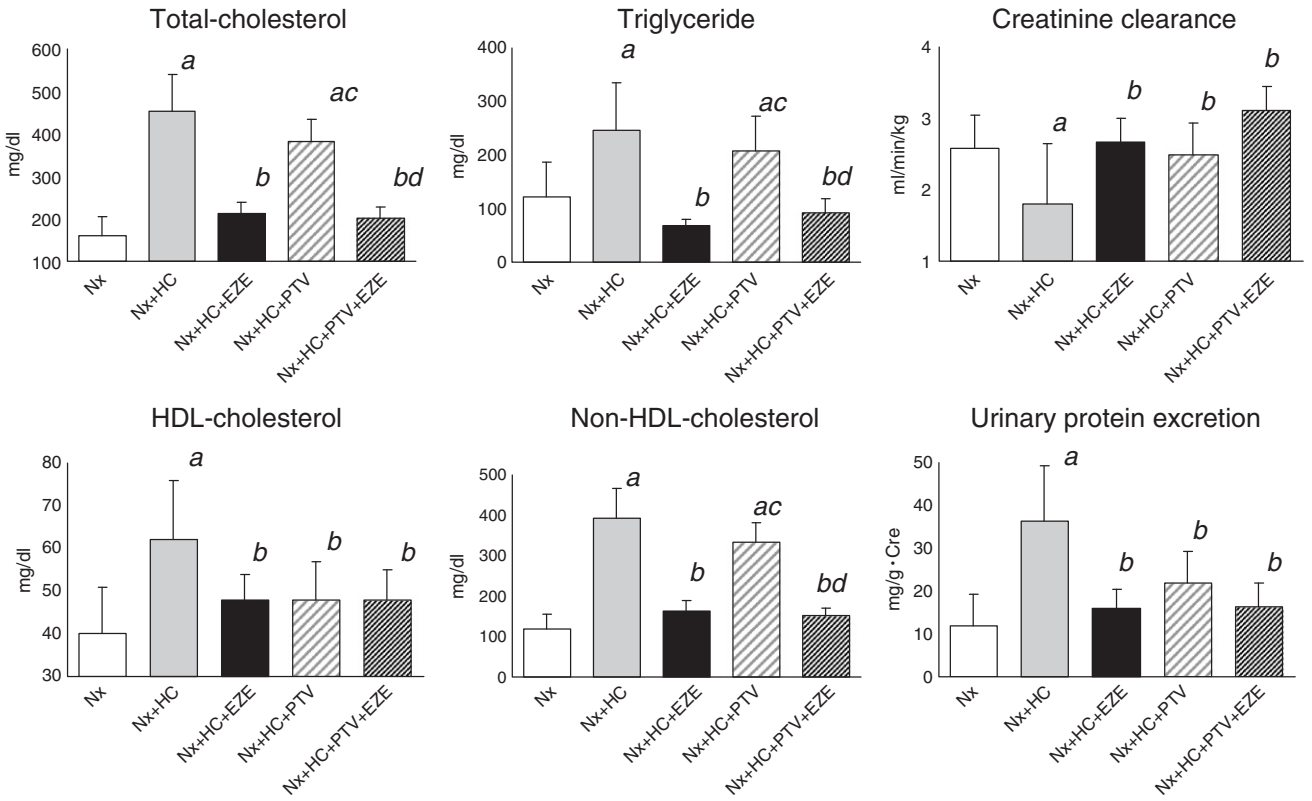

Fig. 3 – Serum lipids and kidney functions in Nx fed normal chow or HC alone, or HC with EZE, with pitavastatin (PTV), or both (EZE + PTV). The rats underwent a 2-step 5/6 nephrectomy consisting of a right nephrectomy followed by the removal of the upper and lower poles of the left kidney. Significance ($P < .05$): a, vs Nx; b, vs Nx + HC; c, vs Nx + HC + EZE; d, Nx + HC + PTV. Each group consisted of 9 animals.

