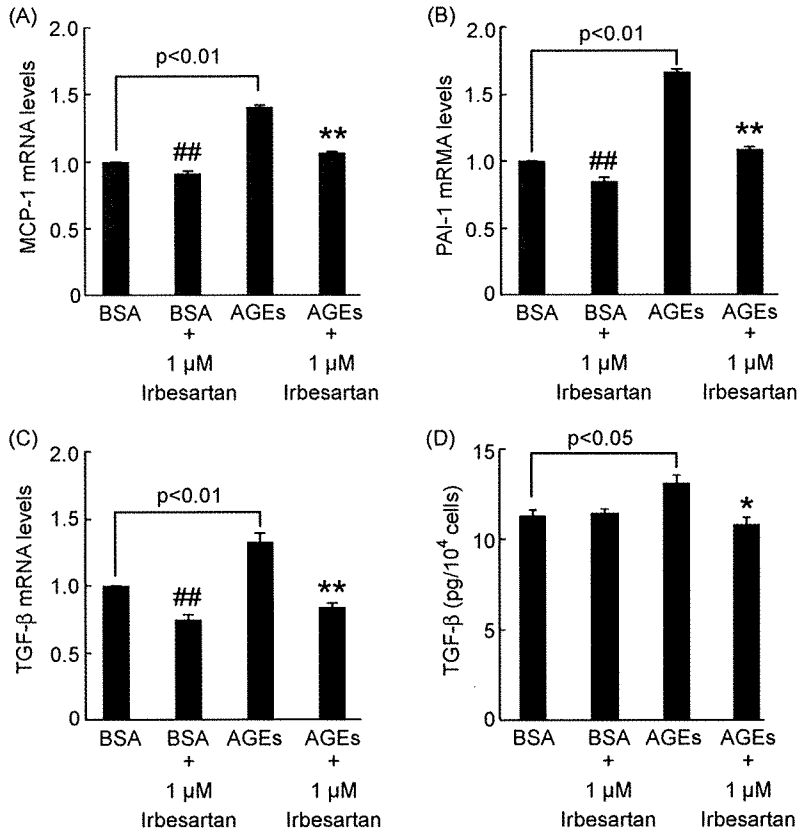


**Fig. 4.** Effects of irbesartan MCP-1 (A), PAI-1 (B), and TGF-β (C and D) expressions in tubular cells. Cells were treated with 100 μg/ml AGE-BSA or non-glycated BSA in the presence or absence of 1 μM irbesartan for 24 h. Then total RNAs were transcribed and amplified by real-time PCR (A–C). Data were normalized by the intensity of β-actin mRNA-derived signals and then related to the value obtained with non-glycated BSA. (D) Conditioned medium were collected and analyzed for TGF-β in an ELISA. $^{\#\#}p < 0.01$ compared to the value with non-glycated BSA alone. $^{**}p < 0.01$ compared to the value with AGEs alone. $N = 3$ per group.

### 2.5. Dihydroethidium (DHE) staining

Tubular cells were treated with 100 μg/ml AGE-BSA or non-glycated BSA in the presence or absence of 1 μM irbesartan for 4 or 24 h, and then the cells were incubated with phenol red free Dulbecco's modified Eagle medium containing 3 μM DHE (Molecular Probes Inc., Eugene, OR, USA). After 15 min, the cells were imaged under a laser-scanning confocal microscope. Intensity of DHE staining in five different field of each sample was analyzed by microcomputer-assisted NIH image.

### 2.6. Measurement of apoptotic cell death of tubular cells

Tubular cells were treated with 100 μg/ml AGE-BSA or non-glycated BSA in the presence or absence of 1 μM irbesartan for 12 or 24 h. Then the cells were lysed and the supernatant was analyzed in an enzyme-linked immunosorbent assay (ELISA) for DNA fragments (Cell Death Detection ELISA plus, Roche Molecular Biochemicals, Mannheim, Germany) as described previously [16]. Caspase-3 activity in the cell lysate was analyzed according to the supplier's recommendation (Roche Applied Science, Mannheim, Germany).

### 2.7. Measurement of TGF-β proteins

Tubular cells were treated with 100 μg/ml AGE-BSA or non-glycated BSA in the presence or absence of 1 μM irbesartan for 24 h. Then TGF-β released into media was measured with an ELISA (R&D Systems Inc., Minneapolis, MN, USA) according to the manufacturer's instructions.

### 2.8. Statistics

All values were presented as means ± standard error (S.E.M.). One-way ANOVA followed by the Scheffe *F*-test was performed for statistical comparisons; $p < 0.05$ was considered significant.

## 3. Results

We first investigated whether irbesartan could inhibit RAGE gene expression in tubular cells. Quantitative real-time RT-PCR analysis revealed that AGE-BSA up-regulated RAGE mRNA levels in tubular cells; compared with non-glycated BSA, 4- and 24-h treatment with AGE-BSA increased RAGE mRNA levels by about 1.7- and 1.2-fold, respectively (Fig. 1A and B). One μM irbesartan significantly blocked the AGE-induced increase of RAGE mRNA levels in tubular cells (Fig. 1A and B).

AGEs exert pleiotropic actions on various types of cells by inducing the generation of intracellular reactive oxygen species (ROS) through the interaction with cell surface receptor, RAGE [7–9,17,18]. So, we next investigated whether irbesartan could block the AGE-elicited ROS generation in tubular cells. As shown in Fig. 2A and B, AGE-BSA increased ROS generation in tubular cells; incubation with AGE-BSA for 24 h increased ROS generation by about 1.4-fold, which was blocked by the treatment with irbesartan.

We next investigated the effects of irbesartan on apoptotic cell death of AGE-exposed tubular cells. As shown in Fig. 3A and B, 12- and 24-h treatment with AGE-BSA induced apoptotic cell death of tubular cells, which were inhibited by irbesartan. Caspase-3 plays a key role in the execution of apoptosis [19]. Therefore, we further examined whether AGEs could increase caspase-3 activity in tubular cells. As shown in Fig. 3C, AGE-BSA significantly increased

caspase-3 activity in tubular cells, which was blocked by the treatment with irbesartan.

Further, irbesartan was found to inhibit the AGE-induced up-regulation of MCP-1, PAI-1 and TGF-β mRNA levels in tubular cells (Fig. 4A–C). TGF-β overproduction by AGE-exposed tubular cells was also blocked by the treatment with irbesartan (Fig. 4D).

## 4. Discussion

In this study, we demonstrated for the first time that irbesartan, an ARB inhibited the AGE-elicited apoptosis and inflammatory, thrombogenic and fibrogenic gene expressions (MCP-1, PAI-1 and TGF-β gene expressions), and TGF-β production in cultured human proximal tubular cells provably by suppressing RAGE levels and subsequent ROS generation. The present findings have extended our previous observations that other types of ARBs such as olmesartan and telmisartan inhibited the AGE-signaling to angiogenesis, inflammation and thrombogenesis by reducing the RAGE expression in cultured endothelial cells [20–23]. Therefore, our present study suggests that there could also exist a pathophysiological crosstalk between the RAS and the AGE-RAGE axis in proximal tubular cell apoptosis and damage. Blockade of the RAS by irbesartan may play a protective role against tubular injury in diabetes by attenuating the deleterious effects of AGEs via down-regulation of RAGE. Chronic tubulointerstitial damage in the kidney, including tubular atrophy and interstitial inflammation fibrosis, is more important than glomerulopathy in terms of renal prognosis in diabetic nephropathy [2,3]. Further, renal MCP-1, PAI-1 and TGF-β overexpressions are involved in tubulointerstitial injury [24–26]. These observations suggest that BP lowering-independent renoprotective effects of irbesartan observed in type 2 diabetic patient could be ascribed, at least in part, to its AGE-RAGE axis blockade properties [6].

*In vitro*-modified AGEs were prepared by incubating BSA with glyceraldehyde for 1 week; this process produces relatively highly modified proteins in comparison to those *in vivo*. However, it is unlikely that extensively modified, unphysiologic AGEs that were formed under the *in vitro*-conditions may exert non-specific and toxic effects on tubular cell damage for the following reasons: we have previously found that immunological epitope of glyceraldehyde-modified AGEs was actually present in serum of diabetic patients and that the concentration (100 μg/ml) of *in vitro*-prepared AGEs used here were comparable with those of the *in vivo* diabetic situation [27,28]. Further, the peak plasma concentration of irbesartan is reported to be about 1–2 μM [29]. So, the concentration of irbesartan having beneficial effects on tubular cells used in the present experiments (1 μM) may also be comparable to the therapeutic levels which are achieved in the treatments for patients with hypertension.

In the present study, we found that irbesartan treatment for 4 h significantly inhibited the AGE-induced up-regulation of RAGE mRNA levels, but not ROS generation in tubular cells (Figs. 1A and 2A). These findings suggest that RAGE suppression is the primary target for the anti-oxidative, anti-apoptotic anti-inflammatory, anti-thrombogenic and anti-fibrogenic effects of irbesartan on proximal tubular cells. Although we did not clarify here the precise molecular mechanisms by which irbesartan reduced RAGE gene expression in tubular cells, irbesartan may reduce RAGE mRNA levels in AGE-exposed tubular cells by blocking the endogenous angiotensin II actions because we have previously found that angiotensin II itself up-regulates RAGE mRNA levels in endothelial cells and vascular pericytes [30,31]. Since AGE-RAGE interaction exerts pleiotropic actions on various cells via ROS generation [7–9,17,18], reduction of RAGE expression by irbesartan may block the AGE-signaling to tubular cell apoptosis and damage by inhibiting the ROS generation. Our present study provides a novel beneficial aspect of irbesartan on diabetic nephropathy; it could work as an agent against the AGE-RAGE axis and may play a protective role against diabetic nephropathy.

