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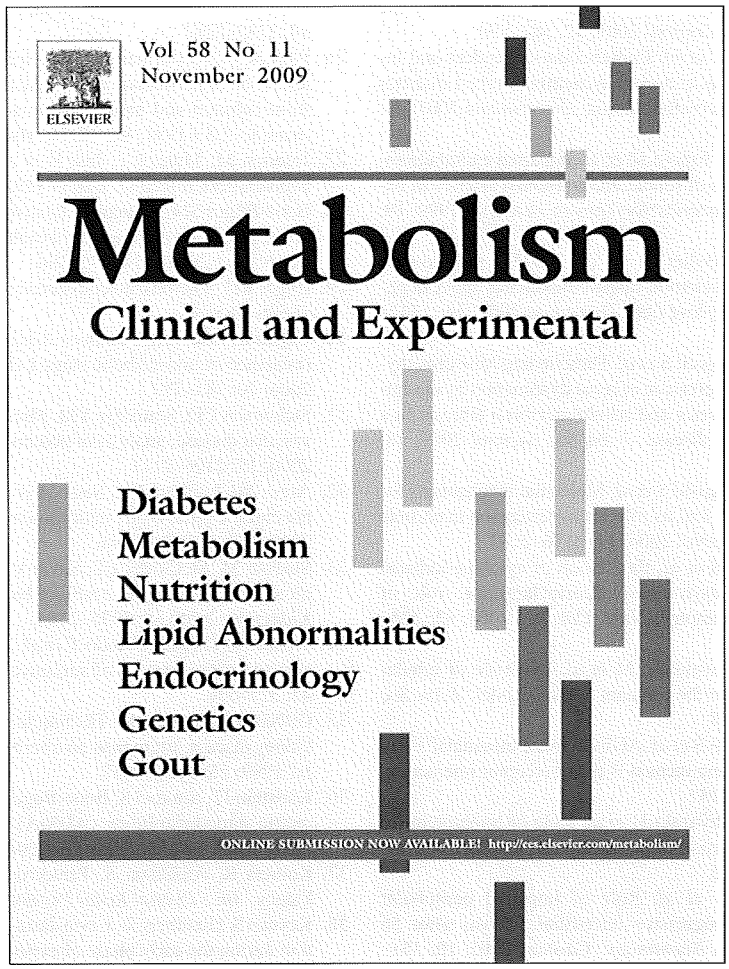

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Positive association of serum levels of advanced glycation end products and high mobility group box–1 with asymmetric dimethylarginine in nondiabetic chronic kidney disease patients

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Abstract

There is accumulating evidence that engagement of the receptor for advanced glycation end products (RAGE) with ligands such as advanced glycation end products (AGEs) and high mobility group box–1 (HMGB-1) elicits vascular inflammation, thus contributing to the increased risk for cardiovascular disease. Furthermore, enhanced accumulation of asymmetric dimethylarginine (ADMA) plays a role in cardiovascular disease in chronic kidney disease (CKD) patients. However, the relationships among serum levels of AGEs, HMGB-1, soluble form of RAGE (sRAGE), and ADMA are largely unknown. The aim of the present study is to determine their relationships in CKD patients. Twenty nondiabetic normotensive CKD patients with dyslipidemia and 20 age- and sex-matched healthy controls were enrolled. All subjects underwent determination of blood chemistries; urinary proteinuria; and serum levels of AGEs, HMGB-1, sRAGE, and ADMA. Serum AGE, HMGB-1, sRAGE, and ADMA levels in CKD patients were significantly higher than those in control subjects. Circulating levels of AGEs in CKD patients were positively associated with sRAGE and ADMA, and HMGB-1 with ADMA, but not sRAGE. There were no significant associations among these markers and serum creatinine, estimated glomerular filtration rate, proteinuria, and lipid levels. In multiple regression analyses, AGEs and HMGB-1 were independently correlated with ADMA. The present study demonstrated that AGE and sRAGE levels were correlated with each other and that AGEs and HMGB-1 were independently associated with ADMA in nondiabetic CKD patients. Elevation of the RAGE ligands may enhance ADMA levels, suggesting the active involvement of AGE/HMGB-1–RAGE–ADMA axis in CKD patients.

1. Introduction

Endothelial dysfunction with reduced nitric oxide production and/or bioavailability is a common feature in patients with apparent coronary atherosclerosis or in high risk-patients with chronic kidney disease (CKD) or diabetes, thereby contributing to the development and progression of cardiovascular disease (CVD) [1,2]. Because asymmetric dimethylarginine (ADMA) is an endogenous nitric oxide synthase inhibitor and its levels are increased in CKD patients [3,4], it is considered that ADMA is a novel emerging risk factor for CVD in patients with CKD. However, the underlying molecular mechanisms for the elevation of ADMA in CKD patients are not fully understood.

Receptor for advanced glycation end products (RAGE) is a member of the immunoglobulin superfamily of cell surface

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molecules capable of interacting with a broad spectrum of ligands including a diverse group of reducing sugar complexes with proteins, lipids, and nucleic acids [5]. Recent studies have shown that engagement of RAGE with ligands such as advanced glycation end products (AGEs) and high mobility group box–1 protein (HMGB-1) elicits oxidative stress generation and subsequently evokes inflammatory and thrombogenic responses in various types of cells, thereby playing an important role in diabetic accelerated atherosclerosis [5-7]. In addition, there are several articles showing that serum levels of AGEs, HMGB-1, and soluble form of RAGE (sRAGE) are elevated in diabetic patients, especially those with coronary artery disease [8-13], thus suggesting that they are novel biomarkers for CVD in diabetes.

