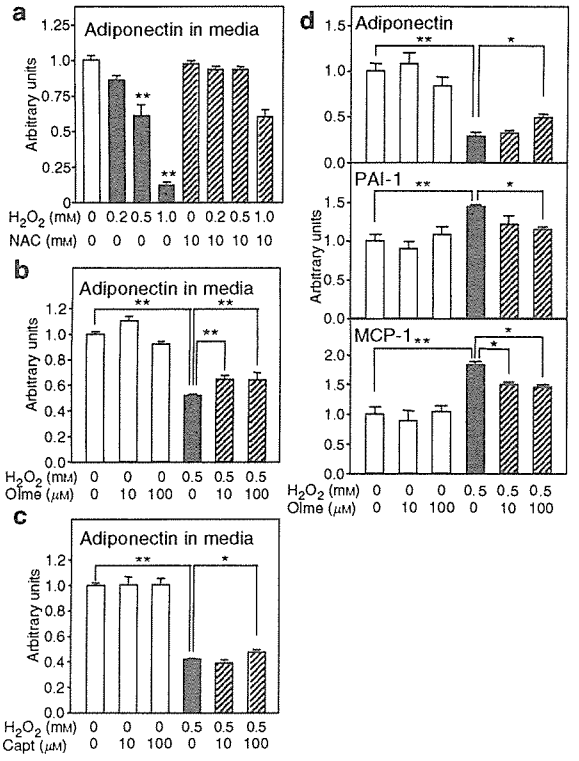

Figure 5 | Effects of olmesartan on production of adipocytokines in adipocytes. (**a**) The secretion of adiponectin from adipocytes exposed to ROS, with or without antioxidant *N*-acetyl cysteine. Differentiated adipocytes derived from SVCs (day 7) were exposed to the indicated concentrations of H_2O_2 and *N*-acetyl cysteine ($n = 3$, each). (**b**) Effects of olmesartan on adiponectin secretion by adipocytes exposed to ROS. Differentiated adipocytes were treated with the indicated concentrations of H_2O_2 and olmesartan (Olme) ($n = 3$, each). Adiponectin secretion in media was analyzed by adiponectin enzyme-linked immunosorbent assay. The values of vehicle-treated adipocytes were arbitrarily set as 1.0. Values are expressed mean ± s.e.m. $^*P < 0.05$, $^{**}P < 0.01$. Similar results were obtained in three other independent experiments. (**c**) Effects of captopril on adiponectin secretion by adipocytes exposed to ROS. Differentiated adipocytes were treated with the indicated concentrations of H_2O_2 and captpril (Capt) ($n = 3$, each). Similar results were obtained in two other independent experiments. (**d**) Effects of olmesartan on mRNA expressions of various adipocytokines. Values are expressed as mean ± s.e.m. after normalization to the level of cyclophilin mRNA. $^*P < 0.05$, $^{**}P < 0.01$.

effect of olmesartan on adiponectin production was observed at mRNA expression level (Figure 5d). The mRNA expression levels of PAI-1 and MCP-1 were elevated in H_2O_2-treated adipocytes. Treatment with olmesartan suppressed the elevation of mRNA expressions of PAI-1 and MCP-1 in H_2O_2-treated adipocytes but not in untreated adipocytes. These results suggest that amelioration of adipocytokine dysregulation by ARB is mediated, at least in part, via its anti-oxidative effect, in addition to reduction of ROS production.

DISCUSSION

In the present study, we demonstrated for the first time that blockade of AT1 receptor ameliorates adipocytokine dysregulation including hypoadiponectinemia using various obese mouse models. Furthermore, ARB ameliorated adipocytokine dysregulation in an obesity-specific manner. We also demonstrated that such action is mediated, at least in part, by targeting oxidative stress in obese adipose tissue.

We found that the ARB, olmesartan, prevented the obesity-related decline in plasma adiponectin concentration. Olmesartan also suppressed the expression of other adipocytokines such as TNF-α, PAI-1, SAA3, and MCP-1, which were upregulated in obesity. These results suggest that olmesartan can improve adipocytokine dysregulation in an obesity-specific manner. Recently, it was reported that activation of RAS by Ang II infusion elicited hypoadiponectinemia.[47] What is (are) the mechanism(s) by which ARB improve adipocytokine dysregulation in adipose tissue? Several studies showed that Ang II promotes and ARBs prevent ROS generation by modulation of NADPH oxidase in atherosclerotic blood vessels and diabetic kidney.[43,48–50] In the present study, we demonstrated that ARB ameliorated TBARS in obese adipose tissues, a marker of LPO, and attenuated the expression levels of all subunits of NADPH oxidase and their upstream transcriptional factor, PU.1, which was upregulated in the obese adipose tissue (Figure 3b and ref.[38]). Inhibition of ROS generation via NADPH oxidase in adipose tissue may be one of the mechanisms to ameliorate of adipocytokine dysregulation by ARB.

We also investigated whether ARB directly affects downstream ROS-mediated adipocytokine dysregulation using cultured adipocytes. The results showed that ARB ameliorated H_2O_2-mediated adipocytokine dysregulation (Figure 5). It was reported that oxidative stress modulates RAS in vascular endothelial cells.[51] Recently, Akishita *et al.*[46] demonstrated that RAS inhibitors, including olmesartan, inhibited H_2O_2-induced apoptosis of endothelial cells. Captopril, an ACE inhibitor, slightly but significantly ameliorated the inhibitory effect of H_2O_2 on adiponectin secretion (Figure 5c), suggesting that RAS inhibition at the level of ACE may ameliorate ROS-mediated impairment of adiponectin secretion. However, captopril had much less effective than ARB, also suggesting that direct antioxidant property of this ARB besides AT1 receptor blockade and/or activation of AT2 under the presence of an ARB showed inhibition against the ROS cascade. In our *in vitro* experiments, sarile ([Sar[1], Ile[8]] angiotensin II), a peptide Ang II receptor antagonist did not show significant effects (data not shown). This data might support the above interpretation. Therefore, the entire molecular mechanism(s) of oxidative stress-related activation of RAS remains unclear. Considered together, amelioration of adipocytokine dysregulation in obesity by ARB is mediated, at least in part, by its anti-oxidative effect, in addition to reduction of ROS production.

Peroxisome proliferator-activated receptor (PPAR)-γ-agonists, thiazolidinediones, were reported to improve adipocytokine dysregulation including adiponectin.[52,53] *In vitro* studies demonstrated that some ARBs, such as telmisartan and irbesartan, had PPAR-γ agonistic effect and enhanced the

production of adiponectin in 3T3-L1 adipocytes.[54–56] Olmesartan might ameliorate adipocytokine dysregulation through PPAR-γ activation. However, this is unlikely because it was reported that olmesartan did not have PPAR-γ agonistic effect *in vitro*.[54] In our present *in vivo* and *in vitro* studies, we could not observe significant changes in mRNA expression levels of PPAR-γ target genes, such as aP2 and CD36, following olmesartan treatment (data not shown). It is also possible that amelioration of adipocytokine dysregulation involves other pathways downstream of ROS such as mitogen-activated protein kinase, and janus kinase-signal transducer, and activator of transcription pathways.[57,58] The effect of AT1 blocker may not be entirely owing to the blockade of AT1. When AT1 is blocked, increase Ang II may act on AT2 receptor. AT2 stimulation by ARB is reported to result in renal production of nitric oxide.[59] It should be also considered that nitric oxide production via AT2 stimulation by ARB may improve adypocytokine dysregulation. Further studies are needed to elucidate the entire mechanism(s) by which ARB improves adipocytokine dysregulation in adipose tissue.

The present study clearly demonstrated that blockade of AT1 receptor ameliorated adipocytokine dysregulation in adipose tissue of obesity. It should be clinically relevant that ARB, which is widely used for the treatment of hypertension, ameliorate adipocytokine dysregulation in obesity, and metabolic syndrome. These results suggest that Ang II signaling and subsequent oxidative stress in adipose tissue may be potential targets for the prevention of atherosclerotic cardiovascular disease in metabolic syndrome and also in metabolic syndrome-based CKD.

MATERIALS AND METHODS

Animals and experimental protocol

All animals were obtained from Clea Japan (Tokyo, Japan). In C57BL/6J and KKAy mice studies, 21-week-old female mice were treated with or without olmesartan for 2 weeks ($n = 4$, each). Mice of the olmesartan group were fed regular chow containing 0.0015% olmesartan medoxomil (kindly provided by Sankyo Co., Tokyo, Japan). We selected the dose of the drug in this study so that it produces a sufficient fall in blood pressure without affecting food intake or body weight. In *db/db* mice studies, 16-week-old female *db/db* mice were treated with or without olmesartan (0.003% olmesartan medoxomil) for 2 weeks ($n = 9$ per group). In diet-induced obesity mice studies, 8-week-old female C57BL/6J mice were fed high-fat/high-sucrose (HF/HS) diet (Oriental Yeast). At the age of 21 weeks, the mice were treated with or without olmesartan (0.003% olmesartan medoxomil) for 3 weeks ($n = 5$ per group). Subcutaneous adipose tissues (inguinal region) were analyzed in these studies. All experimental protocols described in this report were approved by the Ethics Review Committee for Animal Experimentation of Osaka University.

Blood analysis

All blood samples were collected after 6-h fast. Plasma concentrations of adiponectin, glucose, insulin, and H_2O_2 were measured by the adiponectin enzyme-linked immunosorbent assay kit (Otsuka, Tokushima, Japan), the glucose CII-test (Wako Pure Chemical, Osaka, Japan), the insulin measurement enzyme-linked immunosorbent assay kit (Morinaga, Yokohama, Japan), and the Amplex Red hydrogen peroxide assay kit (Molecular Probe Inc., Leiden, The Netherlands), respectively.

Isolation, culture, and differentiation of SVCs

Subcutaneous adipose tissues (inguinal region) were removed from 8-week-old C57BL/6J mice, and SVCs were obtained by using a collagenase digestion procedure.[60] Adipose tissues were incubated with digestion medium (Dulbecco's modified Eagle's medium containing 1 mg/ml type II collagenase (Sigma-Aldrich Co., St Louis, MO, USA, No. C6885)) for 60 min in a shaking water bath at 37°C. After filtration and centrifugation, the pellet SVCs were seeded in 12-well plates, and cultured until confluence in the culture medium (Dulbecco's modified Eagle's medium containing 10% fetal bovine serum and 200 μM ascorbic acid). Two days after confluence (day 0), the medium was switched to differentiation medium (culture medium plus 5 μM troglitazone (kindly provided by Sankyo Co.), 0.25 nM dexamethasone, 0.5 mM 3-isobutyl-1-metyl-xanthin, and 10 μg/ml insulin). Two days later, the differentiation medium was switched to maintenance medium (culture medium plus 10 μg/ml insulin). At day 7, the SVCs were differentiated into adipocytes.