## 5. Limitations

In this study, ROS generation was evaluated with DHE staining. So, we could not determine the source of ROS generation in AGE-exposed tubular cells. However, since we have previously found that AGE-RAGE interaction elicits ROS generation in various types of cells such as endothelial cells and mesangial cells via NADPH oxidase activation [13,18,32,33], it is conceivable that NADPH oxidase is mainly involved in ROS generation in AGE-exposed tubular cells as well. It would be helpful to examine whether expression of the components of NADPH oxidase was increased by the treatment with AGE-BSA in tubular cells and if an inhibitor of NADPH oxidase, diphenylene iodonium, blocked the AGE-evoked ROS generation in these cell type.

In the present experiments, we chose the concentration of AGE-BSA at 100 μg/ml and irbesartan at 1 μM because the concentrations of each agent were comparable with those of the *in vivo* situation as described above. However, it would be better to examine the effects of different concentrations of AGE-BSA and irbesartan on tubular cells.

In the present study, we did not examine the effects of irbesartan on extracellular matrix protein expression in AGE-exposed tubular cells. However, we found here that irbesartan inhibited the AGE-induced TGF-β overproduction by tubular cells. Since TGF-β is a major etiologic agent in tubulointerstitial fibrosis in diabetic nephropathy [15], it is conceivable that irbesartan could also reduce the extracellular matrix protein expression in AGE-exposed tubular cells.

In this study, we did not investigate the effects of angiotensin II on RAGE expression, ROS generation and apoptosis in tubular cells. However, angiotensin II-induced ROS generation was already reported to be involved in apoptotic cell death of proximal tubular epithelial cells, the same cells used in the present experiments [34]. Further, we have previously shown that angiotensin II type 1 receptor-mediated ROS generation elicits RAGE gene overexpression in pericytes, thereby augmenting the AGE-induced pericyte apoptosis, the earliest hallmark of diabetic retinopathy [31]. These observations further support the pathophysiological crosstalk between the AGE-RAGE system and RAS in tubular injury, although effects of angiotensin II on RAGE expression in tubular cells remain unknown.

## Conflict of interest

There is no conflict of interest.

## Acknowledgment

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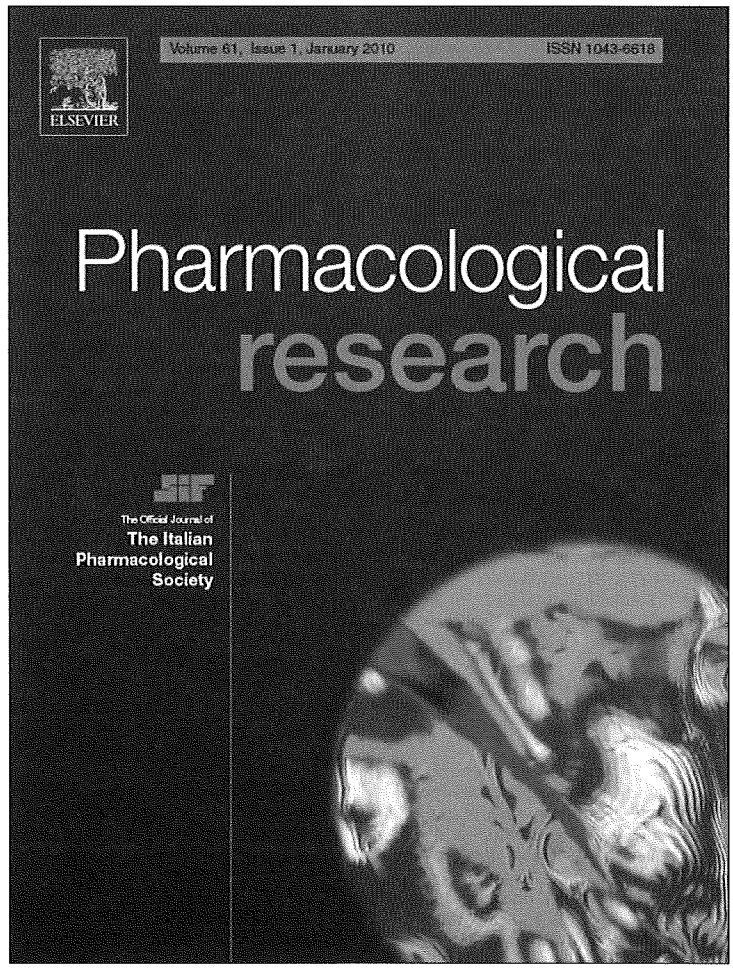

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# Co-administration of ezetimibe enhances proteinuria-lowering effects of pitavastatin in chronic kidney disease patients partly via a cholesterol-independent manner

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ABSTRACT

Since co-administration of ezetimibe, a specific inhibitor of cholesterol absorption into the intestine, has been shown to augment lipid-lowering effects of statins, ezetimibe plus statins is a novel therapeutic strategy for the treatment of dyslipidemia in high-risk patients. Statins have been shown to ameliorate renal function and reduce proteinuria in patients with chronic kidney disease (CKD). However, effects of co-administration of ezetimibe with statins on renal damage and dysfunction in CKD patients remain unknown. In this study, we examined whether co-administration of ezetimibe with pitavastatin could augment renoprotective properties of pitavastatin in non-diabetic CKD patients with dyslipidemia. Total cholesterol, LDL-cholesterol and triglycerides levels were reduced more by co-administration of ezetimibe (10 mg/day) with pitavastatin (2 mg/day) ($n = 10$) than by pitavastatin alone ($n = 10$). In addition, ezetimibe plus pitavastatin treatment produced significant incremental reduction in proteinuria related to pitavastatin therapy alone. In univariate analyses, proteinuria was correlated with plasma levels of total cholesterol, LDL-cholesterol, triglycerides, HDL-cholesterol (inversely), asymmetric dimethylarginine, an endogenous nitric oxide synthase inhibitor, and urinary excretion levels of L-fatty acid binding protein (L-FABP), a marker of tubular injury and 8-hydroxydeoxyguanosine (8-OHdG), an oxidative stress marker. Multiple stepwise regression analysis revealed that LDL-cholesterol ($p < 0.001$) and urinary excretion levels of L-FABP ($p = 0.001$) and 8-OHdG ($p < 0.001$) were independently related to proteinuria ($R^2 = 0.969$). Our present study demonstrated for the first time that co-administration of ezetimibe enhanced proteinuria-lowering effects of pitavastatin in non-diabetic CKD patients partly via a cholesterol-independent manner. Ezetimibe may have pleiotropic actions that could contribute to renoprotective properties of this lipid-lowering agent.



## 1. Introduction

Ezetimibe is a specific inhibitor of cholesterol absorption into the intestine, and a widely used drug for the treatment of dyslipidemia, a well-known traditional risk factor for cardiovascular disease (CVD) [1–3]. Since co-administration of ezetimibe has been shown to efficiently augment lipid-lowering effects of statins [3,4], a dual inhibition of cholesterol absorption and synthesis by ezetimibe and statins may be a novel therapeutic strategy for preventing CVD in high-risk patients with dyslipidemia.

Dyslipidemia contributes not only to CVD but also to the progression of chronic kidney disease (CKD) [5,6]. Indeed, experimental studies have demonstrated that lipid deposition elicits pro-inflammatory and pro-fibrotic reactions in the kidney, thus being involved in the development and progression of CKD [5,6]. Further, clinical studies have shown that dyslipidemia is associated with an increased risk for progressive decline of renal function, and statins ameliorate renal function and reduce proteinuria in patients with CKD [5–9]. These observations suggest that control of dyslipidemia is one of the targets for the treatment of CKD. Since

*Abbreviations:* CVD, cardiovascular disease; CKD, chronic kidney disease; T-Chol, total cholesterol; LDL-C, low-density lipoprotein-cholesterol; TG, triglycerides; HDL-C, high-density lipoprotein-cholesterol; Cr, creatinine; BP, blood pressure; eGFR, glomerular filtration rate; MDRD, modification of diet in renal disease; ADMA, asymmetric dimethylarginine; 8-OHdG, 8-hydroxydeoxyguanosine; ELISA, enzyme-linked immunosorbent assay; L-FABP, L-fatty acid binding protein; SD, standard deviation; DDAH, dimethylarginine dimethylaminohydrolase.

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statins have pleiotropic effects, *that is*, anti-inflammatory and anti-oxidative properties *in vivo* [1], it is generally thought that statins could protect against the development and progression of CKD via both cholesterol lowering-dependent and -independent manners. However, effects of co-administration of ezetimibe with statins on renal damage and dysfunction in CKD patients remain unknown. Therefore, in this study, we examined whether co-administration of ezetimibe with pitavastatin could augment renoprotective properties of pitavastatin in CKD patients. For this, we compared effects of pitavastatin plus ezetimibe treatments on renal function, proteinuria, and urinary excretion levels of 8-hydroxydeoxyguanosine (8-OHdG) and L-fatty acid binding protein (L-FABP) with those of pitavastatin therapy alone in non-diabetic CKD patients with dyslipidemia.

