Original Article

Ezetimibe Ameliorates Early Diabetic Nephropathy in db/db Mice

Yukinori Tamura¹, Toshinori Murayama¹, Manabu Minami¹, Takeshi Matsubara², Masayuki Yokode¹ and Hidenori Arai³

¹Department of Clinical Innovative Medicine, Kyoto University Graduate School of Medicine, Kyoto, Japan

²Department of Nephrology, Kyoto University Graduate School of Medicine, Kyoto, Japan

³Department of Human Health Sciences, Kyoto University Graduate School of Medicine, Kyoto, Japan

Aim: Lipid-lowering medications have been suggested to have a potential benefit in the treatment of chronic kidney disease (CKD) such as diabetic nephropathy. Although ezetimibe has been widely used to lower serum cholesterol levels, the effect of this drug on diabetic nephropathy remains unclear. In the present study, therefore, we examined the protective effect of ezetimibe on diabetic nephropathy in db/db mice.

Method: Db/db mice were fed a standard diet with 0.01% (w/w) of ezetimibe for 8 weeks from 8 weeks of age.

Results: Treatment with ezetimibe did not affect food intake, body weight gain, adiposity, or blood pressure in db/db mice. Ezetimibe also had no effect on glucose metabolism such as fasting plasma glucose and insulin; however, it markedly reduced plasma lipid levels and hepatic lipid contents and reduced the urinary excretion of albumin by 50% in db/db mice, suggesting the effect of ezetimibe on diabetic nephropathy. Furthermore, ezetimibe improved glomerular hypertrophy. Although ezetimibe had no effect on oxidative stress measured by urinary 8-OHdG in db/db mice, the plasma adiponectin level was normalized, and the expression of adiponectin receptor 1 in the kidney was increased by ezetimibe treatment.

Conclusion: Our data suggest that ezetimibe can improve early diabetic nephropathy through its hypolipidemic effect, and the amelioration of adiponectin resistance may also be responsible for the renoprotective effect of ezetimibe as its underlying mechanism.

J Atheroscler Thromb, 2012; 19:608-618.

Key words; Ezetimibe, Diabetic nephropathy, Albuminuria, Adiponectin

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 increasing³. Furthermore, CKD such as diabetic nephropathy is strongly associated with the develop-

ment of cardiovascular disease^{4, 5}; therefore, the establishment of therapeutic strategies for diabetic nephropathy is awaited. Diabetic nephropathy results from complex interactions among 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 preceded by persistent albuminuria and glomerular hypertrophy⁶; therefore, these two manifestations are promising therapeutic targets for the treatment of diabetic nephropathy.

Hypercholesterolemia has been suggested to be associated with the development of diabetic nephropathy⁶. In fact, lipid-lowering therapy using 3-hydroxy-3-methylglutaryl (HMG)-coenzyme A (CoA) reduc-

Address for correspondence: Hidenori Arai, Department of Human Health Sciences, Kyoto University Graduate School of Medicine, 53 Kawahara-cho, Shogoin, Sakyo-ku, Kyoto, 606-8507, Japan

E-mail: harai@kuhp.kyoto-u.ac.jp

Received: August 2, 2011

Accepted for publication: January 17, 2012

Fig. 1. Effect of ezetimibe on body weight, adiposity, and blood pressure in db/db mice at 16 weeks of age. The graphs show body weight (A), food intake (B), liver weight (C), epididymal white adipose tissue (eWAT) weight (D), kidney weight (E), systolic blood pressure (F) in db/m mice and non-treated (Con) or ezetimibe-treated (Eze) db/db mice. Results are expressed as the mean $\pm$ S.D. ** $p < 0.01$ vs. non-treated db/db mice ($n = 6$ in each group). ND: not determined.

tase inhibitors (statins) has been successful for the amelioration of diabetic nephropathy^{7, 8)}. Ezetimibe, another lipid-lowering drug that selectively inhibits cholesterol absorption by inhibiting Niemann-Pick C1-Like 1 (NPC1L1) protein, is also used for the treatment of dyslipidemia^{9, 10)}. In addition to its effect on hyperlipidemia, ezetimibe has been reported to ameliorate renal dysfunction such as non-diabetic nephropathy¹¹⁾ and nephropathy after transplantation¹²⁾; however, the effects of ezetimibe on diabetic nephropathy remain undetermined. In the present study, therefore, we examined the renoprotective effects of ezetimibe on diabetic nephropathy in db/db mice.