Advanced glycation end product–modified proteins are shown to inhibit the enzymatic activity of dimethylarginine dimethylaminohydrolase (DDAH), an enzyme that mainly degrades ADMA in vivo, in cultured endothelial cells [1,2,14]. Furthermore, DDAH activity in endothelial cells is suppressed under oxidative stress conditions [15]. These findings led us to speculate that the activation of RAGE system with ligands such as AGEs and HMGB-1 could be involved in the ADMA elevation in patients with CKD. In this study, we measured serum levels of AGEs, HMGB-1, sRAGE, and ADMA simultaneously and investigated their relationships in early-stage CKD patients without diabetes.

2. Subjects and methods

2.1. Subjects

Twenty nondiabetic stage 1 or 2 CKD patients with dyslipidemia (total cholesterol [T-chol] >220 mg/dL, low-density lipoprotein cholesterol [LDL-C] >140 mg/dL, 150 < triglyceride [TG] < 400 mg/dL, or high-density lipoprotein-cholesterol [HDL-C] <40 mg/dL) (14 men and 6 women; immunoglobulin A nephropathy, n = 13; non–immunoglobulin A type proliferative glomerulonephritis, n = 5; membranous nephropathy, n = 2; mean age, 35.7 ± 5.8 years) and 20 age- and sex-matched healthy controls (14 men and 6 women; mean age, 37.2 ± 6.4 years) were enrolled in the present study. All patients were normotensive (blood pressure <130/80 mm Hg); and none of them received antihypertensive or antihyperlipidemic drugs such as angiotensin-converting enzyme inhibitors, angiotensin II type 1 receptor blockers, and statins. We excluded any patients with chronic pulmonary diseases, liver diseases, and neoplastic disorders and those who had recent (<6 months) acute coronary syndromes, stroke, and any acute infections. Patients who were younger than 20 years old, whose serum creatinine (Cr) level was more than 1.5 mg/dL, or whose proteinuria was more than 3.0 g/d were also excluded. Initially, 24 nondiabetic CKD patients were enrolled; but 4 patients were excluded because of the presence of massive proteinuria (n = 2) or liver diseases (n = 2). 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.

2.2. Data collection

Blood pressure was measured in the sitting position twice after 2 minutes of rest using an upright standard sphygmomanometer. Mean value of blood pressures was used for analysis. Renal function was evaluated by serum Cr levels and estimated glomerular filtration rate (eGFR) according to the Modification of Diet in Renal Disease equation modified for the Japanese population [16]. Serum levels of T-chol, TG, and HDL-C were measured enzymatically at Shinmatsudo Central General Hospital. Low-density lipoprotein cholesterol level was calculated using the Friedewald formula. Serum ADMA level was analyzed by a high-performance liquid chromatography as described previously [17]. Serum levels of AGEs, sRAGE, and HMGB-1 were measured with enzyme-linked immunosorbent assays (ELISAs) as described previously [18-22]. In this study, 1 U of AGEs corresponds to 1 μg of glyceraldehyde-derived AGE–bovine serum albumin as described previously [19]. Intraassay and interassay coefficients of variation of sRAGE ELISA were 7.7% and 5.7, respectively. Intraassay and interassay coefficients of variation of HMGB-1 ELISA were less than 10%, and the detection limit was 0.3 ng/mL [21,22].

2.3. Statistical methods

Data were expressed as mean ± standard deviation. To compare the parameters between CKD patients (n = 20) and healthy controls (n = 20), we used the Wilcoxon signed-rank test. Correlations among serum AGEs, HMGB-1, sRAGE, and ADMA and clinical variables were determined by a

Table 1
Characteristics of CKD patients and age- and sex-matched healthy controls

	CKD patients	Healthy controls	*P* value
Age (y)	36.7 ± 5.8	37.2 ± 6.4	*P* = .699
Sex (male/female)	14/6	14/6	
SBP (mm Hg)	126.7 ± 7.3	122.6 ± 6.2	*P* = .521
DBP (mm Hg)	76.7 ± 4.3	73.4 ± 3.6	*P* = .642
T-chol (mg/dL)	244.7 ± 12.5	162.8 ± 14.0	*P* < .001
LDL-C (mg/dL)	172.6 ± 13.0	102.7 ± 10.5	*P* < .001
HDL-C (mg/dL)	39.1 ± 3.0	59.5 ± 5.8	*P* < .001
TG (mg/dL)	165.7 ± 11.9	102.7 ± 10.5	*P* < .001
Cr (mg/dL)	0.763 ± 0.053	0.709 ± 0.072	*P* < .05
eGFR (mL/min)	86.58 ± 10.43	92.9 ± 10.7	*P* = .062
Proteinuria (g/d)	1.15 ± 0.20	0	
ADMA (nmol/mL)	0.621 ± 0.050	0.361 ± 0.048	*P* < .001
HMGB-1 (ng/mL)	1.20 ± 0.30	ND	
AGEs (U/mL)	13.24 ± 1.33	6.3 ± 1.7	*P* < .001
sRAGE (pg/mL)	1244.0 ± 84.9	506.3 ± 132.2	*P* < .001

SBP indicates systolic blood pressure; DBP, diastolic blood pressure; ND, not detected.

linear regression analysis. To determine independent determinants of serum ADMA levels, multiple stepwise linear regression analysis was performed. Statistical significance was defined as $P < .05$. All statistical analyses were performed with the use of the SAS system (SAS Institute, Cary, NC).