## 2. Subjects and methods

### 2.1.1. Subjects

Twenty non-diabetic stage I or II CKD patients with dyslipidemia (total cholesterol (T-Chol) > 220 mg/dl, low-density lipoprotein-cholesterol (LDL-C) > 140 mg/dl, 150 < triglycerides (TG) < 400 mg/dl, or high-density lipoprotein-cholesterol (HDL-C) < 40 mg/dl) (14 males and 6 females, mean age: 38.9 years old) were enrolled in the present study. All subjects were introduced to our hospital for histopathological diagnosis of the significant proteinuria (ca. 1.5 g/day) (IgA nephropathy $n = 14$, non-IgA type proliferative glomerulonephritis $n = 3$, membranous nephropathy $n = 2$, focal glomerular sclerosis $n = 1$). We excluded any patients with chronic pulmonary disease, collagen disease, liver disease, neoplastic disorders, and those who had recent (<6 months) acute coronary syndromes, stroke and any acute infections. Patients whose age was younger than 20 years or older than 70 years, whose serum creatinine (Cr) level more than 1.2 mg/dl, or whose proteinuria more than 3.0 g/day were excluded. There were no patients with nephrotic syndrome. Twenty patients were randomly assigned into 2 groups: one was a group treated with 2 mg pitavastatin once daily (7 males and 3 females, IgA nephropathy $n = 7$, non-IgA type proliferative glomerulonephritis $n = 2$, membranous nephropathy $n = 1$, mean age; 39.1 years) and the other with 2 mg pitavastatin + 10 mg ezetimibe once daily (7 males and 3 females, IgA nephropathy $n = 7$, non-IgA type proliferative glomerulonephritis $n = 1$, focal glomerular sclerosis $n = 1$, membranous nephropathy $n = 1$, mean age; 38.7 years). These drugs have not been administered to patients before. So, ezetimibe and pitavastatin treatments started all together. In addition, these drugs had not been changed during the study periods as well. Further, all patients received salt restriction (<5 g/day NaCl), but none of them received anti-hypertensive or other anti-dyslipidemic drugs such as angiotensin-converting enzyme inhibitors, angiotensin II type 1 receptor blockers, fibrates, probucol and colestimide during the 6 months because there were no patients whose blood pressure (BP) levels were more than 140/90 mmHg during the study periods. The study protocol was approved by the local ethical committee of Shinmatsudo Central General Hospital, and informed consent was obtained from all study participants. The study complied with the principles of the Helsinki Declaration.

### 2.1.2. Data collection

Blood pressure (BP) was measured in the sitting position twice after 2 min of rest using an upright standard sphygmomanometer. Renal function was evaluated by serum Cr levels and estimated glomerular filtration rate (eGFR) according to the modification of diet in renal disease (MDRD) equation modified for the Japanese population [10]. Serum levels of T-Chol, TG and HDL-C were measured enzymatically at Shinmatsudo Central General Hospital. LDL-C level was calculated using Friedewald's formula. Serum level of asymmetric dimethylarginine (ADMA), an endogenous nitric oxide synthase inhibitor was analyzed by a high-performance liquid chromatography as described previously [11]. Urinary excretion levels of 8-hydroxydeoxyguanosine (8-OHdG), an oxidative stress marker, were measured by an enzyme-linked immunosorbent assay (ELISA) as reported previously [12]. Urinary L-fatty acid binding protein (L-FABP), a marker of tubular injury, was measured with an ELISA kit according to the manufacturer's protocol (CIMC, Tokyo, Japan) [13]. Total urinary protein excretion levels were determined with a pyrogallol red method (Wako, Osaka, Japan).

### 2.2. Statistical methods

Data were expressed as mean ± standard deviation (SD). To compare the parameters, we used the Wilcoxon signed-rank test. A correlation between proteinuria and other clinical variables was determined by a linear regression analysis. To determine independent determinants of proteinuria, multiple stepwise regression analysis was performed. Statistical significance was defined as $p < 0.05$. All statistical analyses were performed with the use of the SAS system (SAS Institute, Cary, NC).

**Table 1**
Clinical variables before and after treatment.

| Characteristics | Pitavastatin | | Pitavastatin + ezetimibe | |
|---|---|---|---|---|
| | Before | After | Before | After |
| Age (years) | 40.0 ± 7.4 | | 38.7 ± 8.8 | |
| Number (male number) | 10(7) | | 10(7) | |
| Systolic BP (mmHg) | 129.8 ± 3.6 | 129.4 ± 2.7 | 128.4 ± 6.1 | 128.0 ± 4.7 |
| Diastolic BP (mmHg) | 79.4 ± 4.2 | 78.8 ± 4.9 | 78.6 ± 4.2 | 78.4 ± 3.4 |
| T-Chol (mg/dl) | 239.0 ± 16.3 | 172.2 ± 22.6* | 243.6 ± 12.8 | 154.2 ± 10.5$^{++\#}$ |
| HDL-C (mg/dl) | 44.3 ± 4.8 | 46.8 ± 3.9* | 43.1 ± 2.9 | 46.2 ± 1.7$^{+}$ |
| LDL-C (mg/dl) | 162.6 ± 12.6 | 97.9 ± 18.4* | 167.9 ± 10.8 | 82.2 ± 10.0$^{++\#}$ |
| TG (mg/dl) | 160.6 ± 10.1 | 140.4 ± 7.9* | 162.8 ± 9.7 | 128.8 ± 6.9$^{++\#\#}$ |
| Serum Cr (mg/dl) | 0.71 ± 0.06 | 0.70 ± 0.05 | 0.71 ± 0.06 | 0.71 ± 0.06 |
| eGFR (ml/min) | 91.2 ± 11.7 | 91.7 ± 12.6 | 91.5 ± 13.6 | 91.8 ± 13.7 |
| 8-OHdG (ng/mg Cr) | 24.2 ± 3.5 | 19.6 ± 3.3* | 24.3 ± 3.6 | 16.7 ± 3.4$^{++}$ |
| L-FABP (μg/g Cr) | 57.8 ± 16.5 | 34.4 ± 14.0* | 57.6 ± 18.7 | 29.4 ± 12.9$^{++}$ |
| Proteinuria (mg/day) | 1512.0 ± 245.7 | 1042.0 ± 229.9* | 1508.0 ± 263.9 | 786.0 ± 219.3$^{++\#}$ |
| ADMA (nmol/ml) | 0.59 ± 0.05 | 0.52 ± 0.03* | 0.60 ± 0.08 | 0.51 ± 0.06$^{++}$ |

Data are shown as mean ± SD, unless otherwise indicated. *$p < 0.001$ vs. before pitavastatin treatment. $^{+}p < 0.01$, $^{++}p < 0.001$ vs. before pitavastatin + ezetimibe treatment. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ vs. after pitavastatin treatment alone.

**Table 2**
Univariate and stepwise multiple regression analyses for determinants of proteinuria.

| Factors | Univariate[a] | | Multivariate[b] | | |
|---|---|---|---|---|---|
| | $\beta$ | $p$ | $\beta$ | $F$ | $p$ |
| Age (years) | −0.019 | 0.905 | | | |
| Sex (%male) | −0.040 | 0.806 | | | |
| Systolic BP (mmHg) | 0.042 | 0.798 | | | |
| Diastolic BP (mmHg) | 0.008 | 0.961 | | | |
| Serum Cr (mg/dl) | 0.130 | 0.425 | | | |
| eGFR (ml/min) | −0.014 | 0.933 | | | |
| T-Chol (mg/dl) | 0.689 | <0.001 | | | |
| LDL-C (mg/dl) | 0.723 | <0.001 | 0.185 | 424.7 | <0.001 |
| TG (mg/dl) | 0.725 | <0.001 | | | |
| HDL-C (mg/dl) | −0.592 | <0.001 | | | |
| 8-OHdG (ng/mg Cr) | 0.968 | <0.001 | 0.557 | 559.7 | <0.001 |
| L-FABP (μg/g Cr) | 0.949 | <0.001 | 0.312 | 377.5 | 0.001 |
| ADMA (nmol/ml) | 0.885 | <0.001 | | | |

$R^2 = 0.969$.
[a] Univariate coefficients. $\beta$: regression coefficients.
[b] A stepwise multivariate regression analysis was performed.

## 3. Results

Clinical variables before and after treatments are shown in Table 1. All patients were normotensive and dyslipidemic. Pitavastatin treatment (2 mg/day) for 6 months significantly ($p < 0.001$) decreased plasma levels of T-Chol, LDL-C, TG and ADMA, while it increased HDL-C levels ($p < 0.001$). Further, pitavastatin treatment significantly ($p < 0.001$) reduced urinary excretion levels of protein, L-FABP and 8-OHdG. T-Chol, LDL-C and TG levels were reduced more ($p < 0.05$, $p < 0.05$ and $p < 0.01$, respectively) by co-administration of ezetimibe (10 mg/day) with pitavastatin (2 mg/day) than by pitavastatin alone. In addition, ezetimibe plus pitavastatin treatment produced significant incremental reduction in proteinuria relative to pitavastatin therapy alone. As shown in Table 2, univariate analyses revealed that proteinuria was correlated with plasma levels of T-Chol, LDL-C, TG, HDL-C (inversely), ADMA and urinary excretion levels of L-FABP and 8-OHdG. Because the parameters could be closely correlated with each other, to determine independent determinants of proteinuria, multiple stepwise regression analysis was performed. This analysis showed that LDL-C ($p < 0.001$) and urinary excretion levels of L-FABP ($p = 0.001$) and 8-OHdG ($p < 0.001$) were independently related to proteinuria ($R^2 = 0.969$) (Table 2).

## 4. Discussion

In the present study, we demonstrated for the first time that co-administration of ezetimibe treatment with pitavastatin for 6 months more ameliorated dyslipidemia and produced significant incremental reduction in proteinuria in non-diabetic CKD patients, compared with pitavastatin monotherapy. Further, we found here that besides LDL-C levels, urinary excretion levels of L-FABP and 8-OHdG, markers of tubular injury and oxidative stress, respectively, were independently correlated with proteinuria in our subjects. Therefore, although further reductions in urinary excretion levels of L-FABP and 8-OHdG by co-administration of ezetimibe therapy were modest and not significant (Table 1), our present study suggests that ezetimibe treatment may decrease proteinuria partly via reduction of urinary excretion of L-FABP and 8-OHdG. There is accumulating evidence that proteinuria is not merely a biomarker for the progression of CKD, but also a mediator of this devastating disorder [14,15]. Further, proteinuria is a strong and independent indicator of CVD in CKD patients as well [16]. These observations suggest that co-administration of ezetimibe could have beneficial effects on cardiovascular-renal systems by reducing proteinuria in non-diabetic CKD patients with dyslipidemia via both cholesterol-dependent and -independent manners. It should be noted that rosuvastatin had no significant effect on composite primary end point of death from cardiovascular causes, nonfatal myocardial infarction, or nonfatal stroke in patients undergoing hemodialysis patients [17]. Therefore, effect of statin plus ezetimibe treatments on cardiovascular disease in patients with severe renal disorder remains unclear.