Methods

Animal Procedure and Experimental Design

Male db/db mice ($n = 12$) and their lean control db/+m ($n = 6$) mice were obtained from Charles River Laboratories Japan, Inc. (Yokohama, Japan) at 6 weeks of age. Db/db mice were fed with normal chow without additional supplementation (non-treated group, $n = 6$) or with chow supplementation with 0.01% (w/w)

ezetimibe ($n = 6$) for 8 weeks from 8 weeks of age. Animals were provided with the diet and water ad libitum and were maintained on a 12-hour light/dark cycle. All animal experiments were conducted according to the Guidelines for Animal Experiments at Kyoto University.

Analysis of Metabolic Parameters

Blood samples were collected after fasting the mice for 16 h. Fasted plasma glucose concentration was measured with Glutest Ace (Sanwa Kagaku Kenkyusho Co, Ltd, Nagoya, Japan). Fasted plasma insulin concentration was measured with an insulin assay kit (Morinaga Institute of Biological Science, Yokohama, Japan). Serum total cholesterol (T-Chol), triglyceride (TG) and cholesterol contents of each lipoprotein fraction were analyzed by Skylight Biotech, Inc. (Tokyo, Japan). Serum adiponectin was measured with a Mouse/Rat Adiponectin ELISA kit (Otsuka Pharmaceuticals, Tokushima, Japan). Serum total protein, creatinine, and BUN were analyzed by SRL, Inc. (Tokyo, Japan).

Fig. 2. Effect of ezetimibe on renal function in db/db mice at 16 weeks of age. Urinary excretion of albumin (A), HE staining of glomeruli (magnification $\times 400$, bar; $50\ \mu\text{m}$) and mean glomerular surface area (B) of db/m mice, non-treated (Con), ezetimibe-treated (Eze) db/db mice. Fifty glomeruli per mouse were analyzed. Results are expressed as the mean $\pm$ S.D. ** $p < 0.01$ vs. non-treated db/db mice ($n = 6$ in each group).

Table 1. Effect of ezetimibe treatment on blood chemistry in db/db mice

	db/m	db/db Con	db/db Eze
Total protein (g/dL)	5.0 ± 0.5	5.9 ± 0.5	5.1 ± 0.7
BUN (mg/dL)	19.1 ± 3.5	20.1 ± 2.4	27.2 ± 7.6
Creatinine (mg/dL)	0.11 ± 0.03	0.08 ± 0.02	0.09 ± 0.03

Results are expressed as the means $\pm$ S.D. ($n = 6$ in each group)

Measurement of Hepatic Lipid Content

Hepatic triglyceride and cholesterol contents were measured with Triglyceride E and Cholesterol E test (Wako Pure Chemical Industries Ltd., Osaka, Japan) as previously described¹³. Tissue triglyceride and cholesterol contents were expressed as mg/mg protein.

Measurement of Urinary Albumin and Creatinine

Urinary albumin and creatinine were measured at 16 weeks age using 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 Inc., Philadelphia, PA). Urinary creatinine was measured with a Hitachi Mode 736 analyzer (Hitachi, Tokyo, Japan). 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 (8OHdG Check; Japan Institute for the Control of Aging, Fukuroi, Japan). Urinary 8-OHdG excretion was expressed as the total amount excreted in 24 h.

Measurement of Glomerular Size

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 $3\ \mu\text{m}$. To measure glomerular size, paraffin sections were stained with hematoxylin and eosin. The glomerular area was measured using Image Pro plus software version 3.0.1 (Media Cybernetics Inc., Bethesda, MD).