Effect of olmesartan on ROS and adipocytokine production in adipocytes

Differentiated adipocytes derived from SVCs (day 7) were incubated with serum-free culture medium containing the indicated reagents or vehicle for 12 h. The medium was replaced with freshly prepared medium containing the same reagents, and further incubated for 12 h. Then, the medium was collected to measure adiponectin secretion using the aforementioned adiponectin enzyme-linked immunosorbent assay kit, and the cells were harvested for RNA analysis. Because olmesartan medoxomil is a prodrug, we performed *in vitro* experiments using RNH-6270, which is the active form of olmesartan medoxomil (kindly provided by Sankyo Co.). Captopril, an ACE inhibitor, was kindly provided by Sankyo Co.

Blood pressure measurement

Systolic blood pressure was measured with an automatic sphygmomanometer (BP98A; Softron, Tokyo, Japan) at the tail artery whereas the animals were restrained.

LPO measurement

The level of TBARS, representing the extent of LPO, was measured in tissue homogenates using the LPO test (Wako Pure Chemical), as described previously.[43]

Insulin tolerance test

After 6-h fast, insulin (3 mU/g body weight) was injected intraperitoneally into KKAy mice.[22] Blood samples were obtained at the indicated time intervals. Insulin sensitivity was estimated by percentage changes in plasma glucose concentrations.

RNA extraction and quantitative reverse transcriptase-polymerase chain reaction

Isolated total RNA was subjected to cDNA synthesis using ThermoScript reverse transcriptase-polymerase chain reaction System (Invitrogen, San Diego, CA, USA). Real-time polymerase chain reaction was performed on an ABI PRISM 7700 using the

SYBR GREEN polymerase chain reaction Master Mix (Applied Biosystems, Warrington, UK). The sequences of primers were designed for cyclophilin, adiponectin, TNF-α, PAI-1, MCP-1, gp91^{phox}, p22^{phox}, p67^{phox}, p47^{phox}, p40^{phox}, Cu, Zn-superoxide dismutases, catalase,[43] AT1a receptor,[61] and SAA3,[40] as reported previously. The polymerase chain reaction primers for PU.1 and 36B4 were as follows; PU.1 5′-CCGCACACCATGTCCACAAC-3′ (forward), 5′-GGACGTGCATCTGTTCCAGC-3′ (reverse), and 36B4 5′-AAGCGCGTCCTGGCATTGTCT-3′ (forward) 5′-CCGCAGGGG CAGCAGTGGT-3′ (reverse).

Statistical analysis

Data were expressed as mean$\pm$s.e.m. of n separate experiments. Differences between groups were examined for statistical significance using Student's t-test or analysis of variance followed by Fisher's protected least significant test for comparisons between two groups or among multiple groups, respectively. A P-value less than 0.05 denoted the presence a statistically significant difference.

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Association of adiponectin and resistin with cardiovascular events in Korean patients with type 2 diabetes: The Korean atherosclerosis study (KAS) A 42-month prospective study

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Abstract

Adiponectin and resistin are proteins that affect insulin resistance and atherosclerosis significantly. We investigated adiponectin and resistin concentrations as predictors of cardiovascular events in Korean patients with type 2 diabetes. The study in 2001 comprised 343 unrelated patients with type 2 diabetes (65 ± 9.2 years old). They were followed up for 42 months. The baseline duration of diabetes, smoking status and history of cardiovascular diseases (CVD) were recorded. BMI, blood pressures, HbA1c, lipid profiles, ECG, creatinine and urine microalbumin were measured. Adiponectin and resistin were measured using enzyme-linked immunosorbent assays. The primary endpoint was defined as one of cardiovascular death, myocardial infarct, CABG, stroke, unstable angina or overt nephropathy. Thirty-eight patients (11.1%) experienced primary endpoint during the follow-up. After adjustment for age, sex, BMI, blood pressure and lipid status, participants in the lowest quartile of adiponectin levels compared with the highest had significantly increased risk of primary endpoint (relative risk = 3.03; 95% CI 1.09–8.41; $p = 0.034$). In contrast, resistin level had no influence on the risk of primary endpoint. A low level of adiponectin, not resistin, was a significant risk factor for the development of cardiovascular events in these Korean patients with type 2 diabetes.

Keywords: Adiponectin; Resistin; Cardiovascular disease; Type 2 diabetes; South Korea

1. Introduction

Adipose tissue is now considered to be endocrinologically active, and secretes a number of biologically active adipokines such as free fatty acids, leptin, tumor necrosis factor alpha (TNFα), plasminogen activator inhibitor-1, adiponectin [1] and resistin [2]. Of these, adiponectin and resistin have attracted considerable attention as novel adipocytokines, which may have critical roles in the development of atherosclerosis [2,3]. Previous studies have reported lower concentrations of serum adiponectin in individuals with type 2 diabetes than in those with normal glucose tolerance in Japanese and Pima Indians [4,5]. Weyer et al. also showed that type 2 diabetes was associated with low plasma adiponectin concentrations in different ethnic groups [6]. In addition, low adiponectin concentration is a risk factor for the subsequent development of diabetes [5,7,8]. However, a study from Japan

Abbreviations: CVD, cardiovascular diseases; CABGcoronary artery bypass graft; RR, relative risk

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reported that adiponectin levels were higher in type 1 diabetic patients than BMI-matched healthy control [9].

In terms of cardiovascular diseases, there have been several cross-sectional studies showing adiponectin level was lower in patients with CVD than in those without [4,10]. However, prospective studies are limited. Zoccali et al. reported that plasma adiponectin level was a predictor of CVD outcome in patients with end-stage renal disease [11], and Pischon et al. found that the incidence of myocardial infarction was associated with a low plasma adiponectin level in a nested case-control study [12]. Recently, Schulze et al. reported that low adiponectin level was prospectively associated with a higher risk of coronary heart diseases in men with type 2 diabetes [13].

Resistin is derived almost exclusively from fat in rodents, and adipose tissue and serum levels are elevated in animal models of obesity and insulin resistance [14,15]. Hyperresistinemia impairs glucose tolerance [2] and induces hepatic insulin resistance in rodents [16], whereas mice deficient in resistin are protected from obesity-associated insulin resistance [17]. In contrast to rodents, in humans resistin is produced primarily in inflammatory cells [18]. Many studies have now reported that circulating resistin levels are increased in people with obesity [19,20], type 2 diabetes [21] and type 1 diabetes [22]. Recent studies have shown that resistin promotes endothelial cell activation and smooth muscle cell proliferation, suggesting a potential role of resistin in atherosclerosis [23,24]. However, several reports have also documented that fasting human resistin concentrations are not significantly correlated with any clinical measure of insulin resistance [25,26], and resistin levels are not different between normal control and type 1 or 2 diabetic patients [27]. Furthermore, there was no prospective study regarding resistin level with cardiovascular events. Thus, the relationship of resistin to inflammation, insulin resistance and atherosclerosis in humans remains largely undetermined. In the present study, we investigated whether adiponectin or resistin concentrations could be predictors of cardiovascular events in Korean patients with type 2 diabetes.

2. Subjects and methods

2.1. Study population

In 2001 the Korean atherosclerosis study (KAS) recruited a cohort of 1542 men and women, 40–70 years of age, from Seoul, Korea. The KAS was planned to investigate the incident rate of cardiovascular events in high risk patients with ischemic heart disease, type 2 diabetes or stroke. Each risk group consisted of about 500 patients. Of these, we chose the 550 patients with type 2 diabetes. In the selection of study subjects, 207 subjects with one or more of the following were excluded from the present analysis: those who had been receiving dialysis for chronic renal failure or had overt proteinuria (>300 mg/24 h), or had any history of myocardial infarction, unstable angina, stroke or cardiovascular revascularization within 6 months before study enrollment. A total of 343 unrelated patients with type 2 diabetes (mean age 65 ± 9.2 years) participated in the study. All subjects were invited to return to clinic visit, at 3 months intervals, at which incident primary endpoint was ascertained by interview and confirmed by hospital records. We confirmed the cardiovascular death by telephone calling when they did not appear on the appointed day. They were followed up over 42 months. At baseline, the duration of diabetes, smoking status and history of cardiovascular diseases were investigated. All subjects provided informed consent and the study protocol was approved by the Ethics Committee of the Institutional Review Board of Seoul National University Hospital.

2.2. Anthropometric assessments and electrocardiogram measurements

Height and weight were measured with the patients barefoot in light clothing, to the nearest 0.1 cm or 0.1 kg, respectively. Body mass index (BMI) was calculated as body weight divided by the height squared (kg/m^2). Blood pressure measurements were made after subjects had remained in the sitting position for 10 min. Measurements were made twice, with a 5-min rest period and mean value of measurements was used. All study participants were fitted with 12-lead electrocardiograms. All electrocardiograms were read independently by a cardiologist in Seoul National University who was blinded to any clinical information. ECG abnormality was defined when the interpretation was of atrial fibrillation, a major ST-T segment change [28], left ventricular hypertrophy or a ventricular conduction defect.

2.3. Laboratory assessments

After 14 h of overnight fasting, venous blood samples were drawn from the antecubital vein of the patients at 08:00–09:00 h. Subjects were asked to refrain from smoking and alcohol for 24 h before blood sampling. Plasma was separated immediately by centrifugation (2000 rpm, 20 min, 4 °C). The fasting plasma concentrations of glucose, total cholesterol, triglyceride and HDL-cholesterol were measured enzymatically using a Hitachi 747 chemistry analyzer (Hitachi, Tokyo, Japan) immediately after blood was drawn. HbA1c were measured by high performance liquid chromatography at Seoul National University Hospital. Adiponectin level was measured using an enzyme-linked immunosorbent assay (ELISA; Otsuka Pharmaceutical Co., Tokyo, Japan) [29] as was resistin (Adipogen®, Seoul, Korea). The intra- and interassay coefficients of variation were 3.3 and 7.4% for adiponectin, and 3.73 and 5.6% for resistin, respectively. Twenty-four hour urine samples were collected from the study participants and assayed by nephelometry (BN II, Dade Behring, Germany) for urinary microalbumin. Microalbuminuria was diagnosed if the 24 h urine albumin was between 30 and 300 mg/day (or the mea-

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sured albumin/creatinine ratio (30–300 mg/g) in a random spot collection when no 24 h urine was available).

2.4. Primary endpoint

The primary endpoint was defined as one of the following: cardiovascular death, the development of myocardial infarction with or without primary coronary intervention, coronary artery bypass graft (CABG), stroke, admission to hospital for unstable angina or dialysis for overt nephropathy.