The present study has extended to our previous finding that ezetimibe decreased serum ADMA levels and improved renal damage in non-diabetic stage I-II CKD patients with dyslipidemia in a cholesterol-independent manner [18]. Although we did not clarify here how ezetimibe co-treatment decreased proteinuria in our CKD patients, anti-oxidative properties of this agent may play some role because (1) ezetimibe was recently shown to suppress oxidative stress generation in the aorta of apoE-deficient mice [19], (2) co-administration of ezetimibe reduced urinary excretion levels of 8-OHdG in our subjects, and (3) there are several papers that oxidative stress is involved in proteinuria and tubular injury in CKD patients [20–22]. ADMA may cause proteinuria via endothelial dysfunction in CKD patients [23,24], and oxidative stress increases ADMA production by various types of cells by inactivating dimethylarginine dimethylaminohydrolase (DDAH), a rate-limiting enzyme that degrades ADMA [25–27]. Ezetimibe co-administration may also block the crosstalk between ADMA and proteinuria via its anti-oxidative properties.

We also showed here first that ezetimibe co-treatment reduced urinary excretion levels of L-FABP. Urinary L-FABP, a marker of tubular damage, is more sensitive than urinary protein in predicting the progression of CKD [13]. Further, changes within tubulointerstitium are shown to be more important than glomerulopathy in terms of renal prognosis in patients with CKD [28]. Co-administration of ezetimibe may have proteinuria-lowering effects in CKD patients by improving tubulointerstitial injury.

## 5. Limitations

Number of this study is small, therefore, further large clinical study is needed to elucidate whether reduction of proteinuria by ezetimibe co-treatment with pitavastatin could be mechanistically related to cardiorenal protection in non-diabetic CKD patients. However, in multiple stepwise regression analysis, besides LDL-C, urinary excretion levels of 8-OHdG was independently related to proteinuria, thus suggesting that renoprotective effects of pitavastatin and ezetimibe co-treatments may be partly ascribed to its anti-oxidative properties. In addition, high salt intake is associated with oxidative stress [29]. Therefore, although all patients received salt restriction (<5 g/day NaCl) during the treatment period, we cannot totally exclude the possibility that daily sodium intake during the treatment period could contribute to or interfere with the results of this study. In this study, we did not evaluate urinary excretion levels of β2-microglobulin and/or N-acetyl-glucosaminidase, classical markers of tubular lesions. However, since urinary excretion levels of L-FABP is reported to accurately reflect the severity of tubulointerstitial damage [30], our present findings suggest that co-administration of ezetimibe may be effective against tubulointerstitial injury in non-diabetic stage I–II CKD patients.

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# SIRT1, a Calorie Restriction Mimetic, in a New Therapeutic Approach for Type 2 Diabetes Mellitus and Diabetic Vascular Complications

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**Abstract:** The rising incidence of diabetes, metabolic syndrome, and subsequent vascular diseases is now a major public health problem in industrialized countries. New therapeutic strategies to prevent these diseases are urgently needed worldwide. It is well known that calorie restriction (CR) can retard the aging process in organisms ranging from yeast to rodents, and delay the onset of numerous age-related diseases including diabetes. Molecules that mimic CR metabolically are therefore potentially new therapeutic targets for age-related diseases. Silent information regulator 2 (Sir2) is an important player in CR-mediated life span extension. There is also increasing evidence that one of the seven mammalian sirtuins, SIRT1, is involved in regulating cellular processes such as apoptosis. SIRT1 has also been implicated in glucose homeostasis and lipid metabolism in various tissues including adipose tissues, liver, pancreas, and skeletal muscle. This review summarizes current understanding of the biological functions of SIRT1, and discusses its potential as a pharmacological target for fighting metabolic and vascular diseases.

**Key Words:** Diabetes, sirtuins, calorie restriction, CR mimetics, vascular complications, SIRT1 activator.

## INTRODUCTION

Obesity is associated with impaired organ function, serious disease, and premature mortality [1, 2]. The worldwide prevalence of obesity has increased markedly over recent decades [3], with a consequent jump in the incidence and prevalence of diabetes. Most of these patients have type 2 diabetes mellitus (T2DM), which represents almost 90% of all diabetes cases. T2DM is a multifactorial disease associated with various microvascular (retinopathy, neuropathy, and nephropathy) and macrovascular (ischemic heart disease, cerebrovascular disease, and peripheral vascular diseases) complications [4-6]. People with T2DM are two to four times more likely to develop cardiovascular disease (CVD) compared with non-diabetics [7]. Lower-extremity amputations are at least 10 times more common in people with T2DM than in nondiabetic individuals in developed countries, and more than half of all nontraumatic lower-limb amputations are due to T2DM [8]. Furthermore, T2DM is the leading cause of end-stage renal failure in many populations in both developed and developing countries. T2DM is also a leading cause of visual impairment and blindness in developed countries [9]. Thus, diabetes is a significant risk factor for macro- and microvascular diseases, and causes considerable disability, premature mortality, loss of productivity, and increased demands on health care facilities. T2DM and its vascular complications are now considered major and growing health issues in most countries, and new therapeutic strategies to prevent T2DM are urgently needed.

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In 1935, McCay *et al.* at Cornell University published the first report of calorie restriction (CR) extending median and maximum lifespan in rats, when implemented after puberty. CR also prevented or at least attenuated the severity of chronic disease. Subsequent data showed that CR retards aging and extends maximum lifespan in several organisms including yeast, flies, worms, fish, and rodents [10, 11]. The effect of CR on longevity in humans is difficult to determine because no validated biomarkers exist as surrogate markers of aging and because it is impractical to conduct randomized, diet-controlled, long-term survival studies in humans [12]. Nonetheless, epidemiological data suggest that CR also has beneficial effects in humans, not only on the pathogenesis of primary and secondary aging, but also on life expectancy.

Several randomized, controlled intervention trials have evaluated the effects of CR on age-related variables in nonobese adults. One such study found that a 25% reduction in calorie intake for 6 months decreased visceral fat mass, insulin resistance, body temperature, metabolic rate, and oxidative stress [13]. Another demonstrated that restricting calorie intake by 20% for 12 months reduced visceral fat mass, decreased levels of circulating inflammatory markers, and improved insulin sensitivity [14]. Thus, CR simultaneously improves multiple metabolic risk factors for cardiovascular disease and other medical abnormalities associated with obesity, promoting CR as a potential cornerstone of therapy for obesity-related diseases including T2DM and its vascular complications.

Despite the demonstrated beneficial effects of CR in humans, restriction of calorie intake seems unlikely to be maintained long term, and there is increasing interest in developing pharmacological agents that can be regarded as "CR mimetics". Such agents could provide the metabolic, hormonal,

and physiological benefits of CR without the practical difficulties of restricting dietary intake or the potential adverse consequences of excessive restriction. Several recent studies have implicated SIRT1 as a common mediator of the observed CR-induced effects in mammals. SIRT1 is one of the seven mammalian sirtuin/Sir2 NAD-dependent deacetylases [15, 16] and it regulates various biological functions in several tissues, including cell survival, mitochondria biogenesis [17], insulin secretion [18, 19], and glucose/lipid metabolism [20, 21]. These findings led to a proposed role for SIRT1 activation in mimicking CR.

This review describes the current understanding of SIRT1 biological functions and discusses potential pharmacological targets based on SIRT1 activity for preventing metabolic diseases such as T2DM and associated vascular complications.

## THE MAMMALIAN SIRTUINS: SIRT1–7

The mammalian sirtuin family consists of seven members, SIRT1–7. Family members are characterized by a conserved 275-amino-acid catalytic core domain and unique N-terminal and/or C-terminal sequences of variable length (Table **1**). Phylogenetic analysis of 60 core domains from different eukaryote and prokaryote sirtuins places the mammalian members into four different classes (I–IV). SIRT1, SIRT2, and SIRT3 are Class I sirtuins, as are all yeast sirtuins and at least one of the Sir2-related proteins in most eukaryotes. Class I is divided into three subclasses: a, b, and c. SIRT1 is a Class Ia protein, along with Sir2 and Hst1 from *Saccharomyces cerevisiae*, *Caenorhabditis elegans* SIR-2.1, and *D. melanogaster 1*. SIRT2 and SIRT3 belong to Class Ib, together with yeast Hst2, fly *D.mel2*, and other fungal and protozoan sirtuins. SIRT4 is a Class II protein, together with sirtuins from bacteria, insects, nematodes, mould fungi, and protozoa. SIRT5 is the sole mammalian Class III sirtuin, a form distributed widely in all prokaryotes of either the bacteria or archaea family. Finally, Class IV contains SIRT6 and SIRT7 in two different subclasses, IVa and IVb, respectively. Sirtuins of this class are not present in prokaryotes, but are broadly distributed in metazoans, plants, and vertebrates (Table **1**) [22].

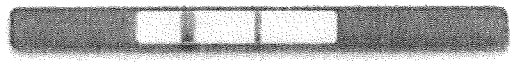
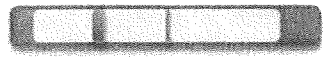
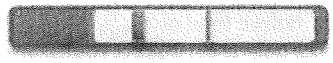
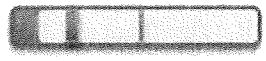
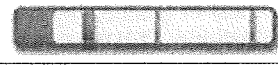
Mammalian sirtuins also differ in their subcellular distributions. SIRT1, SIRT6, and SIRT7 are predominately found in the nucleus (although SIRT1 does have important cytoplasmic functions as well). In the nucleus, most of the SIRT1 is associated with euchromatin, whereas SIRT6 associates with heterochromatin, and SIRT7 localizes at the nucleolus [23]. The sirtuin that resides most prominently in the cytoplasm is SIRT2 [24, 25]. SIRT3, SIRT4, and SIRT5 localize to mitochondria (Table **2**) [26].