Quantitative Real-Time PCR

Total RNA was extracted from frozen adipose tissue (100 mg) and kidney tissue (30 mg) using an

RNeasy mini kit (Qiagen, Valencia, CA). The cDNA was synthesized from total RNA using Super Script III (Invitrogen). Real-time polymerase chain reaction was performed on an ABI PRISM 9700 using the SYBR GREEN polymerase chain reaction Master Mix (Applied Biosystems, Warrington, UK). Primer sets were as follows: TNF alpha forward: CCCAGACCC-TCACACTCAGATC, reverse: GCCACTCCAGCT-GCTCCTC, Nox2 forward: TTGGGTCAGCACT-GGCTCTG, reverse: TGGCGGTGTGCAGTGC-TATC, Nox4 forward: ATTTGGATAGGCTCCAG-GCAAAC, reverse: CACATGGGTATAAGCTTTG-TGAGCA, p22^{phox} forward: GTCCACCATGG-AGCGATGTG, reverse: CAATGGCCAAGCAGAC-GGTC adiponectin receptor 1 (AdipoR1) forward: ACGTTGGAGAGTCATCCCGTA, reverse: CTCT-GTGTGGATGCGGAAGAT, adiponectin receptor 2 (AdipoR2) forward: TGCCAGGAAGATGAAGGG-TTTAT, reverse: TTCCATTTCGTTTCGATAGCA-TGA, β -actin forward: TACCACAGGCATTGTGA-TGG, reverse: TTTGATGTCACGCACGATT. The mRNA levels were normalized relative to the amount of β -actin mRNA and expressed in arbitrary units.

Statistical Analysis

Data are expressed as the mean $\pm$ S.D. Multiple comparisons among the groups were conducted by one-way analysis of variance with Fisher's PLSD test for post-hoc analysis. Pearson's correlation was used to find a correlation between two continuous variables. $P < 0.05$ was considered significant.

Results

Effect of Ezetimibe Treatment on Body Weight, Adiposity, and Systolic Blood Pressure

In db/db mice fed with a standard diet for 8 weeks until 16 weeks of age, body weight, epididymal white adipose tissue (eWAT) weight, liver weight, and kidney weight were increased compared with db/m mice (**Fig. 1A-E**). Although ezetimibe treatment reduced liver weight in db/db mice, body weight, food intake, eWAT weight, and kidney weight were not changed (**Fig. 1A-E**). In addition, there was no difference in systolic blood pressure between ezetimibe-treated and non-treated db/db mice (**Fig. 1F**).

Effect of Ezetimibe Treatment on Renal Dysfunction in db/db Mice

Because albuminuria reflects renal dysfunction at early diabetic nephropathy¹⁴, we measured urinary excretion of albumin in normal chow-fed db/db mice at 16 weeks of age. Urinary excretion of albumin was

Fig. 3. The correlation between the effect of ezetimibe on urinary albumin and glomerular size (A), urinary albumin and serum T-Cho level (B), glomerular size and serum T-Cho level (C) in db/db mice.

markedly increased in db/db mice compared with db/+m mice (**Fig. 2**); however, ezetimibe treatment reduced urinary excretion of albumin by 50% in db/db mice (**Fig. 2**). There was no difference in serum total protein, creatinine, or BUN levels between db/m and non-treated db/db mice. Ezetimibe treatment also had no effect on these variables in db/db mice (**Table 1**). These data suggest that ezetimibe ameliorates early diabetic nephropathy in db/db mice.

Table 2. Effect of ezetimibe treatment on serum lipid in db/db mice

	db/m	db/db Con	db/db Eze
Triglyceride (mg/dL)	50.9 ± 10.2	59.0 ± 23.3	64.8 ± 27.4
Total Cholesterol (mg/dL)	77.8 ± 2.2	150.3 ± 26.8	78.7 ± 23.1**
Chylomicron (mg/dL)	0.8 ± 0.5	1.9 ± 0.6	0.9 ± 0.5*
VLDL cholesterol (mg/dL)	4.2 ± 1.5	7.0 ± 1.6	4.3 ± 3.0
LDL cholesterol (mg/dL)	6.7 ± 1.1	30.4 ± 11.1	7.1 ± 4.8**
HDL cholesterol (mg/dL)	66.1 ± 1.5	111.0 ± 17.6	66.4 ± 16.2**
Small dense LDL cholesterol (mg/dL)	2.4 ± 0.4	5.0 ± 2.1	1.2 ± 0.9**

Results are expressed as the means ± S.D. ($n=6$ in each group)

* $p < 0.05$, ** $p < 0.01$ vs db/db Con group

Effect of Ezetimibe Treatment on Glomerular Hypertrophy in db/db Mice

Glomerular hypertrophy is a marker of diabetic nephropathy along with albuminuria; therefore, we checked glomerular hypertrophy in db/db mice and the effect of ezetimibe by measuring the glomerular surface area. Mean glomerular surface area size in db/db mice was increased compared with db/m mice; however, ezetimibe treatment suppressed glomerular hypertrophy in db/db mice (**Fig. 2B**). Furthermore, there was a significant correlation in the effect of ezetimibe treatment on glomerular hypertrophy and albuminuria in db/db mice (**Fig. 3A**).