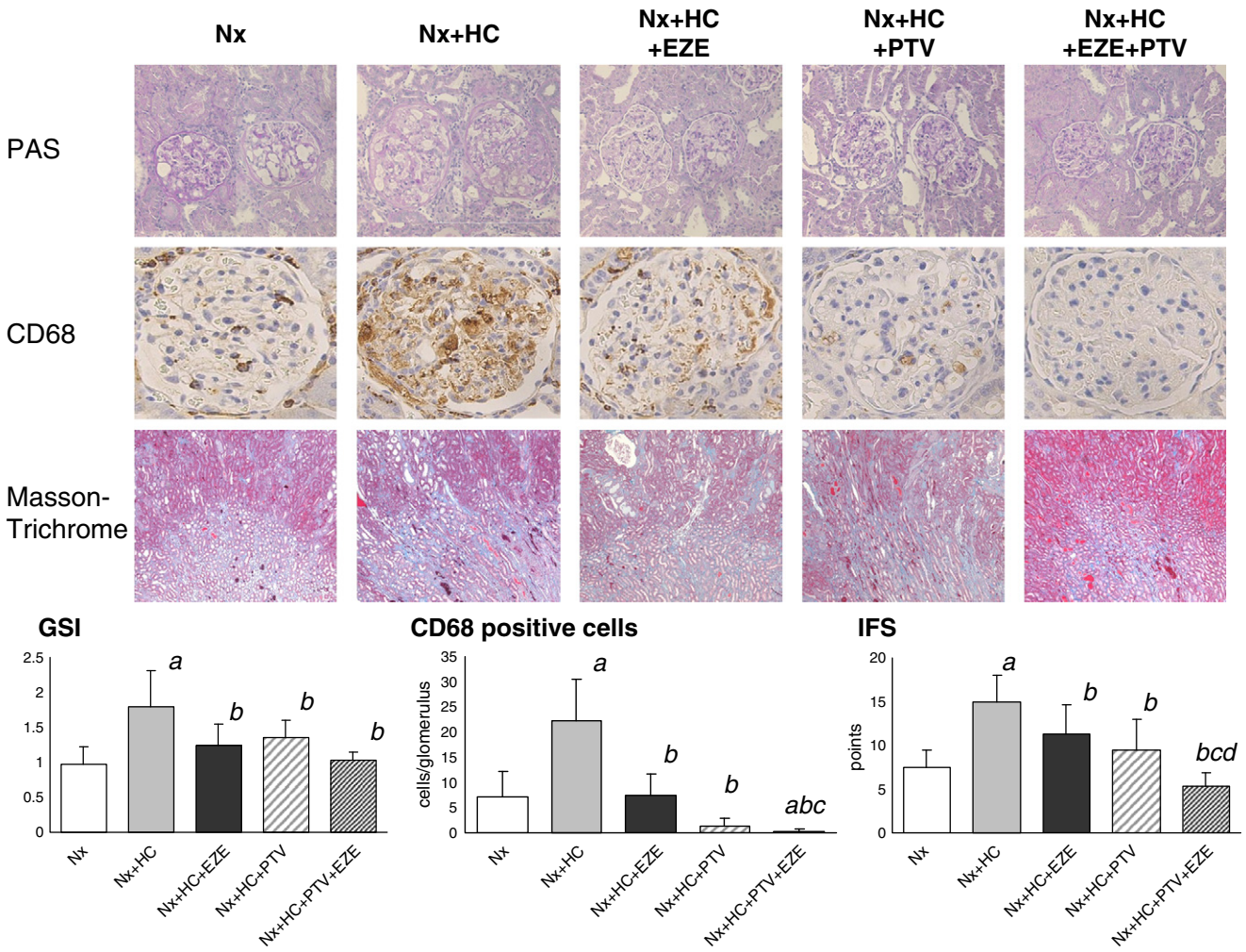

Fig. 4 – Renal histology in Nx fed normal chow or HC alone or with EZE, PTV, or both (EZE + PTV). Significance ($P < .05$): a, vs Nx; b, vs Nx + HC; c, vs Nx + HC + EZE; d, Nx + HC + PTV. The number of each group is 9.

of the Sham (upper panel) and Nx (lower panel) rats. In the Sham group, cholesterol feeding decreased campesterol but increased cholestanol, whereas ezetimibe decreased campesterol but left cholestanol unchanged. Cholesterol feeding increased 8-OHdG, and ezetimibe completely attenuated the increase. Cholesterol feeding and ezetimibe both left the expression of eNOS mRNA essentially unchanged.