In terms of activity (Table **1**), SIRT1 and SIRT5 exhibit robust and weak deacetylase activity, respectively [25, 27]. SIRT4 and SIRT6 are mono-ADP-ribosyl transferases [28, 29], while both deacetylase and mono-ADP-ribosyl transferase activities have been described for SIRT2 and SIRT3 [24-26]. No robust activity has yet been found for SIRT7.

## BIOLOGICAL FUNCTIONS OF SIRT1

When Sir2 was first discovered in yeast 27 years ago, it could hardly have been anticipated how much interest would be taken in this family of proteins. Several studies over the last 8 years have sharpened our understanding of sirtuin

**Table 1. Characteristics of Mammalian Sirtuins**

| Sirtuins | Classification | MW | Shematic of the Structural Domain | Catalytic Activity |
|---|---|---|---|---|
| SIRT1 | Class Ia | 62.0 |  | DAC |
| SIRT2 | Class Ib | 41.5 |  | DAC & ART |
| SIRT3 | Class Ib | 43.6 |  | DAC & ART |
| SIRT4 | Class II | 35.2 |  | ART |
| SIRT5 | Class III | 33.9 |  | DAC |
| SIRT6 | Class IVa | 39.1 |  | ART |
| SIRT7 | Class IVb | 44.8 |  | Unknown |

DAC, Deacetylase; ART, ADP-ribosyl transferase

Catalytic domain

NAD binding site

**Table 2. Characteristics of Mammalian Sirtuins**

| Slirtuins | Chromosomal Location | Localization | Target | Biological Function |
|---|---|---|---|---|
| SIRT1 | 10q21.3 | Nucleus | P53, FOXO, PGC-1α, NF-κB, Smad7, histones, AceCS1 | Anti-apotosis, Metabolism, Neurodegeneration Gene silencing |
| SIRT2 | 19q13.2 | Cytoplasm | H4, α-tubulin | Cell cycle, Tumorigenesis |
| SIRT3 | 11q15.5 | mitochondria | AceCS2 | Metabolism, Thermogenesis |
| SIRT4 | 12q24.31 | Mitochondria | GDH | Insulin secretion, Metabolism |
| SIRT5 | 6q23 | Mitochondria | Unknown | Unknown |
| SIRT6 | 19p13.3 | Nucleus | DNA polymerase β | DNA repair |
| SIRT7 | 17q25.3 | Nucleus | RNA polymerase I | rDNA transcription |

MW, molecular weight; FOXO, forkhead box type O transcription factor; PGC-1α, peroxisome proliferator-activated receptor coactivator 1α; NF-κB, nuclear factor κB; AceCS2, acetyl coemzyme A synthetase 2; GDH, glutamate dehydrogenase.

functions in different organisms. Biological functions now attributed to mammalian sirtuins range from DNA repair to metabolism, with SIRT1 being the most extensively studied.

SIRT1-deficient mice are smaller than their wild-type littermates and frequently have craniofacial abnormalities, although most deficient mice are born live [30-32]. On the 129/J inbred background, all SIRT1-deficient mice die before one month of age. However, if the genetic background is outbred, most SIRT1-deficient mice live for several months [32]. A recent study used SIRT1-deficient mice with a mixed genetic background derived from intercrosses between the CD1 outbred strain and 129/J mice. The findings from this study suggested that SIRT1 is required to promote the beneficial metabolic effects and lifespan expansion observed in mice under CR [33]. The SIRT1-deficient mice were hypermetabolic and showed inefficient mitochondrial function in the liver. When challenged with 40% CR, normal mice maintain their metabolic rate and increase their physical activity, whereas the metabolic rate of these intercrossbred SIRT1-deficient mice dropped and their activity did not increase. Moreover, CR did not extend lifespan in the mutants [33]. While SIRT1-transgenic mice display some phenotypic similarities to littermate control mice on CR [34], they are leaner and more metabolically active. They also have lower blood cholesterol, adipokine levels, insulin, and fasting glucose, and are more glucose tolerant [34]. Thus, SIRT1 is an important regulator of energy metabolism, and might be required for the normal response to CR. Furthermore, SIRT1 is downregulated in insulin-resistant cells and tissues in several experimental models [35] and, even more interestingly, three single nucleotide polymorphisms in the

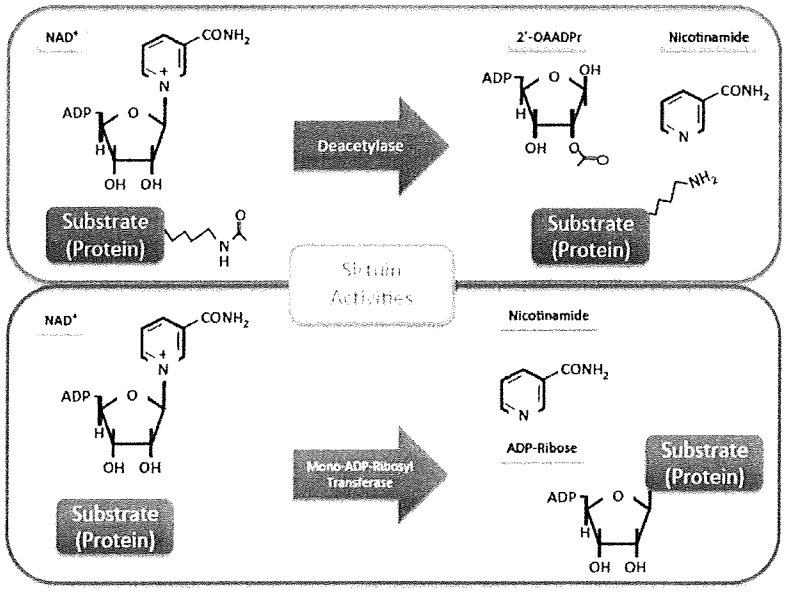



**Fig. (1).** Biological functions of sirtuins. Sirtuins have deacetylase and ADP-ribosyl transferase activities.

SIRT1 gene of Finnish human subjects were significantly associated with systemic energy expenditure [17]. These recent findings implicate SIRT1 as a key regulator of energy and metabolic homoeostasis in both animal models and human subjects. Decreased SIRT1 activity may therefore contribute to the development of obesity-related diseases under excess energy intake, and SIRT1 activation is a potential target for the CR mimetics.

## TISSUE-SPECIFIC FUNCTIONS OF SIRT1

Table **3** and Fig. (**2**) summarize the tissue-specific biological and physiological functions of SIRT1, along with the targets and substrates for SIRT1 deacetylase activity.

### Pancreatic Beta Cells

SIRT1 overexpression in pancreatic β-cells of mice enhanced glucose-stimulated insulin secretion and adenosine triphosphate (ATP) production [18, 19]. These effects were attributed to the SIRT1-mediated transcriptional repression of the uncoupling protein 2 (UCP2) gene, which encodes a mitochondrial inner membrane protein that uncouples $O_2$ consumption from ATP generation to generate heat. High UCP2 expression is observed in SIRT1-deficient mice, along with blunted insulin secretion and lower ATP levels following glucose stimulation of β-cells [18]. Also, by deacetylating FOXO1, SIRT1 promotes activation and transcription of two genes, *NeuroD* and *MafA*, which may preserve insulin secretion and promote β-cell survival *in vivo* [36].

### Adipose Tissues

SIRT1 promotes fat mobilization in white adipose tissue by binding to and repressing genes involved in adipogenesis, such as peroxisome proliferator-activated receptor-γ (PPAR-γ) and fatty acid binding protein (aP2). SIRT1also quenches the PPAR-γ cofactors, nuclear receptor corepressor (NCoR), and silencing mediator of retinoid and thyroid hormone receptors (SMRT) (20). Interestingly, SIRT1 overexpression or pharmacological activation by resveratrol results in PPAR-γ-mediated transcriptional repression, inhibition of adipogenesis, enhanced lipolysis, and the release of free fatty acids [20].

SIRT1 also regulates adiponectin, an adipocyte-derived hormone whose plasma concentrations correlate inversely with adiposity [37, 38]. Adiponectin regulates energy homoeostasis as well as glucose and lipid metabolism [39]. FOXO1 forms a transcriptional complex at the mouse adiponectin promoter with CCAAT/enhancer-binding protein α (C/EBPα). SIRT1 deacetylates FOXO1 and enhances its interaction with C/EBPα, thereby increasing adiponectin concentrations [37]. Administration of adiponectin to high-fat-fed obese mice and to lipoatrophic diabetic mice improved insulin sensitivity while lowering blood glucose, suggesting that adiponectin may protect against the development of insulin resistance and T2DM [40]. Strikingly, CR increases the levels of circulating adiponectin in rats [41], an effect that might be mediated by SIRT1.

### Skeletal Muscle

In mammals, energy and nutrient homeostasis during food deprivation is accomplished *via* regulating the levels of mitochondrial fatty acid oxidation in peripheral tissues. The transcriptional coactivator, PPAR-γ co-activator 1α (PGC-1α) is an important driver of cellular oxidation [42]. Thus, by regulating PGC-1α, SIRT1 modulates mitochondrial function and metabolic homoeostasis, increases consumption of oxygen in muscle fibers, and induces oxidative phosphorylation genes and mitochondria biogenesis [17, 43]. Indeed, fasting-induced PGC-1α deacetylation *via* SIRT1 is required for mitochondrial fatty acid oxidation gene activation in skeletal muscle [43]. SIRT1 activation as a CR mimetic may thus improve insulin resistance by accelerating fatty acid oxidation and mitochondrial biogenesis in skeletal muscle.