Effect of Ezetimibe Treatment on Lipid Metabolism in db/db Mice

To clarify the mechanisms by which ezetimibe improves renal dysfunction, we next examined the effect of ezetimibe treatment on lipid metabolism in db/db mice. Serum TG levels were not affected by ezetimibe treatment in db/db mice (**Table 2** and **Fig. 4**). Serum T-Cho levels were increased in non-treated db/db mice compared with in db/m mice; however, ezetimibe treatment normalized T-Cho levels in db/db mice (**Table 2** and **Fig. 4**). Furthermore, ezetimibe treatment reduced chylomicron, LDL, small dense LDL, and HDL cholesterol levels in db/db mice (**Table 2** and **Fig. 4**). In addition, hepatic TG and T-Cho contents in db/db mice were reduced by ezetimibe treatment (**Fig. 5**), suggesting that ezetimibe treatment improves hepatic steatosis.

Effect of Ezetimibe Treatment on Insulin Resistance in db/db Mice

It has been reported that ezetimibe treatment improves insulin resistance, which is associated with the development of diabetic nephropathy^{15, 16}; therefore, we next examined the effect of ezetimibe treatment on glucose metabolism in db/db mice. Fasted

Fig. 4. Graph of cholesterol (pink line) and triglyceride (blue line) contents in each fraction of lipoprotein in db/m (A), non-treated db/db mice (B) and ezetimibe-treated db/db mice (C). FG: free glycerol.

Fig. 5. Effect of ezetimibe on hepatic steatosis in db/db mice at 16 weeks of age. Hepatic triglyceride content (A), hepatic cholesterol content (B) in db/m mice, non-treated (Con), ezetimibe-treated (Eze) db/db mice. Results are expressed as the mean $\pm$ S.D. ** $p < 0.01$ vs. non-treated db/db mice ($n = 6$ in each group).

plasma glucose, the 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. 6**); however, ezetimibe treatment had no effect on glucose metabolism in db/db mice.

Effect of Ezetimibe Treatment on Oxidative Stress in Kidney of db/db Mice

To examine the effect of ezetimibe treatment on oxidative stress, we measured urinary 8-OHdG levels in db/db mice. Urinary 8-OHdG levels in non-treated db/db mice were significantly higher than those in db/m mice; however, ezetimibe had no effect on urinary 8-OHdG levels in db/db mice (**Fig. 7A**). Furthermore, mRNA expressions of Nox2 and Nox4, the substrate of NADPH oxidase, were not altered by ezetimibe treatment in the whole kidney of db/db mice (**Fig. 8**). These data suggest that ezetimibe has no effect on oxi-

dative stress in the kidney of db/db mice.

Effect of Ezetimibe Treatment on Hypoadiponectinemia

Because hypoadiponectinemia is associated with the development of kidney disease¹⁷, we examined the effect of ezetimibe treatment on serum adiponectin levels in db/db mice. In non-treated db/db mice, serum adiponectin levels were decreased compared with db/m mice; however, ezetimibe treatment normalized serum adiponectin levels in db/db mice (**Fig. 7B**).

Effect of Ezetimibe on Inflammation and Oxidative Stress in Adipose Tissue

Because inflammation and oxidative stress in adipose tissue are a major cause of hypoadiponectinemia¹⁸, we examined the effect of ezetimibe treatment on the expression of inflammatory cytokines and an oxidative stress marker in db/db mice. TNF- α mRNA expression was markedly increased in non-treated db/db mice compared with in db/m mice (**Fig. 9A**); however, ezetimibe treatment reduced TNF- α mRNA expression in db/db mice, suggesting that it suppresses adipose tissue inflammation in db/db mice. Furthermore, oxidative stress markers such as Nox2 and p22^{phox} mRNA expression were decreased by ezetimibe treatment in db/db mice (**Fig. 9B, C**), suggesting that ezetimibe treatment suppresses oxidative stress in adipose tissue of db/db mice.