3. Results

Background of the patients is shown in Table 1. All patients were normotensive and dyslipidemic. Total cholesterol, LDL-C, and TG levels were significantly higher and HDL-C levels were significantly lower in CKD patients compared with those in healthy controls ($P < .0001$). Serum Cr levels in CKD patients were slightly higher than those in healthy controls ($P < .05$), but there was no significant difference of eGFR levels between the 2 groups. Serum AGE, sRAGE, and ADMA levels were significantly elevated in CKD patients compared with those in healthy controls ($P < .001$). Serum HMGB-1 levels were 1.20 ± 0.30 ng/mL in CKD patients, but it was not detected with our ELISA system in healthy controls (<0.3 ng/mL).

In CKD patients, circulating AGE levels were positively associated with serum levels of sRAGE ($r = 0.842$, $P < .001$) and ADMA ($r = 0.575$, $P = .008$), but not HMGB-1 ($r = 0.075$, $P = .753$) (Fig. 1). Furthermore, serum levels of HMGB-1 were positively correlated with ADMA ($r = 0.448$, $P = .048$), but not sRAGE ($r = -0.116$, $P = .626$) (Fig. 2). Because the parameters could be closely correlated with each other, to determine independent determinants of serum ADMA levels, multiple stepwise regression analysis was performed. This analysis showed that AGEs ($P = .006$) and

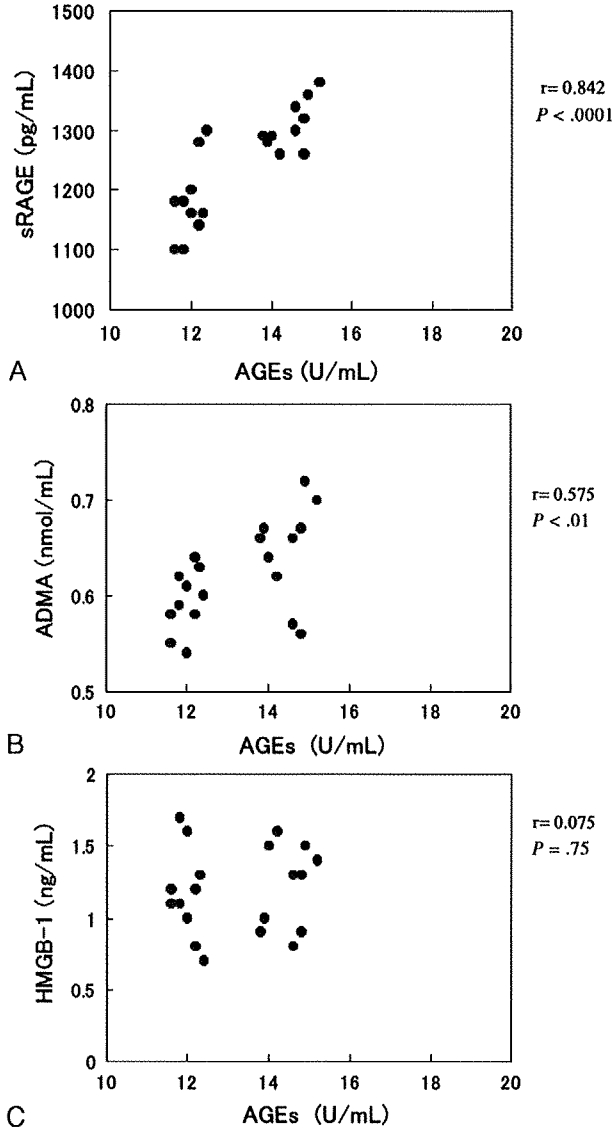

Fig. 1. Correlations between serum levels of AGEs and sRAGE (A), ADMA (B), and HMGB-1 (C) levels in CKD patients.

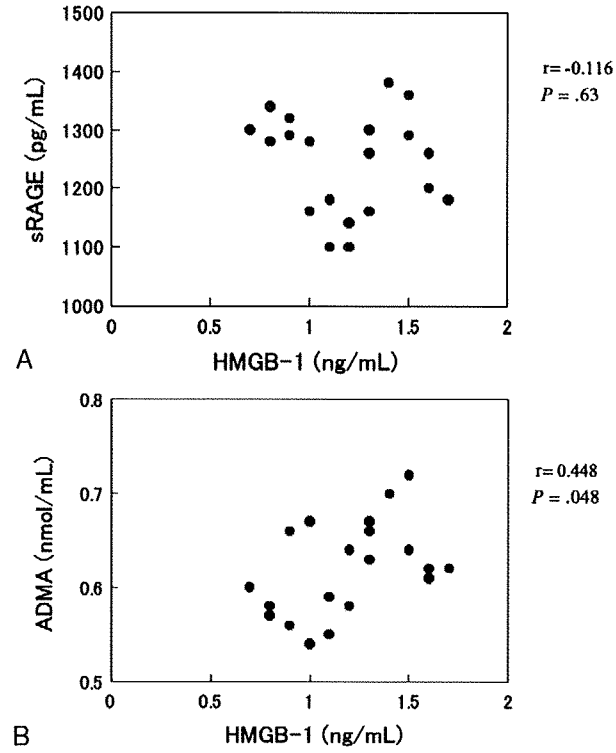

Fig. 2. Correlations between serum levels of HMGB-1 and sRAGE (A) and ADMA (B) in CKD patients.