The Nx group had significantly higher levels of campesterol and cholestanol than the Sham group (36.6 ± 7.0 vs 27.6 ± 1.9μg/mL and 3.89 ± 1.39 vs 2.14 ± 0.19μg/mL, respectively; $P <$ .05). Cholesterol feeding in the Nx group substantially increased the cholestanol but left campesterol unchanged. Ezetimibe significantly reduced both campesterol and cholestanol. Pitavastatin decreased campesterol slightly, but had no effect on cholestanol. The levels of campesterol and cholestanol in the rats treated with pitavastatin and ezetimibe combined were the same as those in the animals treated with ezetimibe alone.

Urinary 8-OHdG excretion was substantially increased in the Nx group compared with the Sham group (765 ± 355 vs 229 ± 80 ng/d, $P < .0001$). The HC doubled 8-OHdG excretion, and ezetimibe completely rectified this effect. Pitavastatin did not reduce urinary 8-OHdG, and the combination of ezetimibe and pitavastatin did not further decrease 8-OHdG compared with ezetimibe alone. The gene expression of eNOS in the kidney was severely downregulated in the Nx group compared with the Sham group. Cholesterol feeding and ezetimibe both left eNOS mRNA expression unchanged. In contrast, pitavastatin increased eNOS mRNA 3-fold, and the combination of ezetimibe and pitavastatin increased eNOS mRNA 6-fold, in the remnant kidney. Campesterol and cholestanol levels were closely associated with 8-OHdG (r = 0.533 and 0.730, respectively; $P < .001$), but not with eNOS mRNA.

Fig. 6 shows the mRNA expressions of TGF-β and CTGF in the kidneys of Sham and Nx rats treated with HC or HC with EZE. The gene expressions of TGF-β and CTGF were significantly increased in the Nx rats but showed no significant changes in response to manipulations of dietary cholesterol or EZE.

Table 3 lists the correlation of kidney damage with various parameters. Simple regression analysis revealed