Effect of Ezetimibe Treatment on the Expression of Adiponectin Receptor (AdipoR) in the Kidney and Adipose Tissue in db/db Mice

We next examined the effect of ezetimibe on the expression of AdipoR1 and AdipoR2 in the kidney

Fig. 6. Effect of ezetimibe on glucose metabolism in db/db mice at 16 weeks of age. Fasted plasma glucose (A), Fasted plasma insulin level (B) and HOMA-IR (C) in db/m mice, non-treated (Con), ezetimibe-treated (Eze) db/db mice. Results are expressed as the mean $\pm$ S.D. * $p < 0.05$ vs. non-treated db/db mice ($n = 6$ in each group).

Fig. 7. Effect of ezetimibe on renal oxidative stress and hypoadiponectinemia in db/db mice at 16 weeks of age. Urinary 8-OHdG level (A) and serum adiponectin level (B) in db/m mice, non-treated (Con), ezetimibe-treated (Eze) db/db mice. Results are expressed as the mean $\pm$ S.D. * $p < 0.05$ vs. non-treated db/db mice ($n = 6$ in each group).

Fig. 8. Effect of ezetimibe on oxidative stress in whole kidney of db/db mice. Expression of Nox4 mRNA (A) and Nox2 mRNA (B) in non-treated (Con) or ezetimibe-treated (Eze) db/db mice. Results are expressed as the mean $\pm$ S.D.

and adipose tissue of db/db mice. AdipoR1, but not AdipoR2, was abundantly expressed in the kidney as well as in adipose tissue (**Fig. 10**). There were no differences in mRNA expression of AdipoR1 and AdipoR2 in adipose tissue and the expression of AdipoR2 in the kidney among db/m mice, non-treated and ezetimibe-treated db/db mice; however, mRNA expression of AdipoR1 was decreased in the kidney of db/db mice compared with db/+m mice. Intriguingly, ezetimibe treatment significantly increased mRNA expression of AdipoR1 in the kidney of db/db mice (**Fig. 10**).

Discussion

In the present study, we showed that ezetimibe treatment improved hyperlipidemia, albuminuria, and glomerular hypertrophy in db/db mice, implying a beneficial role of ezetimibe in early diabetic nephropathy.

thy¹⁹).

Ezetimibe is an anti-hyperlipidemic medication that is used to lower cholesterol levels in addition to statins⁹. Specifically, it appears to bind to a critical mediator of cholesterol absorption, NPC1-L1, on gastrointestinal tract epithelial cells¹⁰. In the present study, we observed a correlation between the effect of ezetimibe on albuminuria and on the T-Chol level in db/db mice (**Fig. 3B**). These data suggest that ezetimibe improves diabetic nephropathy through its hypolipidemic action, and provide further evidence for the importance of hyperlipidemia in the development of diabetic nephropathy. Ezetimibe treatment also markedly reduced LDL cholesterol and small dense LDL cholesterol in db/db mice, consistent with the effect in humans^{8, 20}. In the present study, we found a correlation between the effect of ezetimibe treatment on albuminuria and on the LDL cholesterol level, but not on the small dense LDL cholesterol level.

Fig. 9. Effect of ezetimibe on inflammation and oxidative stress in adipose tissue of db/db mice. The mRNA expression of TNF- α (A), Nox2 (B) and p22^{phox} (C) in db/m mice, non-treated (Con), ezetimibe-treated (Eze) db/db mice. Results are expressed as the mean $\pm$ S.D. * $p < 0.05$ vs. non-treated db/db mice ($n = 6$ in each group).

Fig. 10. Effect of ezetimibe on mRNA expression of adiponectin receptor 1 (AdipoR1) and 2 (AdipoR2) in the kidney and adipose tissue of db/m mice and non-treated (Con) and ezetimibe-treated (Eze) db/db mice. Results are expressed as the mean $\pm$ S.D. * $p < 0.05$, ** $p < 0.01$ ($n = 6$ in each group).

in db/db mice (**Supplementary Fig. 1**); therefore, the effect of ezetimibe on LDL cholesterol might be associated with the amelioration of diabetic nephropathy in this model.

In this study, we found no effect of ezetimibe on the urinary 8-OHdG level and the expression of oxidative stress markers in the kidney of db/db mice, whereas ezetimibe significantly reduced oxidative stress in the adipose tissue of db/db mice, suggesting that the renoprotective effect of ezetimibe is not directly due to reduced oxidative stress in the kidney. Nevertheless, ezetimibe has been reported to have an anti-oxidative effect in non-diabetic individuals¹¹.