2.5. Statistical analyses

Statistical analyses were conducted using SPSS for Windows, Version 11.0 (SPSS, Chicago, IL). Data are expressed as means ± standard deviations or as numbers with percentages. Significant differences between groups were evaluated using ANOVA with post hoc testing. Possible associations between variables were tested using Pearson's correlation. Differences were considered statistically significant at $p < 0.05$. The contribution of adiponectin to the development of a primary endpoint was evaluated using a survival curve. The hazard ratios of potential risk factors were determined using the Cox proportional hazard model, with a primary endpoint as the dependent variable. Kaplan–Meier plot was conducted for incident primary endpoint by the tertiles of adiponectin.

3. Results

The baseline characteristics of the 343 patients are shown in Table 1. The mean age of study participants was 65.0 ± 9.2 years, with a mean duration of diabetes of 10.9 years. When divided into four groups according to the duration of diabetes by 5 years intervals, about 25% of subjects belonged to each group. The mean systolic blood pressure was over 140 mmHg even though 76.4% of subjects had already taken one or more anti-hypertensive agents. Mean total cholesterol level was less than 200 mg/dl, with 26.5% of the patients taking one or more lipid lowering agents. The mean levels of adiponectin and resistin were 5.6 ± 3.7 μg/ml and 7.6 ± 6.5 ng/ml, respectively.

When adiponectin and resistin levels were grouped according to the duration of diabetes, adiponectin levels in the patients with over 15 years of diabetes were significantly higher than in those with shorter durations (data not shown). Resistin level seemed to decrease according to the duration of diabetes, but there was no statistical significance. When adiponectin and resistin levels were grouped according to the tertiles of creatinine level, both adiponectin and resistin levels showed increasing tendencies but only the resistin level in the patients with the highest tertile of creatinine was significantly higher than that in patients with the lowest or mid tertile of creatinine (data not shown).

Of the 343 participants, 38 (11.1%) experienced a primary endpoint in this prospective study: myocardial infarction ($n = 19$), CABG ($n = 5$), stroke ($n = 4$) and admission to hospital for unstable angina ($n = 20$). Nine participants experienced double events. There was no case of cardiovascular death or overt nephropathy.

Table 1
Baseline characteristics of study subjects ($n = 343$)

Age (years)[a]	65.0 ± 9.2
Systolic blood pressure (mmHg)	142.3 ± 7.6
Diastolic blood pressure (mmHg)	84.8 ± 8.9
Weight (kg)	64.5 ± 8.9
Body mass index (kg/m^2)	24.9 ± 2.9
HbA1c (%)	7.9 ± 1.2
Creatinine (mg/dl)	1.0 ± 0.4
Total cholesterol (mg/dl)	197.6 ± 36.6
HDL-cholesterol (mg/dl)	47.2 ± 11.1
Adiponectin (μg/ml)	5.6 ± 3.7
Resistin (ng/ml)	7.6 ± 6.5
Duration of diabetes (years)	10.9 ± 7.3
Sex[b]	
Men	152 (44.3%)
Women	191 (55.7%)
Anti-diabetic agent	
Single	118 (34.4%)
Multiple or insulin	225 (65.6%)
Anti-hypertensive agent	
None prescribed	81 (23.6%)
Single	154 (44.9%)
Multiple	108 (31.5%)
Lipid modifier	
None prescribed	252 (73.5%)
Single	89 (26.0%)
Multiple	2 (0.5%)
Smoking	
Current	65 (19.0%)
Microalbuminuria	
30–300 mg/day	136 (39.7%)
CVD history	
Myocardial infarction, angina, stroke, primary coronary intervention or CABG	81 (23.6%)
ECG	
Abnormal ECG	119 (34.7%)
DM duration (years)	
<5	75 (22.1%)
5≤ to <10	95 (27.9%)
10≤ to <15	80 (23.5%)
15≤	90 (26.5%)

[a] Values are expressed as means ± S.D.
[b] Values are expressed as n (percentages).

From the correlation analysis, adiponectin level was negatively correlated with body weight ($r = -0.146$) and BMI ($r = -0.137$) and positively with serum creatinine ($r = 0.284$) and duration of diabetes ($r = 0.200$) (all $p < 0.05$). In contrast, resistin level was only positively correlated with creatinine level ($r = 0.187$; $p < 0.05$).

In Table 2, the clinical characteristics, biochemical parameters and drug usage have been compared according to the quartiles of adiponectin concentration. Age was different

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Table 2
Comparison of clinical characteristics, biochemical parameters and drug usage by quartiles of adiponectin concentration

	Quartile 1	Quartile 2	Quartile 3	Quartile 4	p-Value[a]
Adiponectin (μg/ml)	2.1 ± 0.8	4.0 ± 0.5	5.9 ± 0.7	10.6 ± 3.5	<0.01
Age (years)	62.8 ± 9.9	65.0 ± 7.6	67.2 ± 9.0	65.1 ± 9.9	0.021
Height (cm)	161.4 ± 7.7	162.1 ± 7.9	160.6 ± 6.5	159.7 ± 8.2	NS
Weight (kg)	65.6 ± 8.5	65.9 ± 8.8	63.6 ± 8.9	63.0 ± 9.4	NS
BMI (kg/m^2)	25.1 ± 2.5	25.1 ± 2.8	24.7 ± 3.2	24.7 ± 3.3	NS
SBP (mmHg)	139.7 ± 19.0	142.2 ± 18.5	145.8 ± 19.0	141.5 ± 19.2	NS
DBP (mmHg)	84.0 ± 13.1	84.5 ± 12.0	88.0 ± 14.7	82.6 ± 11.7	NS
Creatinine (mg/dl)	0.9 ± 0.2	1.0 ± 0.3	0.9 ± 0.3	1.1 ± 0.6	0.017
TC (mmol/l)	5.1 ± 0.9	5.1 ± 1.0	5.2 ± 0.8	5.2 ± 1.1	NS
HDLC (mmol/l)	1.2 ± 0.3	1.2 ± 0.3	1.3 ± 0.3	1.3 ± 0.3	0.008
HbA1c (%)	8.0 ± 1.2	7.8 ± 1.2	7.9 ± 1.2	7.7 ± 1.3	NS
Resistin (ng/ml)	7.3 ± 6.9	7.2 ± 6.0	8.4 ± 6.8	7.8 ± 6.2	NS
DM duration (years)	9.6 ± 6.1	9.3 ± 6.8	12.3 ± 7.4	12.4 ± 8.2	0.003
Thiazolidinedione[b]	0.0%	1.1%	3.5%	2.4%	NS
Statin[b]	29.1%	23.0%	22.4%	25.9%	NS

Data are means ± S.D. or (%). NS, non-significant, SBP, systolic blood pressure; DBP, diastolic blood pressure; TC, total cholesterol; HDLC, HDL-cholesterol.
[a] Statistical significances were tested by one-way analysis of variances between groups.
[b] Percentage of taking thiazolidinedione or statin.

between quartile 1 (lowest) and quartile 3 (second highest) of adiponectin. Concentrations of HDL-cholesterol and creatinine, and duration of diabetes were higher in patients with the highest quartile of adiponectin than among those in the lowest quartile. There were no differences in the usage of thiazolidinedione or statin between the four groups.

When the patients were grouped into tertiles according to the adiponectin levels, Kaplan–Meier analysis showed a higher probability of developing primary endpoints in patients in the lowest than in those in the highest tertile ($p = 0.078$) (Fig. 1).

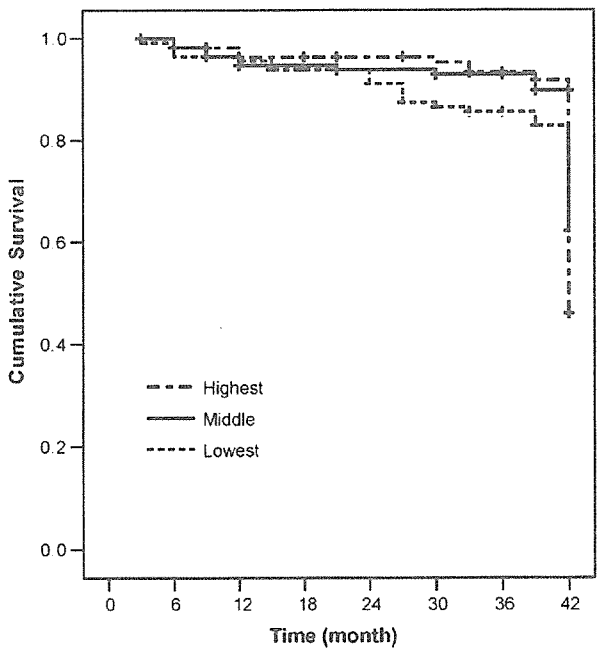

Fig. 1. Kaplan–Meier plot for incident primary endpoint by the tertiles of adiponectin.

Using the Cox proportional hazard model, we further investigated the independent risk of adiponectin or resistin level for the development of primary endpoint during the follow-up period (Table 3). After multivariate adjustment, participants with lower adiponectin levels had a higher risk for primary endpoint (relative risk (RR) for lowest quartiles 3.52; 95% CI 1.28–9.69; $p = 0.015$). Further adjustment for ECG abnormality also maintained significant association between adiponectin level and primary endpoint (RR = 3.61; $p = 0.013$). The relative risk for lowest quartiles of adiponectin after additional adjustment for CVD history was 3.02 (95% CI 1.12–8.17; $p = 0.029$). Further adjustment for BP, smoking and lipid status, HbA1c, microalbuminuria and resistin did not appreciably attenuate the association any further (RR for lowest quartiles 3.03; 95% CI 1.09–8.41; $p = 0.034$).

Resistin level had no influence on the risk of primary endpoint in this study, whether it was analyzed as a continuous variable or as categorical one by dividing into quartiles. When usage of statin was added to this model, there were no changes in significant factors. Similar results were obtained after the five subjects who were taking rosiglitazone or pioglitazone before study enrollment were excluded; these agents are known to increase adiponectin levels [30].

4. Discussion

In this prospective study, a low level of adiponectin was found to be a significant risk factor for the development of cardiovascular events in Korean patients with type 2 diabetes. From the correlation analysis, adiponectin level was negatively correlated with BMI and positively with HDL cholesterol. In contrast, resistin level was not correlated with parameters related to metabolic abnormality and had no significant effect on cardiovascular events.