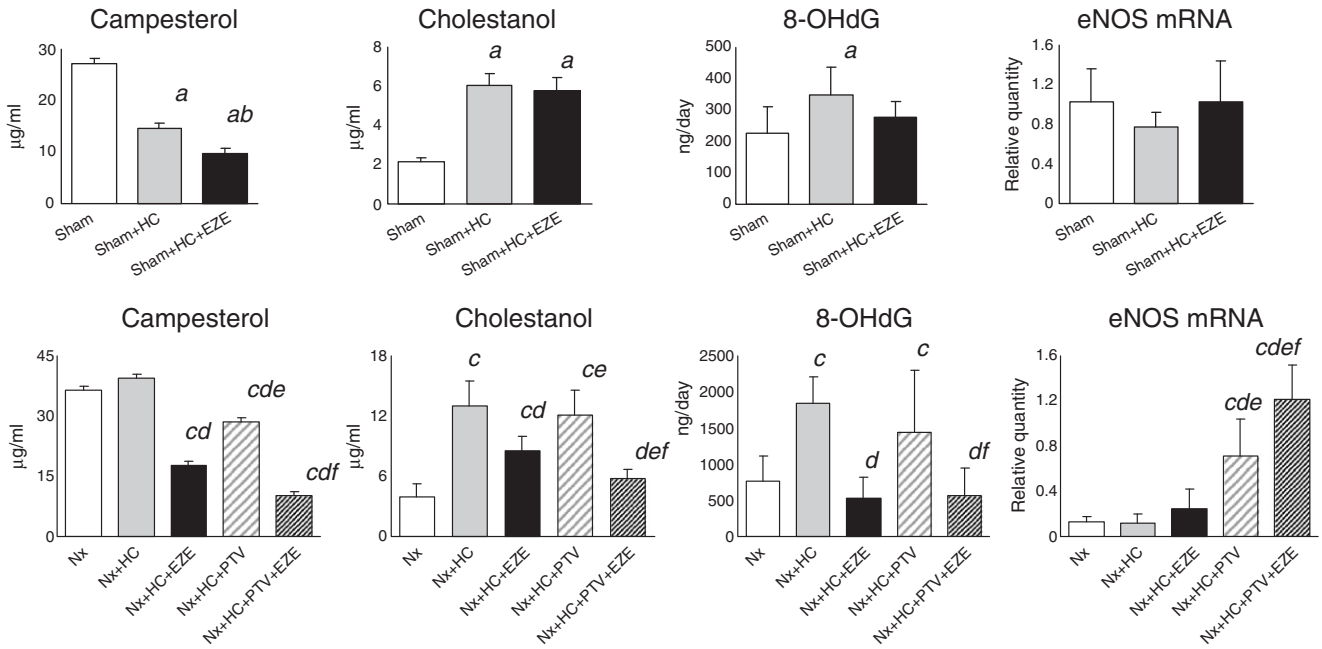

Fig. 5 – Cholesterol absorption markers (campesterol and cholestanol), urinary 8-OHdG, and eNOS mRNA expression in the kidneys of Sham and Nx rats. The upper panels show the Sham (n = 8, each group), and significance (P < .05) is presented as follows: a, vs normal chow fed; b, vs HC. The lower panels show the Nx (n = 9, each group), and significance (P < .05) is presented as follows: c, vs normal chow fed; d, HC; e, vs HC + EZE; f, vs HC + PTV. Relative expressions are values vs Sham-operated rats fed normal chow.

that campesterol, cholestanol, non–HDL-C, triglyceride, and 8-OHdG levels were all associated with kidney damage as assessed by CCr, proteinuria, GSI, and IFG in all of the animals. When campesterol, cholestanol, non–HDL-C, triglyceride, and 8-OHdG were taken as dependent variables for kidney damage in the multiple regression analysis, only campesterol was inversely associated with CCr or IFS. As pitavastatin has essentially no effect on cholesterol absorption, the pitavastatin-treated rats were excluded from the analysis on the possible association between cholesterol absorption and kidney damage. Simple regression analysis revealed that campesterol, cholestanol, non–HDL-C, triglyceride, and 8-OHdG levels were all associated with CCr, proteinuria, GSI, and IFG in rats, excluding the pitavastatin-treated group. Both cholesterol absorption markers were associated with kidney damage in the multiple regression analysis; but non–HDL-C lost its significant association when campesterol, cholestanol, non–HDL-C, triglyceride, and 8-OHdG were taken as dependent variables.

4. Discussion

This study is the first to demonstrate that ezetimibe significantly decreases serum cholesterol levels and attenuates the progression of pathological kidney damage in CKD

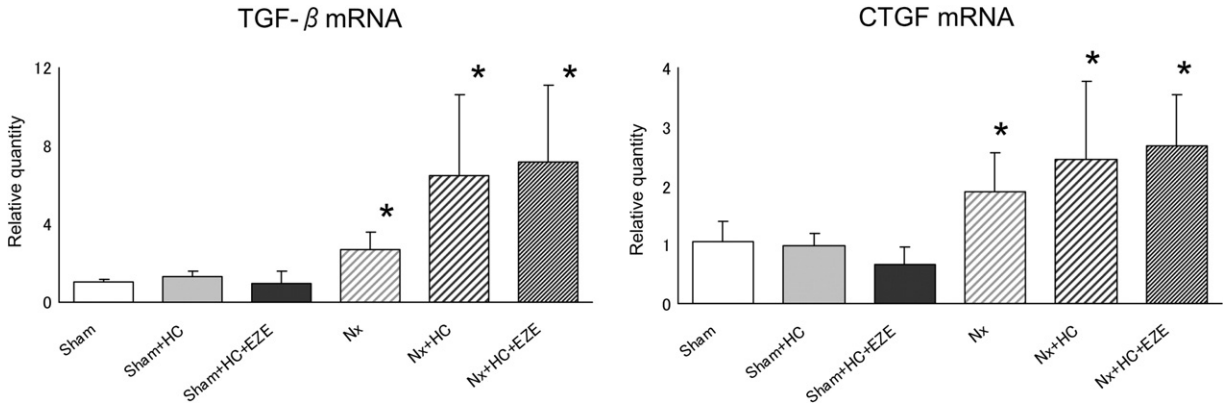

Fig. 6 – Messenger RNA expression of TGF-ß and CTGF in the kidney in Sham and Nx rats treated with HC or HC with EZE. Relative expression is expressed as 1 for Sham operation fed normal chow. n = 8, each Sham group; n = 9, each Nx group. Significant difference (*P < .05) was observed between Sham and Nx rats in the same treatments.