Table 2
Multiple stepwise regression analysis for determinants of serum ADMA levels in CKD patients

Parameters	β	SE	P value
AGEs	.544	0.006	.006
HMGB-1	.407	0.029	.031
sRAGE	.183	0.001	.605

$R^2 = 0.435$. β indicates standardized regression coefficients; SE, standard error.

HMGB-1 ($P = .031$) were independently related to ADMA levels ($R^2 = 0.435$) (Table 2). There were no significant associations among all the parameters assayed here and T-chol, LDL-C, HDL-C, TG, Cr, eGFR, and proteinuria levels in both CKD patients and control subjects.

4. Discussion

In the present study, we demonstrated for the first time that serum levels of AGEs and sRAGE were correlated with each other and that AGE and HMGB-1 levels were independently associated with ADMA in nondiabetic early-stage CKD patients. This study has extended our previous findings that serum AGE levels were positively correlated with sRAGE and that their levels were associated with inflammatory markers and coronary artery disease in type 2 diabetes mellitus patients [9-11]. Although exogenously administered sRAGE was shown to exert atheroprotective properties in diabetic apolipoprotein E–null animals by acting as a decoy receptor for AGEs [23-25], it is questionable that sRAGE in humans could also exert the same biological effects because human serum levels of sRAGE are 1000 times lower than needed for the binding to AGEs [12,15,25]. Therefore, our present findings further support the concept that endogenous sRAGE could not efficiently capture and eliminate circulating AGEs in humans. Recently, most of the sRAGE in human blood has been found to be generated from the cleavage of cell surface RAGE by the action of sheddase, a disintegrin and metalloproteinase 10 [26]. Because AGEs up-regulate tissue RAGE expression [27-30], sRAGE levels in human blood may reflect tissue RAGE expression and ongoing inflammation and be elevated in response to circulating AGEs as a countersystem against the AGE-elicited inflammation in nondiabetic CKD patients.

In this study, serum levels of HMGB-1 were not correlated with sRAGE. This finding was also consistent with the recent observations in patients with diabetes showing that increased serum HMGB-1 levels were associated with coronary artery disease, but not with sRAGE [15]. These observations suggest that the kinetics and regulation of serum AGEs and HMGB-1 could differ. The differences of serum concentrations and binding affinity to RAGE between the 2 could account for the different correlations of these factors with sRAGE [31,32]. Compared with HMGB-1, circulating AGEs may be a stronger stimulant for RAGE expression and subsequent sRAGE generation in humans.

In the present study, we found for the first time that serum levels of AGEs and HMGB-1 were elevated in nondiabetic early-stage CKD patients. Although serum AGE and HMGB-1 levels were reported to increase in diabetic and/or stage 3 to 5 CKD patients [15,33], it is unlikely that blood glucose levels or renal function could affect our present results because (*a*) we enrolled nondiabetic stage 1 or 2 CKD patients without apparently active inflammatory diseases and (*b*) parameters associated with renal dysfunction such as serum Cr, eGFR, and proteinuria levels were not correlated with AGE or HMGB-1 levels. Because serum levels of high-sensitive C-reactive protein and oxidative stress markers are elevated in stage 1 or 2 CKD patients [3,4], increased oxidative stress generation and subclinical inflammation could contribute to the elevation of AGEs and HMGB-1 in our patients.

We also showed here first that circulating levels of AGEs and HMGB-1, but not sRAGE, were independent determinants of serum ADMA levels. Asymmetric dimethylarginine is mainly degraded by DDAH, whose enzymatic activity is suppressed by oxidative stress [15]. Therefore, it is conceivable that the AGE/HMGB-1–RAGE interaction elicits oxidative stress generation and subsequently inactivates DDAH activity, thus leading to increased ADMA production by various types of cells. Therefore, our present findings suggest that the activation of the AGE/HMGB-1–RAGE axis is involved in the elevation of ADMA, which could partly explain the increased risk for CVD in nondiabetic stage 1 or 2 CKD patients [34].

5. Limitations

First, our study was a cross-sectional one and, therefore, does not elucidate the causal relationships among serum AGE, HMGB-1, sRAGE, and ADMA levels. Therefore, we do not know whether circulating levels of AGEs or HMGB-1 could be mechanistically related to vascular inflammation and ADMA elevation. In vitro or in vivo testing in animal models could clarify the causal relationships among these factors and provide the mechanistic insight into how the factors were correlated with each other. Second, further study is needed to strengthen the concept that serum sRAGE is a proteolytically cleaved form from cell surface RAGE that is shed into the bloodstream and therefore serves as a marker for vascular injury in vivo. For this, examining a direct link between serum sRAGE and RAGE expression levels on peripheral blood mononuclear cells or vascular wall cells in patients with nondiabetic CKD would be helpful in a future study. Third, unfortunately, oxidative stress markers were not measured in our subjects because of the lack of serum samples. Therefore, whether oxidative stress markers are correlated with AGEs, HMGB-1, sRAGE, and ADMA in nondiabetic stage 1 or 2 CKD patients remains unclear. Interventional studies with antioxidants would be helpful to clarify whether oxidative stress is involved in positive correlations among these factors.

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RESEARCH ARTICLE

Protective role of pigment epithelium-derived factor (PEDF) in early phase of experimental diabetic retinopathy

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Abstract

Background Pigment epithelium-derived factor (PEDF) is the most potent inhibitor of angiogenesis in the mammalian eye, thus suggesting that PEDF may protect against proliferative diabetic retinopathy. However, a role for PEDF in early diabetic retinopathy remains to be elucidated. We investigated here whether and how PEDF could prevent the development of diabetic retinopathy.