### Liver

During the early stages of fasting, upregulated hepatic glucose output and fatty acid oxidation is essential to sustain energetic balance. Production and oxidation of glucose and

**Table 3. Physiological Roles of SIRT1**

| Organs | Biological Fuction | Target |
|---|---|---|
| Adipose tissue | Fat mobilization, Adiponectin secretion | PPARγ ↓, FOXO ↑ |
| Liver | Gluconeogenesis, Lipolysis | PGC-1α ↑, FOXO ↑, CRTC2↓ |
| Muscle | Mitochondrial biogenesis, Fiber change | PGC-1α ↑ |
| Pancreas | Insulin secretion, β-cell survival | UCP-2 ↓, FOXO ↑ |
| Endothelial Cell | Vasodilation | eNOS ↑ |
| Heart | Anti-apoptosis | p53 ↓, H2A.Z |
| Kidney | Anti-apoptosis | p53 ↓, Smad7 ↓ |
| Others | Acetyl-CoA metabolism<br>Anti-inflammation | AceCS1 ↑<br>NF-κB ↓ |

PPAR γ, peroxisome proliferator-activated receptor-γ; FOXO, forkhead box type O transcription factor; PGC-1α, peroxisome proliferator-activated receptor-γ coactivator 1α; UCP-2, Uncoupling protein-2; NF-κB, nuclear factor κB; AceCS2, acetyl coemzyme A synthetase 2; GDH, glutamate dehydrogenase; CRTC2, CREB regulated transcription coactivator 2.

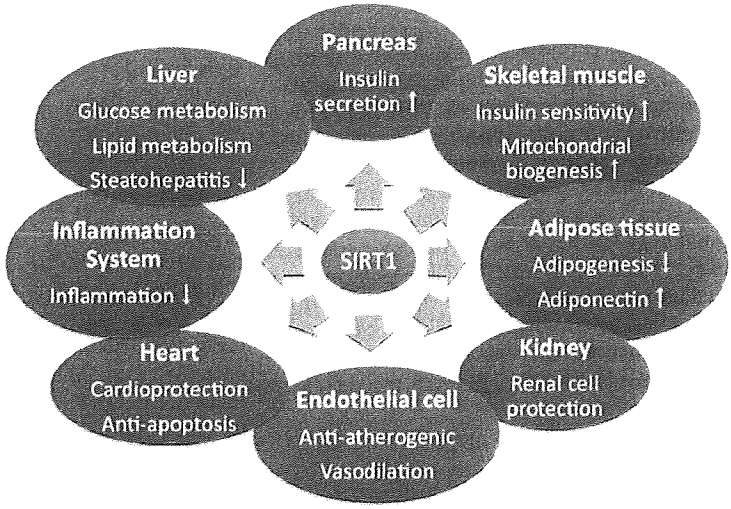



**Fig. (2).** Pleiotropic effects of SIRT1 in diabetes. SIRT1 has multiple effects in organs associated with diabetic mellitus and diabetic vascular complications.

fatty acids in the liver are controlled through a complex network of transcriptional regulators. Amongst these, PGC-1α plays an important role in not only hepatic and systemic glucose metabolism, but also lipid metabolism [42]. SIRT1 regulates genes involved in gluconeogenesis through interaction with and deacetylating PGC-1α [21], which in turn represses glycolysis and increases hepatic glucose output [21]. In contrast to this insight, a recent study demonstrated that SRT1720, an SIRT1 activator, inhibits insulin-induced hepatic glucose production in obese fa/fa rats [44]. More recent evidence also indicates that SIRT1 suppresses hepatic gluconeogenesis in the later phases of fasting [45]. This study also revealed a possible mechanism by which SIRT1 could inhibit gluconeogenesis [45]. In late-phase fasting, gluconeogenesis was suppressed through the inactivation and degradation of the CREB-regulated transcription coactivator 2 (CRTC2, known as TORC2), which positively regulates gluconeogenesis under fasting conditions [45]. SIRT1 is involved in this process through its deacetylase activity. The mechanism underlying SIRT1 activator-mediated inhibition of gluconeogenesis may thus reflect the inhibitory effect of SIRT1 on gluconeogenesis induced late in fasting.

A more recent study revealed that SIRT1 phosphorylates AMP kinase and its substrate ACC, and inhibits FAS induction under high glucose conditions in HepG2 cells , thus inhibiting intracellular lipid accumulation [46]. SIRT1 transgenic mice under a high-fat diet are almost entirely protected from hepatic steatosis [47]. These results highlighted the therapeutic possibilities of SIRT1 activation for improving the fatty liver or steatohepatitis occasionally seen in obese human subjects.

**Inflammatory System**

Growing evidence suggests that inflammation is one of the most important factors underlying the pathogenesis of insulin resistance, with proinflammatory cytokines and other potential mediators of inflammation causally implicated. SIRT1 could participate in the inflammatory process by deacetylating nuclear factor-kappaB (NF-κB). NF-κB subunit, RelA/p65, was identified as the main target of SIRT1 deacetylation [48], while increased acetylation of RelA/p65 has been tightly correlated with a decrease in SIRT1 [49]. Inflammatory markers such as intercellular adhesion molecule 1 (ICAM-1) and tumor necrosis factor (TNF)-α are upregulated during inflammation, which can in turn be inhibited by SIRT1 activation [50]. Interestingly, CR exerts a powerful anti-inflammatory effect in rodents, nonhuman primates, and humans [51], further implicating a role for SIRT1. These recent data together provided useful insights into the roles of SIRT1 in inflammatory pathways. However, there is no direct evidence that SIRT1 reduces inflammation during insulin resistance, and further studies on this particular aspect of SIRT1 function may offer novel and promising targets for anti-inflammatory therapy in such patients.

**Endothelial Cells**

Restricting caloric intake lowers arterial blood pressure in healthy individuals [52, 53], and it improves endothelium-dependent vasodilation in obese and overweight individuals [54]. SIRT1 was also recently implicated in regulating endothelium-dependent vasomotor tone, by demonstrating that it promotes endothelium-dependent vasodilation by targeting endothelial nitric oxide synthase (eNOS) for deacetylation [55]. SIRT1 and eNOS colocalize and coprecipitate in endothelial cells, and SIRT1 deacetylates two lysine residues (Lys496 and 506) of eNOS, thereby stimulating eNOS activity and endothelial nitric oxide (NO) synthesis [55]. These findings implicated SIRT1-mediated deacetylation of eNOS as a possible molecular mechanism that links the effects of CR on the endothelium with those on vascular tone [55]. Thus, SIRT1 activation might promote an anti-atherogenic mechanism through stimulation of eNOS activity in endothelial cells. Indeed, overexpression of endothelial cell-specific SIRT1 attenuated the development of atherosclerotic lesions in apoE knockout mice as well as endothelial dysfunction in diet-induced obese mice [56].

### Kidney

Glomerular cell apoptosis is observed in diabetic kidney [57]. Interestingly, SIRT1 was shown to diminish mesangial cell apoptosis induced by oxidative stress *via* reducing p53 activity [58], and to attenuate transforming growth factor-β (TGF-β)-induced apoptotic signaling mediated by the effector molecule Smad7 [59]. SIRT1-dependent deacetylation of Smad7 at $Lys^{60}$ and $Lys^{70}$ also enhanced the ubiquitin-dependent proteasomal degradation of this effector *via* Smad ubiquitination regulatory factor 1 (Smurf1). Glomerular mesangial cells were thus protected from TGF-β-dependent apoptosis [59]. Both oxidative stress and TGF-β accelerate and contribute to the development diabetic nephropathy [60, 61], strongly implying that SIRT1 could prevent the development of diabetic nephropathy.

### Heart

Myocardial SIRT1 protein expression is decreased in type 2 diabetic *db/db* mouse [62]. Fidarestat, an aldose reductase (AR) inhibitor, attenuated the rise in intracellular $Ca^{2+}$ and prolonged intracellular $Ca^{2+}$ decay observed in cardiomyocytes isolated from *db/db* mice, and this beneficial effect was abrogated by pretreatment with splitomicin, an inhibitor of SIRT1 deacetylase activity [62]. A decrease in SIRT1 could therefore be involved in the pathogenesis of diabetic cardiomyopathy, making SIRT1 activation also a potential therapeutic target for diabetes-associated cardiac dysfunction. An *in vitro* study using cultured cardiomyocytes showed that endogenous SIRT1 is an essential mediator of cardiomyocyte survival under various stress conditions [63]. In addition, a recent study implicated SIRT1 in cardiac hypertrophy using cardiomyocyte-specific SIRT1 transgenic mice [64]. Low to moderate overexpression of SIRT1 in these transgenic mouse hearts attenuated age-dependent and oxidative stress-induced increases in cardiac hypertrophy. These results suggest that SIRT1 could retard aging and confer stress resistance to the heart *in vivo*, although the beneficial effects were observed only with low to moderate doses of SIRT1 [64]. Heart failure is a pathological state in which the heart is unable to pump blood efficiently. It begins with cardiac hypertrophy, followed by increased apoptosis of cardiomyocytes, and culminates in organ failure. Thus, decreasing hypertrophy or apoptosis in cardiomyocytes could ameliorate the disease progression, and there is reason to suspect that SIRT1 activation may be a useful target in this regard.

## STACS (SIRTUIN ACTIVATING COMPOUNDS)

Resveratrol belongs to a group of molecules known as sirtuin activating compounds (STACs). It is a small plant molecule synthesized in response to stress, and was shown to activate mammalian SIRT1 *in vitro* [65]. Consequently, this polyphenolic compound found naturally in the skin of grapes and in a variety of other plants has been shown to extend lifespan in organisms of different phyla, including yeast, worms, flies, and fish [66-69]. Two recent studies addressed the effects of resveratrol in mammals *in vivo*. Baur *et al.* [16] found that administering this polyphenol to middle-aged mice on a high-calorie diet produced a shift in their physiology towards that of mice fed a standard diet, with increased mitochondria, lower blood glucose and insulin, and a hepatic gene expression profile matching that of lean mice and concomitant with increased SIRT1 and PGC1-α activity. Similarly, Lagouge *et al.* [17] showed that mice fed with a high-fat diet supplemented with resveratrol were leaner than control mice not given resveratrol. The supplemented animals also had increased oxidative-type muscle fibers, enhanced resistance to muscle fatigue, lower blood glucose and insulin, and a greater tolerance to cold through the activation of SIRT1 and PGC-1α pathways. Multiple studies also showed that administration of resveratrol could attenuate neuropathy [70-72], cardiac dysfunction [72], endothelial dysfunction [72], and atherosclerotic lesions [73] in diabetic model animals. We also found that resveratrol reversed the development of renal injury in obese type 2 diabetic db/db mice (data not published). Thus, this polyphenolic SIRT1 activator is a potential new therapeutic agent for diabetes and its associated macro- and microvascular complications.