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Table 3
Cox proportional hazard model for primary endpoint

	Quartiles of adiponectin				p-Value
	1	2	3	4	
MV adjusted[a]	1.00	1.38 (0.45–4.26)	2.60 (0.91–7.37)	3.52 (1.28–9.69)	0.015
MV + ECG abnormality[b]	1.00	1.49 (0.48–4.68)	2.76 (0.97–7.88)	3.61 (1.31–10.0)	0.013
MV + ECG abnormality[b] + CVD history[c]	1.00	1.52 (0.49–4.76)	2.70 (0.95–7.65)	3.02 (1.12–8.17)	0.029
MV + ECG abnormality[b] + CVD history[c] + BP, smoking and lipid status, HbA1c, microalbuminuria and resistin[d]	1.00	1.60 (0.50–5.07)	2.71 (0.92–7.95)	3.03 (1.09–8.41)	0.034

Data are relative risk (95% CI). MV, multivariate.

[a] Relative risks adjusted for age, sex, BMI (≥27.0 kg/m^2 vs. <27.0 kg/m^2), creatinine (tertiles) and DM duration (<5, 5–10, 10–15 and >15 years).

[b] ECG abnormality (no vs. yes).

[c] Cardiovascular disease (CVD) history (no vs. yes).

[d] Blood pressure (BP) status (SBP < 140 mmHg and DBP < 90 mmHg vs. SBP ≥ 140 mmHg or DBP ≥ 90 mmHg), smoking status (current vs. non), lipid status (total cholesterol < 240 mg/dl and HDL-cholesterol ≥ 40 mg/dl vs. total cholesterol ≥ 240 mg/dl or HDL-cholesterol < 40 mg/dl) and microalbuminuria (no vs. yes).

Adiponectin appears to be associated with a wide range of biologic effects on glucose and lipid metabolisms and atherosclerosis [3,29,31]. Decreased plasma adiponectin levels have been demonstrated in a variety of insulin-resistant states, including obesity and diabetes, and cardiovascular diseases [4,6,10,32]. In a recent report from the Atherosclerosis Risk in Communities (ARIC) study, subjects with the highest quartile of adiponectin had a ~40% lower risk of developing diabetes than those in the lowest quartile, even after adjustment for various metabolic risk factors [33]. This result indicates that adiponectin could be used as a predictor for the future development of type 2 diabetes in addition to established risk parameters. In addition, several studies also support the idea that adiponectin has direct anti-atherosclerotic effects [30,34,35]. Adiponectin was demonstrated to inhibit strongly the production of various adhesion molecules, including intracellular adhesion molecule-1, vascular cellular adhesion molecule-1 and E-selectin. Adiponectin was also shown to inhibit TNFα-induced nuclear factor-κB activation through the inhibition of IκB phosphorylation [36]. These results suggest that people who have low adiponectin levels are more likely to develop cardiovascular diseases, as in the current study.

In this study, statistical significance was obtained whether adiponectin level was analyzed as a continuous variable or as quartiles. However, in the final Cox proportional model, adiponectin level was analyzed as a categorical variable to show clinical implication although there is still no consensus of cutoff value of adiponectin. When a stratified analysis was conducted after dividing the participants by history of CVD, in the individual with history of CVD ($n = 81$), the lowest quartile of adiponectin level was independent risk factor for primary endpoint with borderline significance (RR = 3.54; $p = 0.099$). In those without history of CVD ($n = 262$), the lowest quartile of adiponectin level showed an increasing tendency of RR for primary endpoint but there was no statistical significance ($p = 0.228$). However, when adiponectin level was inserted as a tertile or continuous variable in the model with the individual having no CVD history, the low level of adiponectin regained borderline statistical significance (RR = 4.02, $p = 0.076$ in lowest to highest tertile of adiponectin; RR = 0.802, $p = 0.075$ in the model where adiponectin was inserted as a continuous variable). In the correlation analysis, adiponectin level was correlated positively with serum creatinine ($r = 0.284$) and the duration of diabetes ($r = 0.200$), and this finding is in accord with a recent report on Pima Indians with type 2 diabetes [37]. Positive correlation between adiponectin level and diabetes duration was still remained after adjustment for renal function ($p = 0.014$). The tendency for adiponectin to increase according to the duration of diabetes is somewhat unexpected and could be caused by the development of adiponectin resistance or reduced clearance of adiponectin. Considering the results of this study and another study showing an inverse correlation between serum adiponectin and creatinine or glomerular filtration rate [38], renal function seems to be involved in the clearance of adiponectin, although the exact mechanism is unknown. Thus, it is prudent to advise that the duration of diabetes and renal function should be included in any analysis of adiponectin in diabetic individuals.

In addition, adiponectin level is known to be increased by thiazolidinedione use. However, there was no significant difference of adiponectin level by thiazolidinedione use in this study. We think this is due to small number of patients with thiazolidinedione ($n = 6$).

Resistin is abundantly expressed in monocytes and macrophages in humans. Recently resistin has been found in atherosclerotic lesions and resistin treatment induces vascular endothelial dysfunction and vascular smooth muscle cell proliferation [39,40]. Therefore, resistin may contribute to atherosclerosis through its effects on vascular endothelial and smooth muscle cells in humans. However, resistin level was not correlated with parameters related to metabolic abnormality and had no significant effect on cardiovascular events in this study. Burnett et al., recently showed that resistin was not independently associated with CVD in the cross-sectional study [41]. There is no clear answer for this discrepancy. Resistin may have less effect on atherosclerosis

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or cardiovascular disease than adiponectin, or it is possible that adiponectin could weaken or abolish the effect of resistin considering the opposing effects of adiponectin and resistin on vascular cells [42].

Our study has some advantages. We prospectively investigated the association between both adiponectin and resistin level and cardiovascular events in type 2 diabetes. In addition to known risk factors, duration of diabetes and renal function were also checked. Nonetheless, the relatively small number of study subjects and short follow-up duration should be considered for a prospective study to draw major conclusions.

In conclusion, this prospective study identified a low level of adiponectin as a significant risk factor for the development of cardiovascular events in Korean patients with type 2 diabetes even after adjustment for known risk factors. In contrast, resistin level seems to have no effect on the development of cardiovascular events, although further larger scale studies are required.

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Angiotensin II-dependent Src and Smad1 signaling pathway is crucial for the development of diabetic nephropathy

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Angiotensin II (Ang II) is known to play a pivotal role in the development of diabetic nephropathy. However, the precise mechanism of Ang II-mediated effects on diabetic nephropathy is still unknown. We have reported that Smad1 plays a key role in diabetic mesangial matrix expansion and directly regulates the transcription of type IV collagen (Col4) *in vitro* and *in vivo*. Here we examined the effect of Ang II on the expression of Smad1 and mesangial matrix expansion in streptozotocin (STZ)-induced diabetic rats *in vivo*, using Ang II type 1 receptor blocker, olmesartan. We also examined the signaling mechanism by which Ang II induces mesangial matrix expansion *in vitro*. Treatment of diabetic rats with low-dose olmesartan for 20 weeks reduced albuminuria and hyperfiltration without affecting blood pressure and inhibited mesangial matrix expansive changes and the expression of Col4 and smooth muscle alpha actin compared with those in untreated rats. Immunohistochemical staining and Western blotting showed that the increased expression of Smad1, phospho-Smad1, and phospho-Src was inhibited by olmesartan. Ang II induced Col4 synthesis and increased expression of phospho-Src and phospho-Smad1 in cultured mesangial cells, which was blocked by olmesartan. PP2, a Src tyrosine kinase inhibitor, and overexpression of dominant negative Src also reduced the phosphorylation of Smad1. Moreover, addition of small-interfering RNA against Src significantly reduced the phosphorylation of Smad1 and synthesis of Col4. Taken together, these results indicate that Ang II can regulate the development of mesangial matrix expansion in the early phase of diabetic nephropathy through Src and Smad1.
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Diabetic nephropathy is the most common cause of end-stage renal disease and a major contributing cause of morbidity and mortality in patients with diabetes in many countries. This disease is characterized by thickening of glomerular basement membrane and mesangial matrix expansion. Moreover, it is well known that mesangial matrix expansion is generally accompanied by various tubulointerstitial damages followed by nephron loss to end-stage renal failure.[1–3] The decline of glomerular filtration rate is associated with the increasing number of obsolescent glomeruli and the severity of mesangial matrix expansion in type I diabetes.[4] The early stage of diabetic nephropathy is associated with glomerular hyperfiltration and glomerular hypertrophy, but not collapse of glomerular capillaries. Therefore, mesangial matrix expansion is one of the most important pathological findings in the early phase of diabetic nephropathy. Interruption of the renin–angiotensin system (RAS) using angiotensin II (Ang II) type 1 receptor blocker (AT1 antagonist) or an angiotensin-converting enzyme inhibitor has been proved clinically effective in slowing the decline in renal function of several nephropathies, including diabetic nephropathy[5–8] and slows the progression of renal disease and delays the need for dialysis or transplantation in diabetic patients.[9] Several reports indicate that RAS is accelerated in early diabetic nephropathy[10] and that intrarenal Ang II expression is already augmented before apparent diabetic nephropathy.[11]

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Ang II can increase vascular tone by inducing contraction of vascular smooth muscle and mesangial cells and promote cellular proliferation and extracellular matrix (ECM) synthesis through direct effects, both hemodynamic and nonhemodynamic, or via induction of growth factors.[12–14] These data support the notion that Ang II can worsen diabetic nephropathy. However, little is known about the precise molecular mechanisms by which Ang II exerts its effects on diabetic nephropathy.

It is well known that mesangial matrix expansion is characterized by increased amounts of ECM, including type IV collagen (Col4), laminin, type I and III collagens, heparan sulfate proteoglycan, and fibronectin.[15–17] Striker *et al*[16] show that various peptide growth factors mediate the regulation of Col4, which is the key component is in the pathophysiology of diabetic nephropathy. We have recently reported that Smad1 is a key molecule for direct transcriptional regulation of Col4 in diabetic nephropathy,[18] and that the glomerular expression of Smad1 is significantly increased in diabetic rats with mesangial matrix expansion.[19] However, the effect of Ang II on Smad1 in the mesangial matrix expansion of diabetic nephropathy is not fully elucidated.

Src is a ubiquitously expressed nonreceptor protein-tyrosine kinase,[20] and is involved in signaling by several cytokines and growth factors such as Ang II[21] and platelet-derived growth factor (PDGF).[22] It is well known that Src may contribute to both cell proliferation and migration.[23,24] PDGF plays a role as a mesangial cell mitogen and causes proliferative glomerulopathy.[25,26] Our previous data also show that the development of glomerulosclerosis from mesangial proliferation is dependent on PDGF, signal transducer and activator of transcription 3, and Smad1 pathway.[27] However, it is not known whether Ang II activates Smad1 or what is the signaling mechanism involved in Ang II-mediated Smad1 activation in mesangial cells. In the present study, therefore, we examined whether Ang II can modulate Smad1-mediated signaling involved in mesangial matrix expansion in diabetic nephropathy *in vivo* and *in vitro*. *In vivo*, we used streptozotocin (STZ)-induced rats receiving low-dose AT1 antagonists and examined the role of Ang II in Smad1-mediated mesangial matrix expansion. *In vitro*, by using primary culture mesangial cells, the effect of Ang II on Smad1 and Src, and the role of Src on Smad1-mediated signaling in mesangial cells were examined.