Methods Streptozotocin-induced diabetic rats were treated with or without intravenous injection of PEDF for 4 weeks. Early neuronal derangements were evaluated by electroretinogram (ERG) and immunofluorescent staining of glial fibrillary acidic protein (GFAP). Expression of PEDF and 8-hydroxydeoxyguanosine (8-OHdG), a marker of oxidative stress, was localized by immunofluorescence. Vascular endothelial growth factor (VEGF) and p22phox expression were evaluated with western blots. Breakdown of blood retinal barrier (BRB) was quantified with fluorescein isothiocynate (FITC)-conjugated dextran. NADPH oxidase activity was measured with lucigenin luminescence.

Results Retinal PEDF levels were reduced, and amplitudes of a- and b-wave in the ERG were decreased in diabetic rats, which were in parallel with GFAP overexpression in the Müller cells. Further, retinal 8-OHdG, p22phox and VEGF levels and NADPH oxidase activity were increased, and BRB was broken in diabetic rats. Administration of PEDF ameliorated all of the characteristic changes in early diabetic retinopathy.

Conclusions Results suggest that PEDF could prevent neuronal derangements and vascular hyperpermeability in early diabetic retinopathy via inhibition of NADPH oxidase-driven oxidative stress generation. Substitution of PEDF may offer a promising strategy for halting the development of diabetic retinopathy. Copyright © 2009 John Wiley & Sons, Ltd.

Keywords diabetic retinopathy; ERG; oxidative stress; PEDF

Introduction

Pigment epithelium-derived factor (PEDF) was first purified from the conditioned media of human retinal pigment epithelial cells with neuronal differentiating activity [1]. Recently, PEDF has been shown to be the most potent inhibitor of angiogenesis in the mammalian eye; it inhibited retinal endothelial cell growth and migration, and suppressed ischaemia-induced retinal neovascularization [1]. Further, PEDF levels in aqueous humour

or vitreous are decreased in diabetic patients, especially with proliferative retinopathy, thus suggesting that a loss of PEDF in the eye may contribute to proliferative diabetic retinopathy [2]. However, a protective role for PEDF in early diabetic retinopathy is not fully understood.

Retinal neuronal cell damage and vascular hyperpermeability are the earliest signs for the development of simple diabetic retinopathy, whose processes are mainly mediated by oxidative stress generation [3–8]. Since we, along with others, have previously shown that PEDF protects retinal neurons and vasculatures from oxidative stress-induced injury [1,6,9–15], it is conceivable that PEDF could play a protective role against neuronal cell damage and vascular hyperpermeability in early diabetic retinopathy. In this study, we investigated whether PEDF could inhibit the development of experimental diabetic retinopathy through its anti-oxidative properties.

Materials and methods

Purification of PEDF proteins

PEDF proteins were purified as described previously [16]. SDS–PAGE analysis of purified PEDF proteins revealed a single band with a molecular weight of about 50 kDa, which showed positive reactivity with monoclonal antibodies (Abs) against human PEDF (Transgenic, Kumamoto, Japan).

Induction of experimental diabetes

Six-week-old male Wister rats received single 60 mg/kg intraperitoneal injection of streptozotocin (Sigma, St. Louis, MO, USA) in 10 mmol/L citrate buffer (pH 4.5). Non-diabetic control rats received citrate buffer alone. Animals with blood glucose levels >250 mg/dL 48 h later were considered diabetic. Diabetic or non-diabetic control rats were injected intravenously with or without PEDF (5 μg PEDF/100 g body weight) three times a week for up to 4 weeks. Diabetic rats were maintained with subcutaneous injections of 2 U insulin (two times per week) (Humalin N, Eli Lilly, IN, USA). Rats were killed on the day of 4 weeks, and the eyes were removed and fixed for immunohistochemical and biochemical analyses. All animal procedures were conducted according to the guidelines provided by the Kurume University Institutional Animal Care and Use Committee under an approved protocol.

Immunohistochemical staining of PEDF and glial fibrillary acidic protein

Rat eyes were fixed for 1 h in 4% paraformaldehyde. Then the eyes were embedded in optimal cutting temperature (OCT) compound (Tissue-Tek, Sakura Finetechnical Co, Ltd, Tokyo, Japan) to freeze and cut into 10-μm sections. The frozen sections were incubated with polyclonal Abs raised against human PEDF (5 μg/mL; BioProducts, Middletown, MD, USA) or cow glial fibrillary acidic protein (GFAP) (1 : 20; Dako, Carpinteria, CA, USA). After exposure to Alexa fluo 488 goat anti-rabbit IgG (Invitrogen, Eugene, OR, USA), images were obtained by a confocal laser-scanning fluorescence microscopy. Intensity of PEDF and GFAP staining in three different fields of each sample was analysed by microcomputer-assisted image J.

Electroretinogram

Electroretinogram (ERG) was monitored as described previously [17]. Briefly, rats were anesthetized with an intramuscular injection of ketamine hydrochloride (50 mg/kg) and xylazine (10 mg/kg), and then the pupils were maximally dilated. A built-in LED contact lens electrode (Mayo, Aichi, Japan) was placed on the right eye, and the stimulus was controlled by a white LED stimulator (Mayo). The retinal signals were amplified with a bandpass 1–1000 Hz for scotopic responses.