More recently, small-molecule activators of SIRT1 that are 1,000-fold more potent than resveratrol have been identified [44]. These compounds bind to the SIRT1 enzyme-peptide substrate complex at an allosteric site N-terminal to the catalytic domain and lower the Michaelis constant for acetylated substrates [44]. One of these compounds, SRT1720, improved insulin sensitivity, lowered plasma glucose, and increased mitochondrial capacity [44] in diet-induced obese and genetically obese mice. Hyperinsulinemic-euglycemic clamp studies in Zucker fa/fa rats also demonstrated that such SIRT1 activators improve whole-body glucose homeostasis and insulin sensitivity in adipose tissue, skeletal muscle, and liver [44]. Interestingly, SRT1720 also significantly suppressed insulin-induced glucose production in obese fa/fa rats. These newly identified small-molecule SIRT1 activators thus also look promising in novel therapeutic approaches for treating diseases of aging such as type 2 diabetes.

Nicotinamide phosphoribosyltransferase (Nampt) has also been presumed to be a cytokine (PBEF) or hormone (visfatin). Nampt synthesizes nicotinamide mononucleotide (NMN) from nicotinamide in a mammalian NAD+ biosynthetic pathway and is required for SIRT1 activity *in vivo* [74, 75]. Glucose restriction (GR) was associated with activation of AMP-activated protein kinase (AMPK), and activated AMPK was required to promote GR-induced transcription of Nampt [76]. Indeed, GR augmented Nampt activity and modified the intracellular [NAD+]:[NADH] ratio and nicotinamide levels in skeletal muscle [76]. Skeletal myoblasts derived from $SIRT1^{+/-}$ heterozygous mice were resistant to the effects of either GR or AMPK activation, suggesting that the crosstalk among AMPK, Nampt, and SIRT1 may be important in some CR and GR-associated biological functions [76]. An extracellular or intracellular form of Nampt (eNampt/iNampt) could therefore pose as an endogenous regulator of SIRT1 activity in normal and pathological conditions such as obese type 2 diabetes.

## PERSPECTIVES

SIRT1 has been implicated in numerous metabolic pathways including adipogenesis, insulin secretion, gluconeogenesis, and mitochondria biogenesis, suggesting that SIRT1 activation may have beneficial effects in human subjects with obesity or T2DM. However, many questions remain to

be answered before such an approach could be instituted. *1)* Exactly how does SIRT1 contribute to the beneficial effect of CR and how does activation of SIRT1 promote health benefits in humans? *2)* Does SIRT1 activity decrease under excess energy intake and is this effect responsible for obesity-related diseases such as T2DM and metabolic syndrome? *3)* What are the target molecules that regulate SIRT1 expression and its up-or downregulation? *4)* What is the tissue-specific mechanism that regulates SIRT1 activity? *5)* What are the other functions of SIRT2-7 proteins since they are known to have multiple targets and affect many biological functions?

Future studies in conditional knockout and transgenic mice, as well as in disease models, are needed to address all of these questions and ultimately provide a clear perspective as to the possible success of SIRT1 activation as a CR mimetic. Such challenges must be met to provide novel and urgently needed therapeutic strategies for the treatment of type 2 DM and metabolic syndrome, and to prevent the associated vascular complications.

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# Correlation Between Albuminuria and Spontaneous Platelet Microaggregate Formation in Type 2 Diabetic Patients

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**OBJECTIVE** — Albuminuria in type 2 diabetic patients is a risk factor for cardiovascular disease. We investigated the correlation between albuminuria and spontaneous microaggregation of platelets (SMAP) formed under shear stress.

**RESEARCH DESIGN AND METHODS** — The study subjects were 401 type 2 diabetic individuals (252 with normoalbuminuria and 149 with albuminuria) who were examined for SMAP under conditions of shear stress only (no agonist stimulation) and the reversibility of platelet microaggregation after stimulation with 1 μmol/l ADP, measured by a laser light-scattering method. Active glycoprotein IIb/IIIa (GPIIb/IIIa) and P-selectin expression levels on platelets as an index of platelet activation were measured by whole-blood flow cytometry.

**RESULTS** — SMAP formation was noted in 53% of diabetic patients. All patients with SMAP showed an irreversible pattern of platelet microaggregates by a low dose of ADP. SMAP was observed in 75% of diabetic subjects with albuminuria and in 39% of those with normoalbuminuria. Multivariate logistic regression analysis identified urinary albumin excretion rate and brachial-ankle pulse-wave velocity as independent factors associated with SMAP. The degree of SMAP correlated with active GPIIb/IIIa ($\gamma = 0.59$, $P < 0.001$) and P-selectin ($\gamma = 0.55$, $P < 0.001$) expression levels. These early-activated platelet profiles were significantly inhibited in albuminuric patients with aspirin intake, although the effect was incomplete.

**CONCLUSIONS** — Our study demonstrated an independent association between albuminuria and early changes in activated platelet profiles of type 2 diabetic patients. Further follow-up and intervention studies are needed to establish whether the inhibition of SMAP affects the course of cardiovascular disease in type 2 diabetic patients.

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There is growing evidence that increased urinary albumin excretion rate (AER) in type 2 diabetic patients is both a predictor of progression to chronic renal failure and an independent risk factor for cardiovascular disease (1). The U.K. Prospective Diabetes Study (UKPDS) group demonstrated that type 2 diabetic patients with microalbuminuria or overt proteinuria have a two- to three-fold greater risk of cardiovascular death, compared with those patients with normoalbuminuria (2). Furthermore, our group and others recently reported that a reduction of AER in type 2 diabetic patients was associated with a decreased occurrence of cardiovascular complications (3,4). Thus, albuminuria is considered an important therapeutic target for cardiovascular protection. However, the pathophysiological factors underlying this cardiorenal interaction remain unknown. Identifying such factors and developing tools to identify patients at higher risk of both conditions might allow the design of new therapeutic strategies to improve outcomes in type 2 diabetic patients.

Platelet activation and aggregation are fundamental processes in the development of atherosclerosis and thrombosis, both of which contribute to cardiovascular risk (5,6). Platelets aggregate when activated, to form microaggregates of only a few cells as the initial response to various stimuli but large, tighter platelet aggregates appear with prolonged stimulation (7). In diabetic patients, platelets tend to hyperaggregate (8), although previous platelet aggregation measurements by optical density (9) or impedance methods (10) generally reflected the formation of large platelet aggregates in response to exogenous stimulation with various agonists. These conventional methods do not measure platelet microaggregate formation, which occurs at the initial process of platelet activation. Recently, a particle-counting method based on laser light scattering was developed to effectively detect platelet microaggregate formation (11). This new method also measures spontaneous microaggregation of platelets (SMAP), which occurs with a stirring force only and has no requirement for exogenous agonist stimulation. Using this new method, Matsuno et al. (12) reported SMAP as the predominant platelet aggregation event in type 2 diabetic patients. However, this study did not address the association between SMAP and albuminuria.

We therefore investigated the association between changes in early-activated platelet profiles and increased albuminuria in type 2 diabetic patients. The incidence of SMAP was investigated in relation to albuminuria status. To further evaluate the active state of SMAP, we simultaneously measured the cell surface expression levels of two markers of platelet activation: active fibrinogen receptor glycoprotein IIb/IIIa (GPIIb/IIIa), an

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important player in platelet adhesion, and P-selectin (CD62p), a transmembrane protein present in the α- (or dense) granules.

## RESEARCH DESIGN AND METHODS

The diabetic subjects were recruited from type 2 diabetic patients who regularly visited the outpatient clinic of the Department of Medicine, Shiga University of Medical Science, in 2006–2007. Patients were clinically diagnosed with type 2 diabetes in accordance with the criteria of the World Health Organization. Patients were excluded based on the following criteria: complicating cancer, liver disease, infectious disease, collagen disease, nondiabetic kidney disease confirmed by renal biopsy, intake of nonsteroidal anti-inflammatory drugs within the previous 2 weeks, and intake of antiplatelet drugs with the exception of aspirin. Eligible patients were informed of the study protocol in oral and written forms. A total of 401 patients were finally enrolled in this study. Thirty healthy volunteers were also enrolled as the healthy control group to confirm the results of our previous study (17 male and 13 female, aged 60 ± 7 years [mean ± SD], BMI 23.1 ± 2.9 kg/m$^2$, systolic blood pressure 131 ± 9 mmHg, total cholesterol 199 ± 20 mg/dl, triglycerides 109.8 ± 26.4 mg/dl, and taking no medication). Each individual provided a blood sample for biochemical analysis and detection of platelet microaggregation under fasting conditions and underwent standard physical examinations; brachial-ankle pulse-wave velocity (baPWV) was also measured. AER was determined by an immunoturbidimetry assay (Hitachi 7070E; Hitachi High-Technologies, Tokyo, Japan) in a single 24-h urine sample collected on the same day as the blood sample was collected. AER <20 μg/min was considered normoalbuminuria, and AER ≥20 was classified as albuminuria. The group with albuminuria comprised 120 patients with microalbuminuria (20 μg/min ≤ AER < 200 μg/min) and 29 patients with overt proteinuria (200 μg/min ≤ AER). aPWV was measured by an automatic device (BP-203RPE; Colin, Komaki, Japan). The estimated glomerular filtration rate (eGFR) was calculated using the simplified prediction equation proposed by the Japanese Society of Nephrology: eGFR (milliliters per minute per 1.73 m$^2$) = 194 × (age [years])$^{-0.287}$ × (serum creatinine [milligrams per deciliter])$^{-1.094}$ × 0.739 (if female).