Table 3 – Simple and multiple correlations of kidney damage with various parameters

Variables	CCr		UPE		GSI		IFS	
	Simple	Multiple	Simple	Multiple	Simple	Multiple	Simple	Multiple
All groups								
Campesterol	−0.57*	−0.49*	0.57*	NS	0.48*	NS	0.47*	0.44*
Cholestanol	−0.51*	NS	0.79*	NS	0.74*	NS	0.72*	0.78*
Non–HDL-C	−0.6*	NS	0.843*	NS	0.77*	NS	0.71*	NS
Triglyceride	−0.46*	NS	0.75*	NS	0.57*	NS	0.45*	−0.42*
8-OHdG	−0.51*	NS	0.64*	NS	0.6*	NS	0.59*	NS
The groups excluded pitavastatin and combination treatment								
Campesterol	−0.69*	−1.08*	0.68*	0.51*	0.57*	0.67*	0.43*	0.45*
Cholestanol	−0.51*	−0.94*	0.86*	0.9*	0.75*	1.06*	0.77*	0.85*
Non–HDL-C	−0.6*	NS	0.91*	NS	0.77*	NS	0.77*	NS
Triglyceride	−0.46*	0.56*	0.78*	NS	0.56*	NS	0.43*	−0.47*
8-OHdG	−0.57*	NS	0.79*	NS	0.67*	NS	0.65*	NS

Kidney damage is assessed using CCr, urinary protein excretion, GSI, and IFS as dependent variables, and markers of cholesterol absorption, serum lipids, and urinary 8-OHdG as independent variables. NS indicates not significant; UPE, urinary protein excretion.
* Significance ($P < .05$).

models. We previously reported that a representative CKD model, the Nx rat, became hyperlipidemic with a decline of kidney function in part due to the downregulation of peroxisome proliferator-activated receptor–α gene expression [31]. Diamond [31] has proposed that many features of glomerulosclerosis share biological properties with atherosclerosis. Several experiments have shown that Nx animals fed cholesterol over long periods develop glomerulosclerosis with increased macrophages and foam cells concomitantly with elevated serum cholesterol [32,33]. In the study we report here, an HC stimulated macrophage infiltration into the glomeruli of normal (sham-operated) rats; and ezetimibe remarkably suppressed this macrophage infiltration while reducing serum cholesterol levels. The inhibition of cholesterol absorption associated with ezetimibe-mediated renoprotection may be partly independent of the cholesterol-lowering action of ezetimibe in plasma. Nakamura et al [34] reported that ezetimibe reduced urinary excretion along with reduced urinary 8-OHdG in proteinuric patients. We found that urinary 8-OHdG excretion was remarkably increased by cholesterol-feeding in both normal (Sham) and Nx rats and that ezetimibe attenuated this oxidative stress marker. Ezetimibe inhibits the absorption of not only cholesterol but also oxidized cholesterol (5-epoxycholesterol, etc), an aggravator of atherosclerosis [35]. No measurements of oxidized cholesterol were taken in our present experiments. We cannot discount the possibility, however, that an HC aggravates stress in the kidney by supplying oxidized cholesterol and that the inhibition of cholesterol absorption by ezetimibe rectifies this action. Kasiske et al [36] have reported that fibrate (clofibric acid) and the specific cholesterol synthesis inhibitor mevinolin reduce serum cholesterol and ameliorate kidney damage in Nx rats. Given that these agents have no effects on cholesterol absorption, it would be reasonable to assume that plasma cholesterol reduction is also involved in the mechanism of the ezetimibe-induced renoprotection. Eto et al [37] demonstrated that remnant lipoproteins stimulate the accumulation of cholesteryl ester in human mesangial cells. Ezetimibe reduces remnant-like particle cholesterol levels in dyslipidemic subjects [38]. Thus, the benefits of ezetimibe might be mediated by a reduction in the delivery of remnant lipoproteins to the kidney. Considering these points in sum, we speculate that the renoprotective effect of ezetimibe is conferred via both the inhibition of cholesterol absorption and the reduction of serum cholesterol concentration.