Immunohistochemical staining of 8-hydroxydeoxyguanosine

Rat eyes were fixed for 1 day in 4% paraformaldehyde. Then the eyes were embedded in paraffin wax for sectioning. Five micrometre paraffin sections were incubated with monoclonal Abs raised against 8-hydroxydeoxyguanosine (8-OHdG) (10 μg/mL; Japan Institute for the Control of Aging, Shizuoka, Japan). After exposure to peroxidase-labelled secondary anti-mouse Abs, the sections were incubated with 3,3′-diaminobenzidine solution for visualizing 8-OHdG immunoreactivity. Immunoreactivity of 8-OHdG staining in five different fields of each sample was analysed by microcomputer-assisted image J.

Western blot analysis

Fifty micrograms of protein extracted from rat retinas was subjected to SDS–PAGE and western blotting with specific primary Abs raised against human PEDF (BioProducts), human GFAP (Santa Cruz Biotechnology, Santa Cruz, CA, USA), human p22phox (Santa Cruz Biotechnology), human vascular endothelial growth factor (VEGF) (Santa Cruz Biotechnology), or α-tubulin (Sigma) and a horseradish peroxidase-conjugated secondary Abs (Promega, Madison, WI, USA). Detection was performed by enhanced chemiluminescence (Amersham Pharmacia Biotech, Buckinghamshire, UK).

NADPH oxidase assay

NADPH oxidase activity of the retinas was measured by luminescence assay in 50 mmol/L phosphate buffer, pH

7.0, containing 1 mmol/L ethylene glycol tetraacetic acid (EGTA), 150 mmol/L sucrose, 5 μmol/L lucigenin as the electron acceptor and 100 μmol/L NADPH as a substrate as described previously [6].

Quantification of blood retinal barrier

Breakdown

Blood retinal barrier (BRB) breakdown quantification was determined as described previously [6]. Briefly, after deep anaesthesia, the rats received intravenous injection with FITC-conjugated dextran (4.4 kDa, Sigma). After 10–15 min, a blood sample was collected and then each rat was perfused with phosphate-buffered saline (PBS). After perfusion, the retinas were carefully removed, weighted and homogenized to extract the FITC-conjugated dextran. BRB breakdown was calculated using the following equation:

$$\frac{\text{Retinal FITC-dextran (μg)/retinal weight (g)}}{\text{Plasma FITC-dextran concentration (μg/μL)}} \times \text{circulation time (h)}$$

Statistical analysis

All values were presented as means ± SEM. Unless otherwise indicated, one-way ANOVA followed by the Scheffe F test was performed for statistical comparisons. In Figure 3B, Mann–Whitney U test was performed; $p < 0.05$ was considered significant.

Results

As shown in Figure 1A and B, body weight was lower and blood glucose levels were higher in diabetic rats, compared with those in non-diabetic control rats. PEDF treatment did not affect body weight or blood glucose levels in both non-diabetic and diabetic rats.

We examined whether retinal PEDF levels were decreased in diabetic rats. As shown in Figure 2A, PEDF immunoreactivity in the ganglion cell layer, the inner plexiform layer and pigment epithelium was dramatically decreased in diabetic rats, compared with non-diabetic control rats. PEDF administration significantly restored the decrease in retinal PEDF levels in diabetic rats (Figure 2A). Western blotting analysis also confirmed that retinal PEDF levels were decreased in diabetic rats, which were restored by PEDF injections (Figure 2B).

Then we investigated the effects of PEDF administration on retinal neuronal injury in diabetic rats. As shown in Figure 3A and B, ERG responses of the diabetic rats were reduced in amplitude, compared with those recorded from non-diabetic control rats; the amplitude of b-wave was more affected than that of a-wave. PEDF treatment significantly ameliorated the decrease in amplitudes of a- and b-wave in diabetic rats. Further, although GFAP expression was restricted to the innermost retinal layer, where astrocytes are located in control rats, it was also detected in the endfeet of the Müller cells in diabetic rats (Figure 3C). PEDF treatment significantly reduced the expression levels of GFAP in the Müller cells of diabetic retinas (Figure 3C). Western blotting analysis also confirmed that retinal GFAP levels were increased in diabetic rats, which were blocked by PEDF injections (Figure 3D).

Oxidative stress could contribute to early phase of diabetic retinopathy [3–8]. So, we next examined the effects of PEDF on retinal levels of 8-OHdG, an oxidative stress marker in diabetic rats. As shown in Figure 4A, immunohistochemistry of 8-OHdG showed intense staining in the nuclei of cells in the inner and outer plexiform layers of diabetic rat retinas. In addition, expression levels of p22phox, a membrane component of NADPH oxidase [6], and its enzymatic