Several human and animal studies have reported that hypoadiponectinemia is associated with renal dysfunction²¹⁻²³. In the present study, we observed the improvement of hypoadiponectinemia by ezetimibe treatment in db/db mice; however, we did not find a correlation between the effect of ezetimibe on albuminuria and on adiponectin in db/db mice (data not shown), suggesting that improvement of hypoadiponectinemia may not be responsible for the renoprotective effect of ezetimibe in db/db mice. In contrast, we found a negative correlation between the effect of ezetimibe on albuminuria and AdipoR1 expression in the kidney of db/db mice (**Supplementary Fig. 2A**).

Furthermore, we also observed a negative correlation between the effect of ezetimibe on AdipoR1 expression in the kidney and the LDL cholesterol level in db/db mice (**Supplementary Fig. 2B**). Guo *et al.* reported decreased adipoR1 expression in the kidney of diabetic rats, suggesting the existence of adiponectin resistance in diabetic nephropathy²⁴. Taken together, whether renoprotection by ezetimibe occurs through alteration of the adiponectin effect remains to be determined.

We also observed that ezetimibe treatment suppressed the expression of pro-inflammatory cytokines such as TNF- α in adipose tissue of db/db mice. TNF- α can dose-dependently reduce the expression of adiponectin in adipocytes by suppressing its promoter activity²⁵; however, we did not find any correlations among the effect of ezetimibe on the serum adiponectin level, TNF- α expression in adipose tissue, and serum lipid profiles in db/db mice. We thus speculate that ezetimibe has pleiotropic effects, which might not be mutually interrelated.

Insulin resistance is also 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 non-diabetic subjects¹⁶. In the present study, we observed marked elevation of plasma glucose and insulin in db/db mice, indicating the development of insulin resistance. Because it has been reported that ezetimibe improved hepatic insulin resistance¹⁵, we examined the effect of ezetimibe on the glucose metabolism; however, ezetimibe had no effect on systemic insulin resistance in db/db mice despite the increase in serum adiponectin. These data indicate that the renoprotective effect of ezetimibe in db/db mice was independent of systemic insulin-sensitizing action.

Ezetimibe can be used for hypercholesterolemic patients who exhibit statin resistance or suffer adverse effects of statin treatment²⁶. Recently, combination therapy with a statin and ezetimibe showed efficacy and safety compared with high-dose statin therapy in patients with hypercholesterolemia²⁷. Therefore, combination therapy of statins and ezetimibe as well as ezetimibe monotherapy might be useful for the treatment of diabetic nephropathy associated with hyperlipidemia²⁸.

In conclusion, our data suggest that ezetimibe can improve diabetic nephropathy through its hypolipidemic action, and the amelioration of adiponectin resistance may be responsible for the renoprotective effect of ezetimibe as its underlying mechanism.

Acknowledgements

We thank Nami Yamaga (Kyoto University) for excellent technical assistance. This study was supported by Takeda Science Foundation.

Disclosure

None.