Materials and methods

Animals

Male Sprague–Dawley rats weighing 170–200 g were used in study. Rats were purchased from Shimizu Laboratory Animal center (Hamamatsu, Japan). The animals were housed under specific pathogen-free conditions at the Animal Facility of Kyoto University, Faculty of Medicine. All the animal experiments were performed in accordance with institutional guidelines, and the Review Board of Kyoto University granted ethical permission to this study.

Induction of STZ Diabetic Rat

Male rats weighing 170–200 g were made diabetic by a single intravenous injection of STZ (Wako, Osaka, Japan) (55 mg/kg body weight) in 0.05 mol/l citrate buffer (pH 4.5). Rats receiving an injection of citrate buffer were used as controls. The levels of blood glucose were determined 2 days after injection of STZ or vehicle, and rats with blood glucose levels more than 16.7 mmol/l were used as diabetic.[28]

Protocol of Treatment with Ang II Type 1 Receptor Blocker in STZ Rats

The rats were divided into four groups for the investigation: *group 1*, control rats treated with vehicle; *group 2*, control rats treated with olmesartan (Sankyo Co., Tokyo, Japan) (0.6 mg/kg/day); *group 3*, STZ-diabetic rats treated with vehicle; *group 4*, STZ-diabetic rats treated with olmesartan (0.6 mg/kg/day). The dosage of olmesartan was determined based on the previous reports.[29] Olmesartan or vehicle was administered 4 weeks after the injection of STZ or vehicle. At 24 weeks after STZ injection, the rats were weighted and killed. Blood was collected at the time of killing. Serum creatinine and blood urea nitrogen were measured with a Hitachi Mode 736 autoanalyzer (Hitachi, Tokyo, Japan).[30] The levels of blood glucose and HbA1c were measured using One touch ultra (Johnson & Johnson, Tokyo, Japan) and DCA2000 analyzer (Bayer Medical, Tokyo, Japan). Before killing, creatinine and albumin were measured from 24-h urine collection.

Histological Examination

Light microscopy
After removal of the kidney, tissue blocks for light microscopy examination were fixed in methyl Carnoy's solution and embedded in paraffin. Sections (2 μm) were stained with periodic acid-Schiff methenamine (PASM).

Immunohistochemistry
Kidney sections were processed for immunohistochemistry following standard procedures. To study Col4 and Smad1, ethyl Carnoy's solution-fixed and paraffin-embedded tissue blocks were used. Kidney sections were rehydrated and treated with 0.3% hydrogen peroxide in methanol for 30 min. To eliminate nonspecific staining, sections were incubated with the appropriate preimmune serum

for 60 min at room temperature, and then incubated with AvidinD and Biotin blocking solutions (Vector, Burlingham, CA, USA) for 15 min each. Sections were incubated with anti-Col4 antibody (1:200 dilution) (PROGEN BIOTECHNIK GMBH, Heidelberg, Germany) or anti-Smad1 antibody (1:100 dilution) (Santa Cruz Biotechnology, CA, USA) overnight at 4°C, and then incubated with the appropriate biotinylated secondary antibodies followed by incubation with the avidin–biotin peroxidase complex (Vectastain Elite ABC kit, Vector). Peroxidase conjugates were subsequently localized using diaminobenzidine tetrahydrochloride. To examine the expression of smooth muscle alpha actin (α-SMA), the tissues were snap-frozen in cold acetate in OCT compound (Sakura Finetechnical, Tokyo, Japan), and then cut in 4-μm-thick sections and fixed in acetone for 10 min, and treated with 0.3% hydrogen peroxide in methanol for 30 min. Sections were treated in the same manner as Col4 and Smad1 with anti-α-SMA antibody (1A4 at 1:100 dilution) (Sigma, St Louis, MO, USA).

Quantitation of light microscopy
Glomerular morphometry was evaluated in PASM-stained tissues. The glomerular surface area and the PASM-positive area/glomerular area (%) were measured using an image analyzer with microscopy (IPAP, Image Processor for Analytical Pathology; Sumitomo Chemical Co., Osaka, Japan) as described[31] For each animal, 50 glomeruli were analyzed.

Immunofluolescence staining and quantitation of glomerular Col4 expression
Immunofluorescence staining for Col4, pSmad1, and pSrc was performed using frozen kidney sections. Sections (4 μm) were fixed in acetone, blocked with 10% donkey serum, and incubated overnight with anti-Col4 antibody (1:200 dilution) (PROGEN), anti-phospho Smad1/5/8 antibody (1:100 dilution) (Cell Signaling Technology, Beverly, MA, USA), or anti-phospho Src (Cell Signaling) (1:50 dilution).

To quantificate the expression of Col4, the Col4-positive area in glomeruli was measured using Image-Pro PLUS (Media Cybernetics, silverspring, MD, USA) as described.[32] For each animal, 50 glomeruli were evaluated.

Isolation of Glomeruli

Rat glomeruli were isolated from renal cortex of rats using the differential sieving method. The purity of the glomeruli was >90%.[33]

Cell Culture

We previously reported that cultured rat mesangial cells changed their phenotypes during multiple passages.[34] Therefore, we freshly isolated mouse mesangial cells from normal 4-week-old mice (C57BL/6J × SJL/J) and identified according to the method previously described.[35] The mesangial cells were maintained in B medium (a 3:1 mixture of minimal essential medium/F12 modified with trace elements) (Nissui Pharmaceutical Co., Ltd., Tokyo, Japan) supplemented with 1 mM glutamine, penicillin at 100 U/ml, streptomycin at 100 μg/ml (Invitrogen, Carisbad, CA, USA), and 20% fetal calf serum (FCS) (Invitrogen). The cultured cells fulfilled the criteria generally accepted for glomerular mesangial cells. These cells were plated on 100-mm plastic dishes (Nalge Nunc International, Roskilde, Denmark) and maintained in B medium/20% FCS. After 48 h incubation, cells were starved in DMEM containing 0.5% FCS before treatment. Stimulation with Ang II (Sigma), olmesartan (Sankyo), PP2 (Calbiochem, Darmstadt, Germany), or U0126 (Calbiochem) was carried out in DMEM containing 0.5% FCS at 37°C for the indicated times.

Cell Transfection

Mesangial cells (1.0×10^5) were seeded into six-well plates (Nunc). After 6 h, the cells were transfected with 2 μg of expression vector encoding dominant negative Src (Upstate Biotechnology, Charlottesville, VA, USA) or with 2 μg of mock vector (pUSEamp(−); Upstate) using Fugene6 transfection reagent (Roche, Penzberg, Germany) according to the manufacturer's instructions with some modifications. After 24 h of transfection, cells were starved in DMEM containing 0.5% FCS before treatment. After 48 h incubation, cells were stimulated with or without Ang II (Sigma). The cells were harvested in lysis buffer after stimulation and Western blotting was performed as reported below.

Small-Interfering RNA

Mesangial cells (1.0×10^5) were seeded into six-well plates (Nunc) and were grown until 60–80% confluent. The c-Src small-interfering RNA (si RNA) (SMARTpool; Dharmacon, Lafayette, CO, USA) or control siRNA (SMARTpool; Dharmacon) was combined with DharmaFECT transfection reagent (Dharmacon), and the cells were transfected according to the recommended protocol with siRNA (100 nM final concentration). After 48 h of transfection, cells were starved in DMEM containing 0.5% FCS before treatment. After 48 h incubation, cells were stimulated with or without Ang II. The cell lysates were then prepared for Western blot analysis.

Western Blotting

Isolated glomeruli or harvested mesangial cells were suspended in RIPA buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 1% Nonindet P-40, 0.25% SDS, 1 mM Na_3VO_4, 2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml of aprotinin), and incubated for

1 h at 4°C. After centrifugation, the supernatants were used as total cell lysates. In all, 30 μg of each sample was applied to SDS-PAGE. After electrophoresis, the proteins were transferred to nitrocellulose filters (Schleicher & Schuell, Keene, NH, USA). The blots were subsequently incubated with anti-Smad1 (Upstate), anti-phospho Smad1/5/8 (Cell Signaling), anti-Col4 antibody (PROGEN) (Biodesign, Saco, ME, USA), anti-nonphospho Src (Cell Signaling), anti-phospho Src (Cell Signaling), anti-p44/42 mitogen-activated protein (MAP) kinase antibody (Oncogene, San Diego, CA, USA), anti-phospho p44/42 MAP kinase antibody (Cell Signaling), anti-β-actin antibody (Cell Signaling), or anti-α-SMA antibody (Sigma), following by incubation with horseradish peroxidase-conjugated goat anti-rabbit IgG, and sheep anti-mouse IgG (Amersham Biosciences, Piscataway, NJ, USA). The immunoreactive bands were visualized using horseradish peroxidase-conjugated secondary antibody and the enhanced chemiluminescent system (Amersham Biosciences). These bands were quantificated by imaging densitometer, Science Lab 99 Image Gauge (Fujifilm, Tokyo, Japan).

Glomerular Filtration Rate and the Measurement of Urinary Albumin Excretion

Urine volume (Vu) was measured at 24 weeks by 24 h urine collection from rats housed in individual metabolic cages. During the urine collection, the rats were allowed free access to food and water. Serum and urine creatinine concentrations (Cp and Cu) were measured, and glomerular filtration rate (GFR) was calculated by the following equation: GFR = (Cu/Cp) × Vu/body weight.[36] The albumin concentration in the urine was measured by Nephrat (Exocell Inc., Philadelphia, PA, USA).

Statistical Analysis

The data are expressed as the mean ± the standard deviation (s.d.). Comparison among more than two groups was performed by one-way analysis of variance (ANOVA) followed by the *post hoc* analysis (Bonferroni/Dunn test) to evaluate statistical significance between the two groups. All analyses were performed using StatView (SAS Institute, Cary, CA, USA). Statistical significance was defined as $P<0.05$.

Results

Characteristics of Four Groups

Table 1 shows the characteristics of four groups of rats at 24 weeks after STZ injection. Blood sugar (BS) and HbA1c were increased, and the body weight (WT) was decreased in both diabetic groups compared with those in nondiabetic groups. There was no significant difference in systolic blood pressure (BP) between the four groups.