The study protocol and informed consent procedure were approved by the Ethics Committee of Shiga University of Medical Science. All participants provided written informed consent.

### Detection of platelet microaggregation

Platelet-rich plasma was obtained from blood collected into sodium citrate (14 μmol/l) by immediate centrifugation at 155*g* for 12 min at room temperature. The platelet-rich plasma was removed and centrifuged at 1,750*g* for 10 min at room temperature in the presence of prostaglandin $I_2$ (20 μmol/l). The resultant platelets were washed with HEPES-Tyrode buffer and resuspended to 4 × 10$^8$ cells/ml. In this platelet suspension, platelets did not form the microaggregates without stirring force or any exogenous agonists. Platelet microaggregation was determined by measuring the light-scattering intensity (13) on a PA-200 aggregometer (Kowa, Tokyo, Japan). SMAP was observed under low shear stress conditions at a stirring speed of 1,000 rpm (26 dyn/cm$^2$) without stimulation with an exogenous agonist. This degree of low shear stress is considered to be the same degree of shear stress occurring in the arterial stream, suggesting that this system provides a condition that mimicked the state of platelets in the artery. The reversibility of platelet microaggregation in response to a low dose of ADP (1 μmol/l) was also investigated. The data were recorded as a two-dimensional graph showing the change in total light intensity over time, expressed as a cumulative summation at 10-s intervals of scattered light intensity (Ii) and the number of particles corresponding to that intensity (Ni) in terms of particle size (intensity) (ΣIiNi) (volts × counts per second). Particles with an intensity of 25–400 mV represented small aggregates (9–25 μm). The degree of SMAP was described by measurement of the area under the curve (AUC) of each detection line for 5 min. AUC data were expressed as ×10$^5$ particles. Each measurement was performed twice. All individuals showed identical results as to whether SMAP was formed. The mean values of two AUC measurements as the degree of SMAP in each individual were used for the analysis to minimize the variability.

### Quantification of GPIIb/IIIa and P-selectin expression levels

The platelet surface expression levels of GPIIb/IIIa and P-selectin were assessed by whole-blood flow cytometry using a fluorescein isothiocyanate-conjugated PAC-1 antibody and a phycoerythrin-conjugated CD62p antibody, as described previously (14,15). In brief, a saturating antibody concentration in 50 μl was added to 50 μl of whole blood. Negative control samples contained prepared IgG and IgM. The samples were incubated for 15 min at room temperature and then were analyzed within 2 h by flow cytometry. The platelets were identified based on particle size and complexity using a peridinin-chlorophyll-conjugated CD61 antibody. Fluorescence data from 10,000 platelet events were collected. The expression levels were expressed as a percentage of the positively identified platelets. All antibodies were purchased from BD Biosciences (San Jose, CA).

### Statistical analysis

Categorical variables were compared between the two groups using $\chi^2$ tests, an unpaired Student *t* test was used for normally distributed variables, and the Mann-Whitney *U* test was used for nonnormally distributed variables. For the nonparametric comparison among four groups, a Kruskal-Wallis test was first performed to find a measure of the aggregate degree to which the group means differed and then the Mann-Whitney *U* test with Bonferroni correction was used to compare the differences between groups. A Spearman correlation coefficient was used to analyze the association between two variables. A multivariate logistic regression model was applied to evaluate the independence of factors that were significantly different between the groups, using logarithmic transformed values of nonnormally distributed variables. All data were analyzed using the SPSS software package (version 11; SPSS, Chicago, IL). $P < 0.05$ was considered statistically significant.

## RESULTS

### Platelet microaggregation in diabetic patients

SMAP was detected in 211 (52.6%) of 401 patients with type 2 diabetes (Fig. 1*A*) but not in any of the 30 healthy volunteers tested here (Fig. 1*B*) or in our previous study (12). The diabetic patients with SMAP showed an abnormal and irrevers-

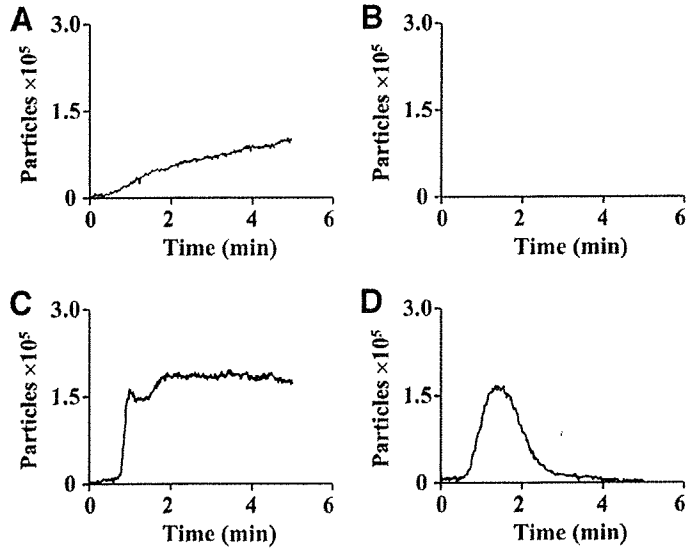


**Figure 1**—*Typical patterns of SMAP and reversibility of platelet microaggregation by low-dose ADP, measured by a laser light-scattering system. The spontaneous formation of microaggregated platelets under low shear stress alone without any exogenous agonists was observed in 52.6% of diabetic patients (A), whereas it was not observed in 47.4% of diabetic patients and in any healthy volunteers (B). Diabetic patients with SMAP showed irreversible platelet microaggregation in response to ADP (1 μmol/l) (C), whereas the others showed the typical reversible pattern of platelet microaggregation within 5 min (D).*

ible pattern of platelet microaggregation, even after stimulation by a low dose of ADP (Fig. 1*C*), whereas a normal reversible pattern was observed in patients without SMAP and in all healthy volunteers (Fig. 1*D*). Table 1 provides details of the clinical characteristics of the diabetic subgroups according to the formation of SMAP. Waist-to-hip ratios, the frequency of taking renin-angiotensin system inhibitors, urinary AER, eGFR, and baPWV were significantly different between diabetic patients with SMAP and those without such events. Multiple logistic regression analysis including these factors identified the levels of AER (odds ratio 3.4 [95% CI 2.2–5.3], $P < 0.001$) and baPWV (15.3 [1.1–227.5], $P < 0.05$) as independent factors associated with SMAP detection.

### Correlation between platelet microaggregation and albuminuria

Next, the frequency and degree of SMAP were analyzed according to albuminuria status (>20 μg/min). The frequency of the formation of SMAP in diabetic patients with albuminuria was significantly higher than in those with normoalbuminuria (75% of 149 patients with albuminuria vs. 39% of 252 patients with normoalbuminuria, $P < 0.001$). Similarly, the degree of SMAP evaluated by AUC was significantly higher in those with albuminuria than in those with normoalbuminuria (0.52 [interquartile range 0.01–1.46] vs. 0.00 [0.00–0.16], $P < 0.001$). In addition, the degree of SMAP was significantly correlated with the levels of urinary AER as continuous variables (Spearman $\gamma = 0.43$, $P < 0.001$). Regardless of the status of albuminuria, the platelet microaggregates in all patients with SMAP showed the irreversible pattern after stimulation by a low dose of ADP, and those in the patients without SMAP were reversible.

### Effect of aspirin medication on platelet microaggregation

We evaluated the effect of aspirin medication on the frequency and degree of SMAP. Of the diabetic patients, 163 (41%) were prescribed 100 mg/day aspirin. Patients with albuminuria and aspirin intake had a significantly lower frequency of SMAP than those without it (50 of 76 patients taking aspirin vs. 62 of 73 patients not given aspirin), whereas the SMAP rates were similar in the two subgroups in patients with normoalbuminuria (37 of 87 patients taking aspirin vs. 62 of 165 patients without aspirin intake). The degree of SMAP among the patients with albuminuria was similarly significantly lower with than without aspirin (0.33 [0.00–1.27] vs. 0.88 [0.17–1.76]), whereas patients with nor-

**Table 1**—*Clinical characteristics of the study subjects according to the formation of SMAP*

| | Diabetic patients without SMAP | Diabetic patients with SMAP |
|---|---|---|
| *n* | 190 | 211 |
| Sex (male/female) | 110/80 | 119/92 |
| Age (years) | 64 ± 11 | 65 ± 9 |
| Duration of diabetes (years) | 17 ± 11 | 16 ± 9 |
| BMI (kg/m$^2$) | 24.6 ± 4.3 | 24.7 ± 3.8 |
| Waist-to-hip ratio | 0.93 ± 0.07 | 0.95 ± 0.08* |
| A1C (%) | 7.2 ± 0.8 | 7.2 ± 1.0 |
| Diabetes treatment (%) (diet/oral agents/insulin) | 11/55/34 | 7/49/44 |
| Systolic blood pressure (mmHg) | 133 ± 17 | 134 ± 14 |
| Diastolic blood pressure (mmHg) | 73 ± 10 | 72 ± 10 |
| Hypertension (%) | 66 | 74 |
| Taking renin-angiotensin system inhibitors (%) | 41 | 59* |
| Total cholesterol (mg/dl) | 194 ± 31 | 197 ± 29 |
| Triglycerides (mg/dl) | 86 (61–116) | 92 (62–134) |
| HDL cholesterol (mg/dl) | 54 (46–65) | 52 (45–62) |
| Taking statins (%) | 52 | 48 |
| Urinary albumin excretion rate (μg/min) | 9 (6–16) | 23 (8–87)† |
| eGFR (ml/min per 1.73 m$^2$) | 74 ± 23 | 70 ± 20* |
| baPWV (cm/s) | 1,606 (1,416–1,800) | 1,726 (1,521–1,987)† |
| Taking aspirin (%) | 40 | 41 |
| Current smoking (%) | 22 | 21 |

Data are means ± SD for normally distributed continuous variables or medians (25th–75th interquartiles) for skewed continuous variables. *$P < 0.05$. †$P < 0.01$ vs. diabetic subjects without SMAP.