References

- 1) Mauer SM, Steffes MW, Ellis EN, Sutherland DE, Brown DM, Goetz FC: Structural-functional relationships in diabetic nephropathy. *J Clin Invest*, 1984; 74: 1143-1155
- 2) Nagai K, Arai H, Yanagita M, Matsubara T, Kanamori H, Nakano T, Iehara N, Fukatsu A, Kita T, Doi T: Growth arrest-specific gene 6 is involved in glomerular hypertrophy in the early stage of diabetic nephropathy. *J Biol Chem*, 2003; 278: 18229-18234
- 3) Coresh J, Selvin E, Stevens LA, Manzi J, Kusek JW, Eggers P, Van Lente F, Levey AS: Prevalence of chronic kidney disease in the United States. *JAMA*, 2007; 298: 2038-2047
- 4) Yokoyama H, Sone H, Saito K, Yamada D, Honjo J, Haneda M: Flow-mediated dilation is associated with microalbuminuria independent of cardiovascular risk factors in type 2 diabetes - interrelations with arterial thickness and stiffness. *J Atheroscler Thromb*, 2011; 18: 744-752
- 5) Kim KJ, Lee BW, Kim HM, Shin JY, Kang ES, Cha BS, Lee EJ, Lim SK, Lee HC: Associations between cardio-ankle vascular index and microvascular complications in type 2 diabetes mellitus patients. *J Atheroscler Thromb*, 2011; 18: 328-336
- 6) Kwan BC, Beddhu S, Kronenberg F, Cheung AK: Does statin therapy improve cardiovascular outcomes in patients with type 2 diabetes receiving hemodialysis? *Nat Clin Pract Nephrol*, 2006; 2: 76-77
- 7) Tonolo G, Velussi M, Brocco E, Abaterusso C, Carraro A, Morgia G, Satta A, Faedda R, Abhyankar A, Luthman H, Nosadini R: Simvastatin maintains steady patterns of GFR and improves AER and expression of slit diaphragm proteins in type II diabetes. *Kidney Int*, 2006; 70: 177-186
- 8) Sharp Collaborative G: Study of Heart and Renal Protection (SHARP): randomized trial to assess the effects of lowering low-density lipoprotein cholesterol among 9,438 patients with chronic kidney disease. *Am Heart J*, 2010; 160: 785-794 e710
- 9) Rizzo M, Rini GB, Spinaz GA, Berneis K: The effects of ezetimibe on LDL-cholesterol: quantitative or qualitative changes? *Atherosclerosis*, 2009; 204: 330-333
- 10) Garcia-Calvo M, Lisnock J, Bull HG, Hawes BE, Burnett DA, Braun MP, Crona JH, Davis HR Jr, Dean DC, Detmers PA, Graziano MP, Hughes M, Macintyre DE, Ogawa A, O'Neill K A, Iyer SP, Shevell DE, Smith MM, Tang YS, Makarewicz AM, Ujjainwalla F, Altmann SW,