AT1 Antagonist Olmesartan Ameliorates the Renal Function of STZ Rats

Figure 1 shows the creatinine clearance and urinary albumin excretion of four groups of rats determined at the end of the experiment. The diabetic rats treated with vehicle (group 3) showed increased creatinine clearance and urinary albumin excretion compared with control rats (group 1 and group 2). However, the diabetic rats treated with olmesaran (group 4) showed less creatinine clearance and urinary albumin excretion than untreated diabetic rats. The glomerular hypertrophy was recognized in untreated diabetic rats, whereas olmesartan ameliorated it. In treated control rats, olmesartan also ameliorated hyperfiltration, albuminuria, and glomerular hypertrophy owing to aging.

Olmesartan Inhibits Mesangial Matrix Expansion in STZ Rats

To examine the effect of olmesartan on diabetic mesangial matrix expansion, we assessed mesangial matrix expansion by measuring PASM-positive areas in the four groups of rats. Untreated diabetic rats showed increased degree of mesangial matrix area and mesangial matrix expansion compared with control rats. When we treated diabetic rats with olmesartan for 20 weeks, both mesangial matrix area and mesangial matrix expansion were significantly ameliorated (Figure 2A). Figure 2B shows a representative light microscopic picture in each group. To investigate the effect of olmesartan on the morphological changes seen diabetic mesangial matrix expansion, such as increased Col4 and α-SMA expression, we examined their

Table 1 Physiological characteristics of control and diabetic rats with or without olmesartan treatment

	Number	*Body WT (g)*	*HbA1c (%)*	*BS (mg/dl)*	*Systolic BP (mmHg)*
Control	6	627 ± 10	2.7 ± 0.3	114 ± 12	113 ± 8
Control+Olm	6	631 ± 12	2.6 ± 0.1	119 ± 11	112 ± 3
Diabetes	7	320 ± 75*	7.0 ± 1.1*	382 ± 97*	112 ± 2
Diabetes+Olm	7	352 ± 53*	6.4 ± 0.9*	459 ± 64*	111 ± 4

The rats were separated into control and diabetic groups with or without olmesartan treatment. Rats with olmesartan treatment (Olm) were administered 0.6 mg/kg olmesartan. After 24 weeks of STZ injection, systolic blood pressure (Systolic BP) was measured by the cuff-tailed method, and the rats were weighed (Body WT) and killed. Blood was taken to evaluate blood sugar (BS) and HbA1c. The data are expressed as the means ± s.d. *$P<0.05$ *vs* control rats.

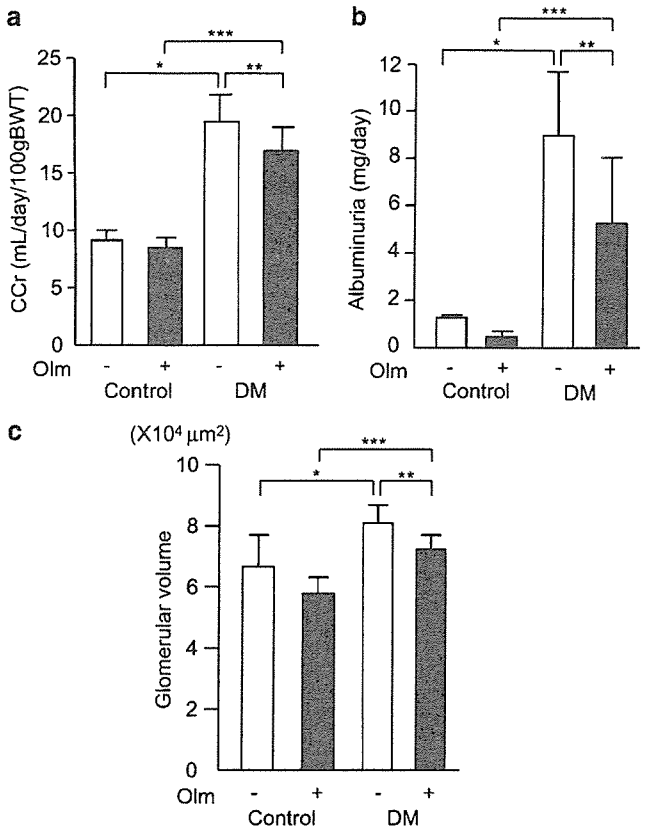

Figure 1 Effects of olmesartan on creatinine clearance and albuminuria in STZ rats. (**a**) Creatinine clearance (Ccr) was calculated and normalized as described in Materials and methods. (**b**) Albuminuria was measured by Nephrat. The data are expressed as the means ± s.d. ($n=6$ in the control group and $n=7$ in the diabetic group). (**c**) Glomerular volume was detected by IPAP as described in Materials and methods. $^{*}P<0.05$ *vs* control rats treated with vehicle (group 1). $^{**}P<0.05$ *vs* diabetic rats treated with vehicle (group 3). $^{***}P<0.05$ *vs* control rats treated with olmesartan (group 2).

expression in the four groups. In untreated diabetic rats, the glomerular expression of Col4 was significantly increased compared with that in control rats and their expression was mostly localized at the mesangial area by immunohistochemical analysis. Olmesartan treatement inhibited the glomerular expression of Col4 by approximately 30% compared with untreated diabetic rats (Figure 3C). We also examined the glomerular expression of α-SMA by Western blotting and found that α-SMA was almost undetectable in nondiabetic rats. However, the expression of α-SMA was significantly increased in untreated diabetic rats, compared with control rats. The expression was also decreased by olmesartan treatment (Figure 3A). Figure 3D shows a representative light microscopic picture in each group. In untreated control rats, slight mesangial matrix expansion due to aging was recognized and olmesartan ameliorated this matrix expansion. The decreased glomerular surface area as a denominator of mesangial matrix fraction in treated control rats led that they were larger than untreated control rats (Figure 2A). The same phenomenon was recognized in Col4-positive staining area (Figure 3C).

Glomerular Expression of Smad1, Phospho-Smad1 (pSmad1), Src, and Phospho-Src (pSrc) is Decreased by Olmesartan

We next examined the glomerular expression of Smad1 in these rats. By Western blotting and immunohistochemistry, Smad1 expression was remarkably increased in untreated diabetic rats compared with nondiabetic rats. However, its expression was decreased by olmesartan treatment (Figure 4). We also examined whether the phosphorylation and translocation of Smad1 were affected by olmesartan treatment in diabetic rats. By Western blotting and immunohistochemistry, pSmad1 was hardly detected in nondiabetic rats, whereas pSmad1 was markedly increased and its expression was localized in the nucleus in untreated diabetic rats. As shown in the Smad1 expression, the glomerular expression of pSmad1 was significantly decreased by olmesartan treatment (Figure 4). To investigate the role of Src in mesangial matrix expansion in diabetic nephropathy, we examined the glomerular expression of Src along with phosphorylated Src by Western blotting. The glomerular expression of Src and pSrc was markedly increased, compared with control rats. The increased expression was also decreased by olmesartn treatment as in Smad1 and pSmad1 (Figure 5A and B). By immunohistochemistry, we also found that increased pSrc was localized at the mesangial area. As shown in the Smad1 and pSmad1 expression, the glomerular expression of pSrc was significantly reduced by olmesartan treatment (Figure 5C).

In Vitro Effect of Olmesartan, PP2, and U0126 on Ang II-Mediated Signaling in Mesangial Cells

To determine whether Ang II can activate Smad1 and increase the synthesis of Col4, and whether this effect can be blocked by olmesartan, cultured mesangial cells were incubated with 0.1 μM Ang II for of the indicated times or with various concentrations of Ang II for 15 min. As shown in Figure 6a, Ang II caused the phosphorylation of Smad1 in a time-dependent manner, peaking at 15 min. Stimulation of mesangial cells with various concentrations of Ang II showed a dose-dependent increase in pSmad1 reaching a plateau at 1 μM (Figure 6b). Ang II also stimulated the phosphorylation of Src and the synthesis of Col4 (Figure 6c). Next, we examined whether olmesartan can prevent the Ang II-induced phosphorylation of Smad1, Src, and the synthesis of Col4. As shown in Figure 6c, olmesartan inhibited the phosphorylation of Smad1 and Src and the expression of Col4 after Ang II stimulation. To show the interaction between Src and Smad1, we examined whether PP2 (10 μM), an Src tyrosine kinase