A number of experimental studies with a recent model of CKD have already revealed that statins confer renoprotective effects without altering serum lipid levels [6-9]. Pitavastatin is an oil-soluble superstatin with a strong cholesterol-lowering action that has been associated with regression of coronary lesions in a human study [39]. Pitavastatin, like the other statins, essentially lacked a cholesterol-lowering effect in our experiments. It did, however, significantly ameliorate kidney dysfunction and pathological changes of the remnant kidney of cholesterol-fed Nx rats. The combination of ezetimibe and statin treatment is well known to substantially reduce low-density lipoprotein (LDL) cholesterol levels in humans [40,41]. In our rats, however, the cholesterol-lowering effect of the combination of ezetimibe and statin was comparable to that of ezetimibe alone. In comparison with the ezetimibe-alone treatment, the combination treatment of ezetimibe and pitavastatin synergistically improved kidney dysfunction and the pathological changes of the remnant kidney of the cholesterol-fed Nx. This was most likely attributable to both the cholesterol-lowering effect of the ezetimibe and the pleiotropic effect of the pitavastatin. Unlike ezetimibe, pitavastatin did not affect urinary 8-OHdG excretion. In view of the high correlation between 8-OHdG and plasma cholesterol or cholesterol absorption, we strongly doubt that statin can reduce oxidative stress without conferring a cholesterol-lowering action. In the study by Nakamura et al [42], pitavastatin alone or in combination with ezetimibe reduced urinary 8-OHdG in proteinuric human subjects. Given that pitavastatin reduces serum cholesterol in human, we suspect that the pitavastatin-mediated 8-OHdG reduction is closely associated with cholesterol reduction.

Endothelial nitric oxide synthase is a key enzyme for the generation of NO, a compound that protects against vascular endothelial damage [43,44]. The downregulation of eNOS is associated with the development of glomerulosclerosis [45,46] and interstitial fibrosis [47]. Indeed, eNOS mRNA levels were

severely downregulated in the remnant kidney in our experiments. Although unresponsive to treatment with ezetimibe alone, eNOS mRNA increased significantly in response to pitavastatin and further increased in response to the combination of pitavastatin and ezetimibe. In the absence of any significant effect of pitavastatin on cholesterol reduction, we can reasonably assume that pitavastatin upregulates eNOS gene expression through its pleiotropic effects and that the effects might be more pronounced under a cholesterol-reduced state.

In this study, we measured the mRNA levels of TGF-β and CTGF in glomeruli in association with fibrotic parameters. The gene expressions of TGF-β and CTGF were both increased in the Nx rats, as has been reported previously. Neither of these gene expressions, however, showed any significant change in response to manipulations of dietary cholesterol and EZE. The fibrotic changes induced by dietary cholesterol might be independent of TGF-β or CTGF. The present observation, however, would be too preliminary to support a convincing conclusion.

The recently opened findings of the Study of Heart and Renal Protection trial demonstrate that the combination of ezetimibe and simvastatin did not significantly suppress the progression to end-stage renal disease in advanced CKD [48]. We note, however, that the baseline serum LDL cholesterol levels in their human CKD patients were fairly low (<110 mg/dL), whereas our animal model was severely hypercholesterolemic (around 500 mg/dL). We can presume, therefore, that the benefit of cholesterol-lowering therapy would not be manifested in CKD patients with low cholesterol levels. The rat model in this study is of only limited use in expanding our understanding of human pathophysiology. In rats, for example, LDL is not a major cholesterol-carrier lipoprotein; and statins confer no cholesterol-lowering effect. According to a recent analysis of the Fenofibrate Intervention and Event Lowering in Diabetes study, fenofibrate reduced albuminuria and slowed the eGFR loss over a 5-year period in patients with type 2 diabetes mellitus who initially manifested reversible increases of plasma creatinine [49]. This tells us that the renoprotective effect of the lipid-regulating drugs varies with the nature of the subjects and the duration of treatment. Nevertheless, our study suggests that excess cholesterol intake hinders kidney function and that the suppression of cholesterol absorption attenuates the lipotoxicity on the kidney.

In conclusion, although it remains to be definitively proven, our results suggest that ezetimibe and pitavastatin confer renoprotective effects via different mechanisms in our rat model of CKD: the former by cholesterol reduction via inhibition of cholesterol absorption; the latter by a cholesterol-independent pleiotropic effect, such as upregulation of eNOS production.

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Conflict of Interest

Yusaku Mori and Tsutomu Hirano disclosed no conflict of interest.

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