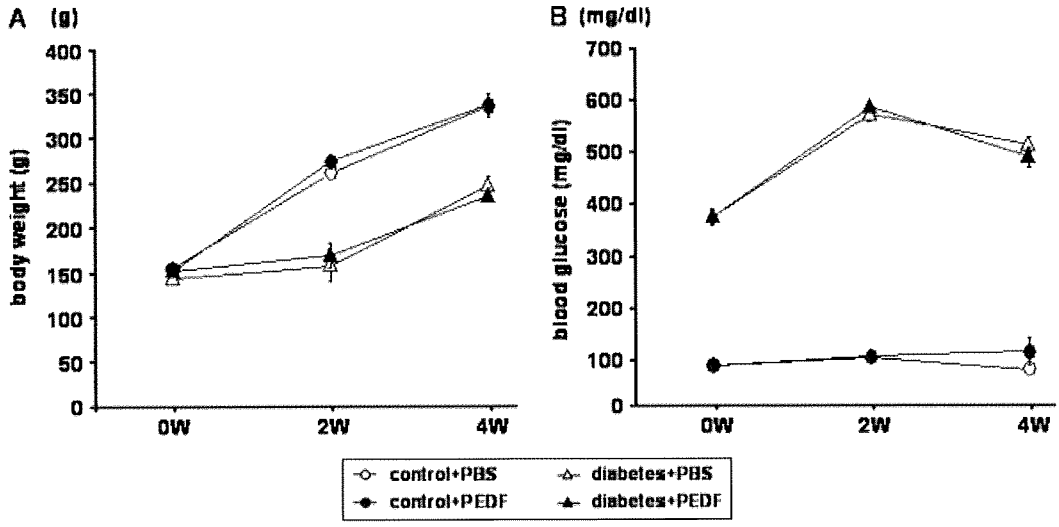

Figure 1. Effects of pigment epithelium-derived factor administration on body weight (A) and blood glucose levels (B) in diabetic or non-diabetic control rats. $N = 6–7$ per group

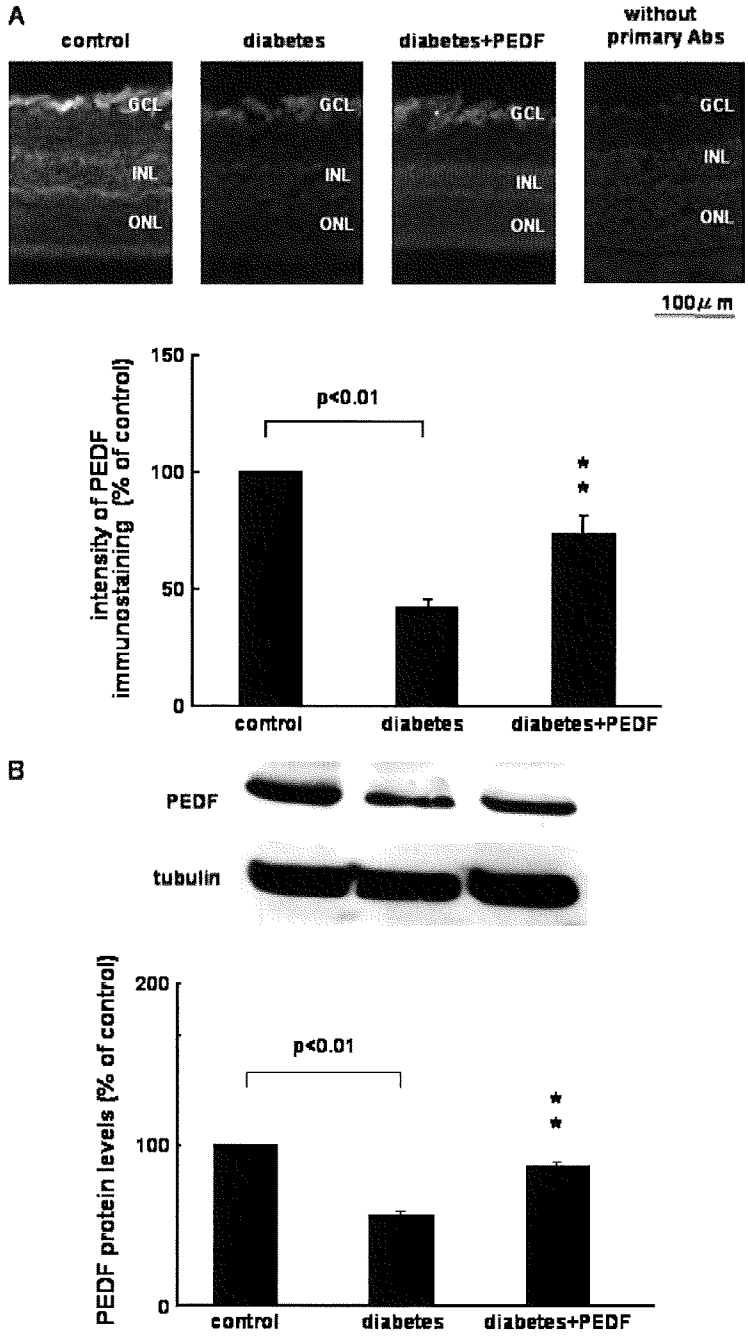

Figure 2. Effects of pigment epithelium-derived factor (PEDF) administration on retinal PEDF levels in diabetic or non-diabetic control rats. $N = 3$ per group. (A) Upper panel shows a typical immunohistochemical staining of PEDF in rat retinas. GCL, ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer. Lower panel shows quantitative data. PEDF levels were evaluated as fluorescent intensity on a confocal microscope. Intensity of PEDF staining was analysed by microcomputer-assisted image J. (B) Western blotting analysis for PEDF. Upper panel shows typical bands. Lower panel shows quantitative data normalized by the intensity of tubulin bands. $^{**}p < 0.05$ compared with the value of diabetic rats. This figure is available in colour online at www.interscience.wiley.com/journal/dmrr

activity were increased in diabetic rats (Figure 4B and C). Administration of PEDF decreased retinal levels of 8-OHdG and p22phox and NADPH oxidase activity in diabetic rats (Figure 4A–C).

To further investigate the protective role of PEDF against early diabetic retinopathy, we examined the effects of PEDF on VEGF expression and vascular permeability in diabetic rats. As shown in Figure 5A and B, retinal VEGF expression was increased, and BRB function was disturbed in diabetic rats, both of which were ameliorated by the treatment of PEDF.