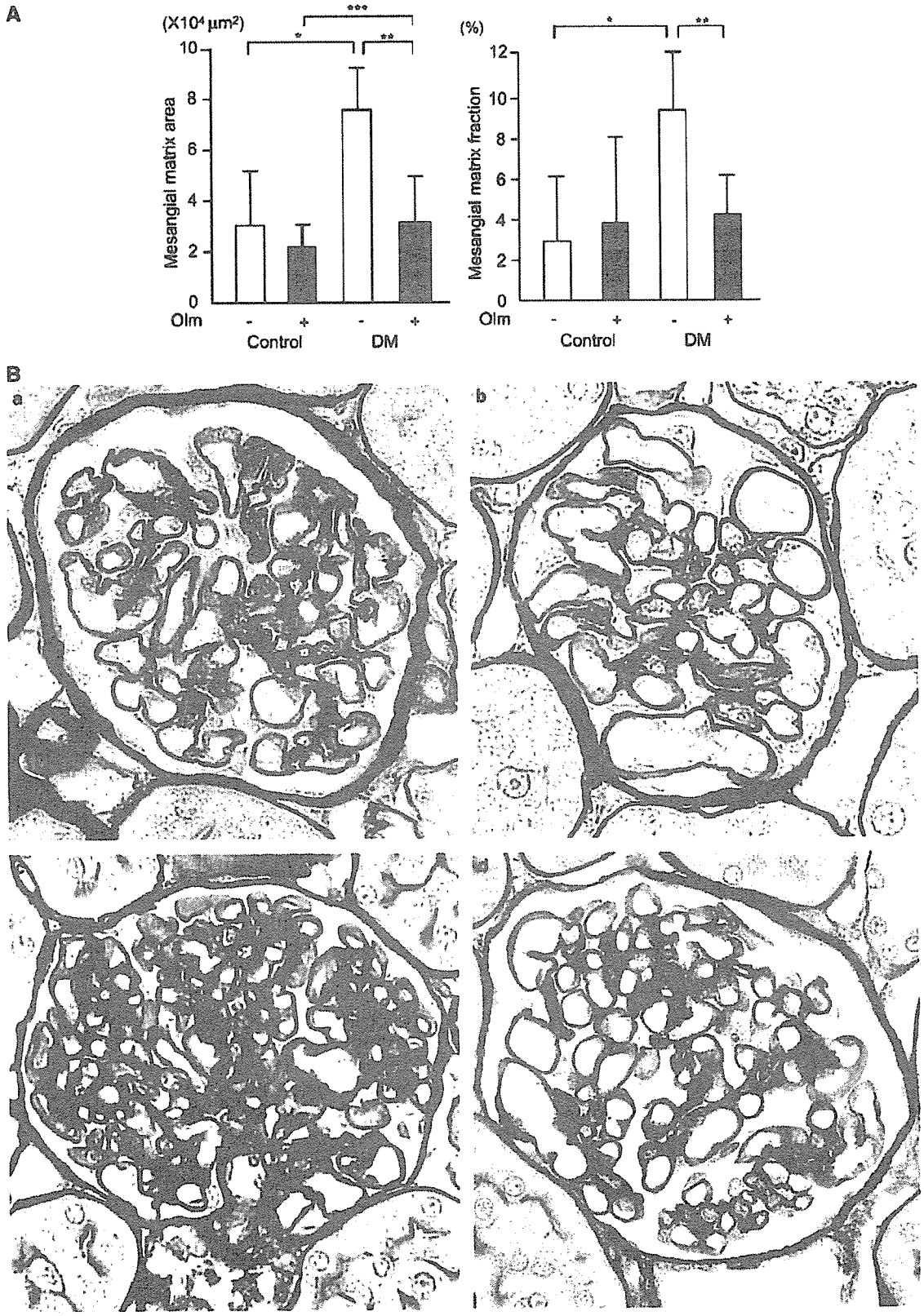

Figure 2 Mesangial matrix fraction in four groups. (**A**) Glomerular surface area and PASM-positive area were determined as described in Materials and methods. The mesangial sclerotic fraction was determined as percentage of mesangial matrix area per total glomerular surface area. The mesangial matrix area (left) and matrix fraction (right) in four groups were shown. $^{*}P<0.05$ *vs* control rats treated with vehicle (group 1). $^{**}P<0.05$ *vs* diabetic rats treated with vehicle (group 3). $^{***}P<0.05$ *vs* control rats treated with olmesartan (group 2). (**B**) Representative light microscopic pictures of glomeruli (PASM staining, × 400 original magnification) from control rats treated with vehicle (group 1) (**a**), control rats treated with olmesartan (group 2) (**b**), diabetic rats treated with vehicle (group 3) (**c**), and diabetic rats treated with olmesartan (group 4) (**d**).

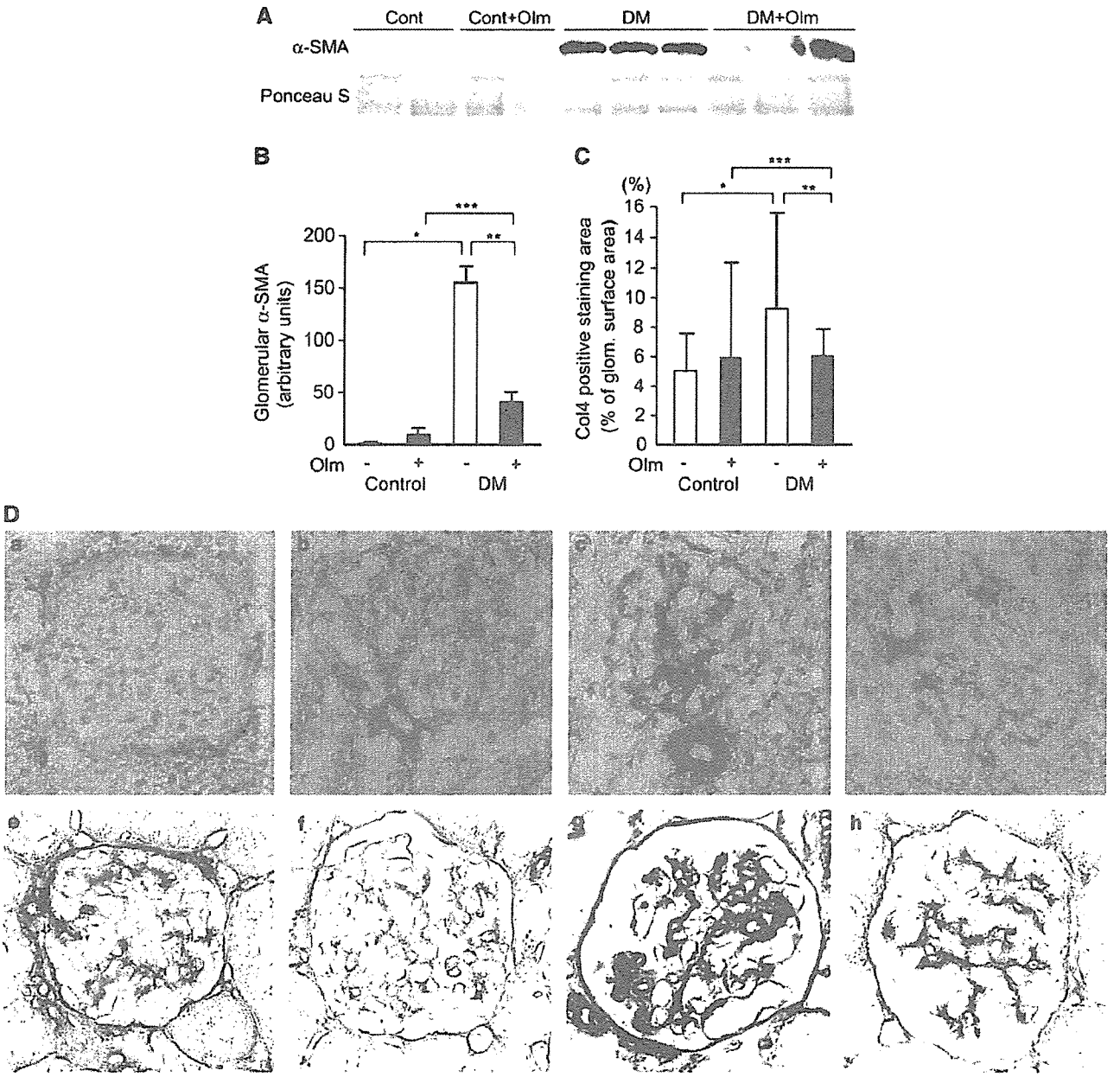

Figure 3 Glomerular expression of α-SMA and Col4 in four groups. (**A**) Immunoblots for α-SMA in the four groups are shown. Protein (30 μg) obtained from each glomerular lysates was loaded. Each lane represents a representative Western blotting for the glomerular lysates from each rat. (**B**) Optical densitometry of glomerular expression of α-SMA in Western blotting. $*P<0.05$ *vs* control rats treated with vehicle (group 1). $**P<0.05$ *vs* diabetic rats treated with vehicle (group 3). $***P<0.05$ *vs* control rats treated with olmesartan (group 2). (**C**) Morphometric analysis of the glomerular expression of Col4. The glomerular staining area of Col4 was measured as described in Materials and methods. $*P<0.05$ *vs* control rats treated with vehicle (group 1). $**P<0.05$ *vs* diabetic rats treated with vehicle (group 3). $***P<0.05$ *vs* control rats treated with olmesartan (group 2). (**D**) Immunohistochemistry of α-SMA and Col4 from glomeruli of control rats with vehicle (group 1) (**a**, **e**), control rats treated with olmesartan (group 2) (**b**, **f**), diabetic rats treated with vehicle (group 3) (**c**, **g**), and diabetic rats treated with olmesartan (group 4) (**d**, **h**). Representative light microscopic appearance of glomerulus is shown. The original magnification was × 400. **a–d**, α-SMA; **e–h**, Col4.

inhibitor, can affect Ang II-induced phosphorylation of Src and Smad1. As shown in Figure 6d, PP2 inhibited the phosphorylation of Src along with Smad1. We also examined the effect of a MEK inhibitor, U0126 on Ang II-induced phosphorylation of p44/42 MAP kinase. In contrast to the effect of PP2, U0126 did not affect the phosphorylation of Smad1 (Figure 6e).

Dominant Negative Src and siRNA Against Src Inhibit Ang II-Mediated Phosphorylation of Smad1 and Synthesis of Col4 in Mesangial Cells

To elucidate the interaction between Src and Smad1, we used dominant negative Src to block the activation of Src in mesangial cells. Transfection of dominant negative Src decreased the expression of