- Chapman KT, Thornberry NA: The target of ezetimibe is Niemann-Pick C1-Like 1 (NPC1L1). *Proc Natl Acad Sci U S A*, 2005; 102: 8132-8137
- 11) Nakamura T, Sato E, Fujiwara N, Kawagoe Y, Ueda Y, Suzuki T, Ueda S, Adachi H, Okuda S, Yamagishi S: Ezetimibe decreases serum levels of asymmetric dimethylarginine (ADMA) and ameliorates renal injury in non-diabetic chronic kidney disease patients in a cholesterol-independent manner. *Pharmacol Res*, 2009; 60: 525-528
- 12) Buchanan C, Smith L, Corbett J, Nelson E, Shihab F: A retrospective analysis of ezetimibe treatment in renal transplant recipients. *Am J Transplant*, 2006; 6: 770-774
- 13) Tamura Y, Sugimoto M, Murayama T, Ueda Y, Kanamori H, Ono K, Ariyasu H, Akamizu T, Kita T, Yokode M, Arai H: Inhibition of CCR2 ameliorates insulin resistance and hepatic steatosis in db/db mice. *Arterioscler Thromb Vasc Biol*, 2008; 28: 2195-2201
- 14) Dronavalli S, Duka I, Bakris GL: The pathogenesis of diabetic nephropathy. *Nat Clin Pract Endocrinol Metab*, 2008; 4: 444-452
- 15) Nomura M, Ishii H, Kawakami A, Yoshida M: Inhibition of Hepatic Neiman-Pick C1-Like 1 Improves Hepatic Insulin Resistance. *Am J Physiol Endocrinol Metab*, 2009
- 16) Jauregui A, Mintz DH, Mundel P, Fornoni A: Role of altered insulin signaling pathways in the pathogenesis of podocyte malfunction and microalbuminuria. *Curr Opin Nephrol Hypertens*, 2009; 18: 539-545
- 17) Tesaro M, Canale MP, Rodia G, Di Daniele N, Lauro D, Scuteri A, Cardillo C: Metabolic syndrome, chronic kidney, and cardiovascular diseases: role of adipokines. *Cardiol Res Pract*, 2011; 2011: 653182
- 18) Maury E, Brichard SM: Adipokine dysregulation, adipose tissue inflammation and metabolic syndrome. *Mol Cell Endocrinol*, 2010; 314: 1-16
- 19) Sharma K, McCue P, Dunn SR: Diabetic kidney disease in the db/db mouse. *Am J Physiol Renal Physiol*, 2003; 284: F1138-1144
- 20) Kalogirou M, Tsimihodimos V, Gazi I, Filippatos T, Saougos V, Tselepis AD, Mikhailidis DP, Elisaf M: Effect of ezetimibe monotherapy on the concentration of lipoprotein subfractions in patients with primary dyslipidaemia. *Curr Med Res Opin*, 2007; 23: 1169-1176
- 21) Ix JH, Sharma K: Mechanisms linking obesity, chronic kidney disease, and fatty liver disease: the roles of fetuin-A, adiponectin, and AMPK. *J Am Soc Nephrol*, 2010; 21: 406-412
- 22) Ohashi K, Iwatani H, Kihara S, Nakagawa Y, Komura N, Fujita K, Maeda N, Nishida M, Katsube F, Shimomura I, Ito T, Funahashi T: Exacerbation of albuminuria and renal fibrosis in subtotal renal ablation model of adiponectin-knockout mice. *Arterioscler Thromb Vasc Biol*, 2007; 27: 1910-1917
- 23) Yilmaz MI, Saglam M, Qureshi AR, Carrero JJ, Caglar K, Eyileten T, Sonmez A, Cakir E, Oguz Y, Vural A, Yenicesu M, Stenvinkel P, Lindholm B, Axelsson J: Endothelial dysfunction in type-2 diabetics with early diabetic nephropathy is associated with low circulating adiponectin. *Nephrol Dial Transplant*, 2008; 23: 1621-1627
- 24) Guo Z, Zhao Z: Effect of N-acetylcysteine on plasma adiponectin and renal adiponectin receptors in streptozotocin-induced diabetic rats. *Eur J Pharmacol*, 2007; 558: 208-213
- 25) Maeda N, Takahashi M, Funahashi T, Kihara S, Nishizawa H, Kishida K, Nagaretani H, Matsuda M, Komuro R, Ouchi N, Kuriyama H, Hotta K, Nakamura T, Shimomura I, Matsuzawa Y: PPARgamma ligands increase expression and plasma concentrations of adiponectin, an adipose-derived protein. *Diabetes*, 2001; 50: 2094-2099
- 26) Wierzbicki AS, Doherty E, Lumb PJ, Chik G, Crook MA: Efficacy of ezetimibe in patients with statin-resistant and statin-intolerant familial hyperlipidaemias. *Curr Med Res Opin*, 2005; 21: 333-338
- 27) Foody JM, Brown WV, Zieve F, Adewale AJ, Flaim D, Lowe RS, Jones-Burton C, Tershakovec AM: Safety and efficacy of ezetimibe/simvastatin combination versus atorvastatin alone in adults ≥ 65 years of age with hypercholesterolemia and with or at moderately high/high risk for coronary heart disease (the VYTELD study). *Am J Cardiol*, 2010; 106: 1255-1263
- 28) Baigent C, Landray MJ, Reith C, Emberson J, Wheeler DC, Tomson C, Wanner C, Krane V, Cass A, Craig J, Neal B, Jiang L, Hooi LS, Levin A, Agodoa L, Gaziano M, Kasiske B, Walker R, Massy ZA, Feldt-Rasmussen B, Krairittichai U, Ophascharoensuk V, Fellstrom B, Holdaas H, Tesar V, Wiecek A, Grobbee D, de Zeeuw D, Gronhagen-Riska C, Dasgupta T, Lewis D, Herrington W, Mafham M, Majoni W, Wallendszus K, Grimm R, Pedersen T, Tobert J, Armitage J, Baxter A, Bray C, Chen Y, Chen Z, Hill M, Knott C, Parish S, Simpson D, Sleight P, Young A, Collins R: The effects of lowering LDL cholesterol with simvastatin plus ezetimibe in patients with chronic kidney disease (Study of Heart and Renal Protection): a randomised placebo-controlled trial. *Lancet*, 2011; 377: 2181-2192

Supplementary Fig. 1. The correlation between the effect of ezetimibe on urinary albumin and LDL cholesterol (A), urinary albumin and small dense LDL cholesterol (B), glomerular size and LDL cholesterol (C), glomerular size and small dense LDL cholesterol (D) in db/db mice.

Supplementary Fig. 2. The correlation between the effect of ezetimibe on urinary albumin and AdipoR1 expression in the kidney (A), LDL cholesterol and AdipoR1 expression in the kidney (B) of db/db mice.