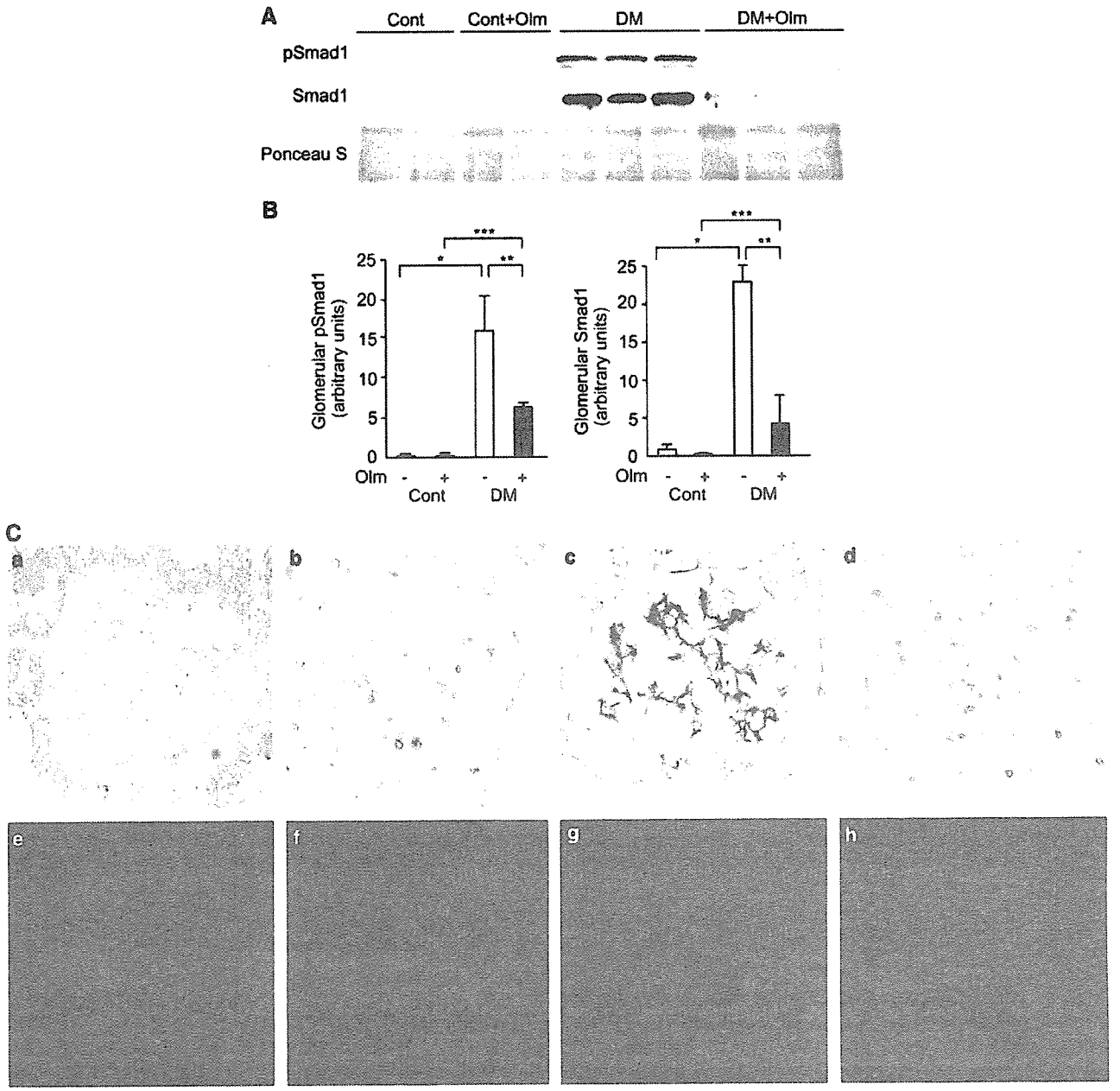

Figure 4 Glomerular expression of Smad1 and phospho-Smad1 (pSmad1) in four groups. (**A**) Immunoblots for Smad1 and pSmad1 in the four groups are shown. Glomerular lysates were prepared as described in Figure 3. In total, 30 μg of each sample was analyzed. Each lane represents a representative Western blotting for the glomerular lysates from each rat. (**B**) Optical densitometry of glomerular expression of Smad1 and pSmad1 in Western blotting. $^{*}P<0.05$ *vs* control rats treated with vehicle (group 1). $^{**}P<0.05$ *vs* diabetic rats treated with vehicle (group 3). $^{***}P<0.05$ *vs* control rats treated with olmesartan (group 2). (**C**) Immunohistochemistry of Smad1 and pSmad1 in control rats treated with vehicle (group 1) (**a**, **e**), control rats treated with olmesartan (group 2) (**b**, **f**), diabetic rats treated with vehicle (group 3) (**c**, **g**), and diabetic rats treated with olmesartan (group 4) (**d**, **h**). Representative light microscopic appearance of glomerulus is shown. The original magnification was $\times 400$. **a–d**, Smad1; **e–h**, phospho-Smad1.

pSmad1 and Col4 under Ang II stimulation compared with mock vector. In contrast, β-actin protein levels, used as an internal control, were invariant across the samples (Figure 7). Next, mesangial cells were transfected with siRNA against Src or an equimolar amount of negative control siRNA and then treated with Ang II. Addition of siRNA against Src suppressed Ang II-induced phosphorylation of Src and Smad1, and synthesis of Col4. In contrast, β-actin protein levels, used as an internal control, were invariant across the samples (Figure 8).

Discussion

In this study, we demonstrate that the AT1 antagonist ameliorates mesangial matrix expansion in the early phase of diabetic nephropathy through

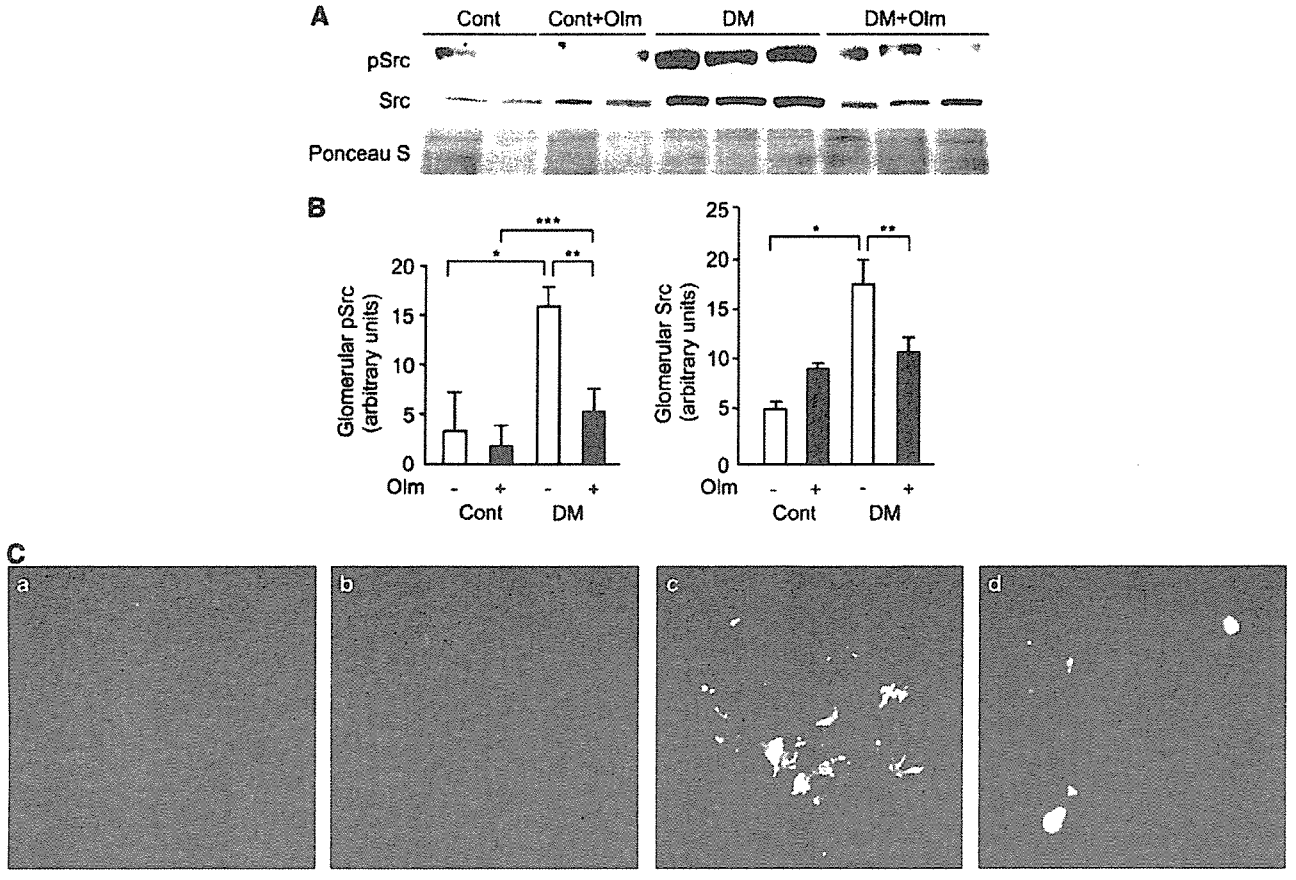

Figure 5 Glomerular expressions of Src and phospho-Src (pSrc) in four groups. (**A**) Immunoblots for Src and pSrc in the four groups are shown. Glomerular lysates were prepared as described in Figure 3. In all, 30 μg of each sample was analyzed. Each lane represents a representative Western blotting for the glomerular lysates from each rat. (**B**) Optical densitometry of glomerular expressions of Src and pSrc in Western blotting. $^{*}P<0.05$ *vs* control rats treated with vehicle (group 1). $^{**}P<0.05$ *vs* diabetic rats treated with vehicle (group 3). $^{***}P<0.05$ *vs* control rats treated with olmesartan (group 2). (**C**) Immunohistochemistry of pSrc in control rats treated with vehicle (group 1) (**a**), control rats treated with olmesartan (group 2) (**b**), diabetic rats treated with vehicle (group 3) (**c**), and diabetic rats treated with olmesartan (group 4) (**d**). Representative light microscopic appearance of glomerulus is shown. The original magnification was $\times$ 400.

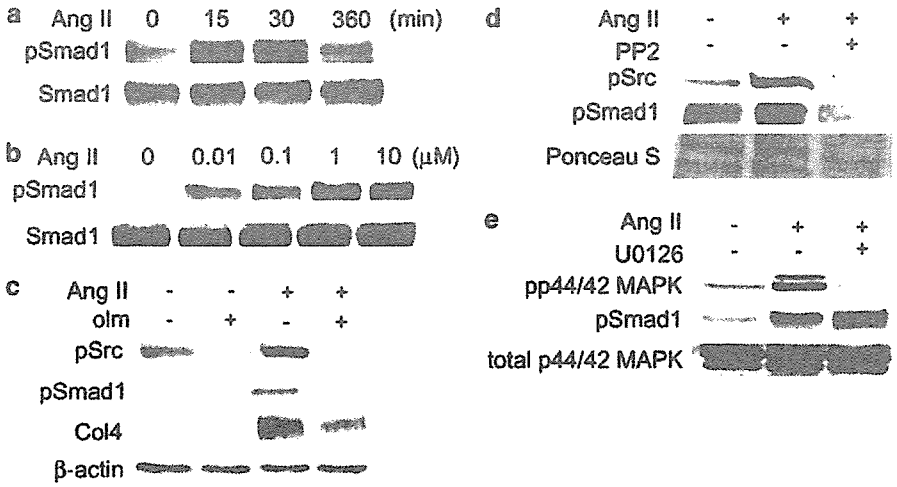

Figure 6 Role of Smad1 and Src activation by Ang II in mesangial cells. (**a**) Time course of phosphorylation of Smad1 by Ang II. Serum-deprived mesangial cells were treated with 0.1 μM Ang II for the indicated times. (**b**) Dose response of phosphorylation of Smad1 by Ang II. Cells were treated with various concentrations of Ang II (0–10 μM) for 15 min. (**c**) Effect of olmesartan on pSmad1, pSrc, and Col4. Mesangial cells were preincubated with olmesartan (10 μM) for 48 h before exposure to Ang II (0.1 μM, 15 min). One of three independent experiments is shown. (**d**) Effect of PP2 on pSmad1 and pSrc. Mesangial cells were preincubated with PP2 (10 μM) for 48 h before exposure to Ang II (0.1 μM, 15 min). A representative data from three independent experiments is shown. (**e**) Effect of U0126 on pSmad1 and phospho-p44/42 MAP kinase (pp44/42 MAP kinase). Mesangial cells were preincubated with U0126 (10 μM) for 48 h before exposure to Ang II (0.1 μM, 15 min). A representative data from three independent experiments is shown.