Discussion

One early phase of diabetic retinopathy involves neuronal cell derangements and vascular hyperpermeability, which precede any visible morphological abnormalities in

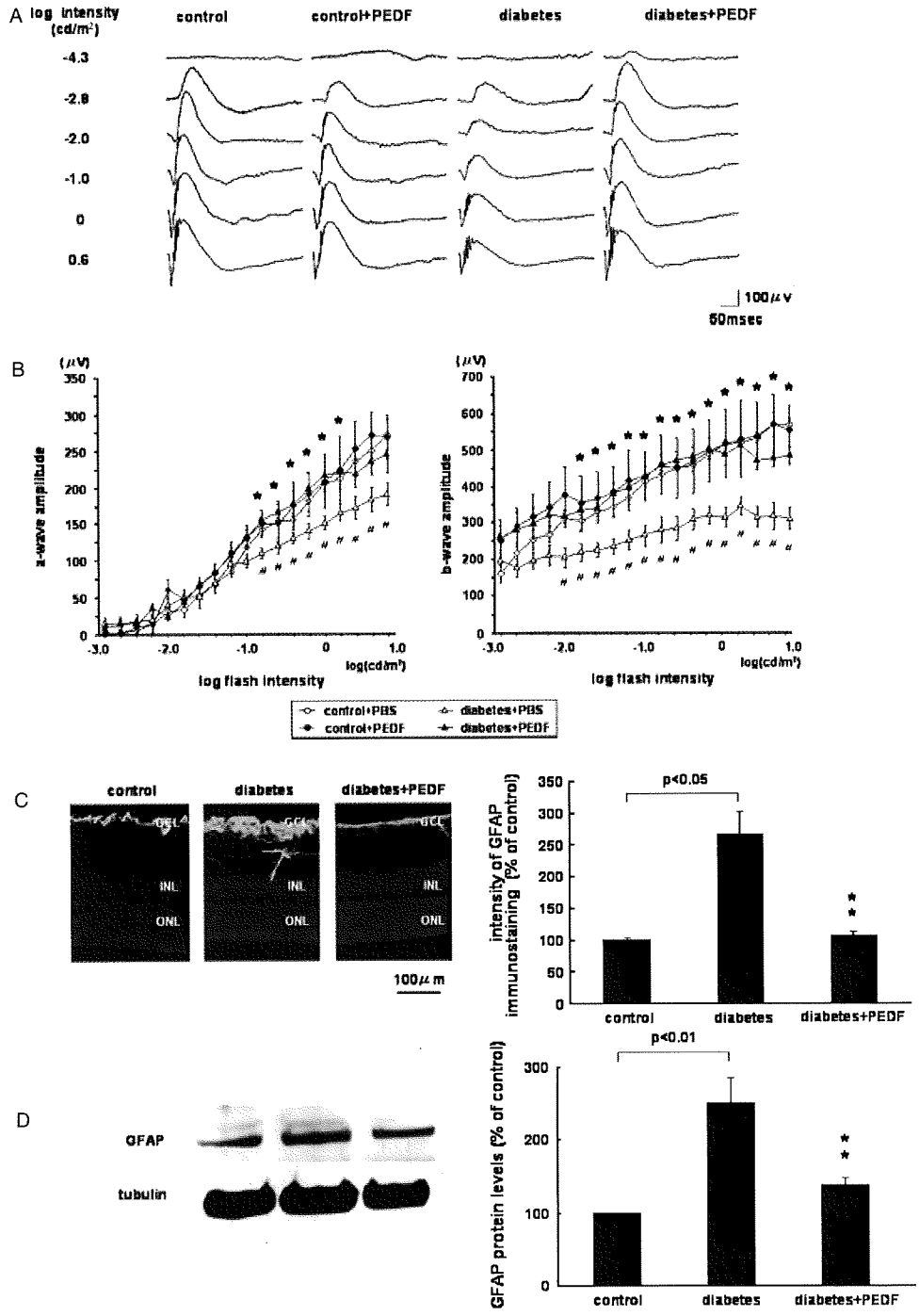

Figure 3. Effects of pigment epithelium-derived factor (PEDF) administration on retinal neuronal cell damage in diabetic or non-diabetic control rats. (A) Typical scotopic electroretinogram responses. (B) Intensity-response curves. Amplitudes of a- and b-wave in diabetic or non-diabetic control rats treated with or without PEDF. $^{*}p < 0.01$ compared with the value of diabetic rats. $^{\#}p < 0.01$ compared with the value of non-diabetic control rats. $N = 5$ per group. (C) Upper panel shows a typical immunohistochemical staining of glial fibrillary acidic protein (GFAP) in rat retinas. GCL, ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer. Lower panel shows quantitative data. GFAP levels were evaluated as fluorescent intensity on a confocal microscope. Intensity of GFAP staining was analysed by microcomputer-assisted image J. (D) Western blotting analysis for GFAP. Upper panel shows typical bands. Lower panel shows quantitative data normalized by the intensity of tubulin bands. $^{**}p < 0.05$ compared with the value of diabetic rats. This figure is available in colour online at www.interscience.wiley.com/journal/dmrr

diabetic retinas [3–8]. There is accumulating evidence that these processes are predominantly mediated by oxidative stress generation [3–8]. Indeed, anti-oxidants such as lipoic acid and cannabidiol have been shown to prevent the decrease in amplitude of ERG, retinal neuronal cell death and vascular leakage in experimental diabetic retinopathy [4,5]. In this study, we demonstrated for the first time that PEDF administration restored the decrease in amplitudes of a- and b-wave of ERG, which was associated with suppression of GFAP expression in

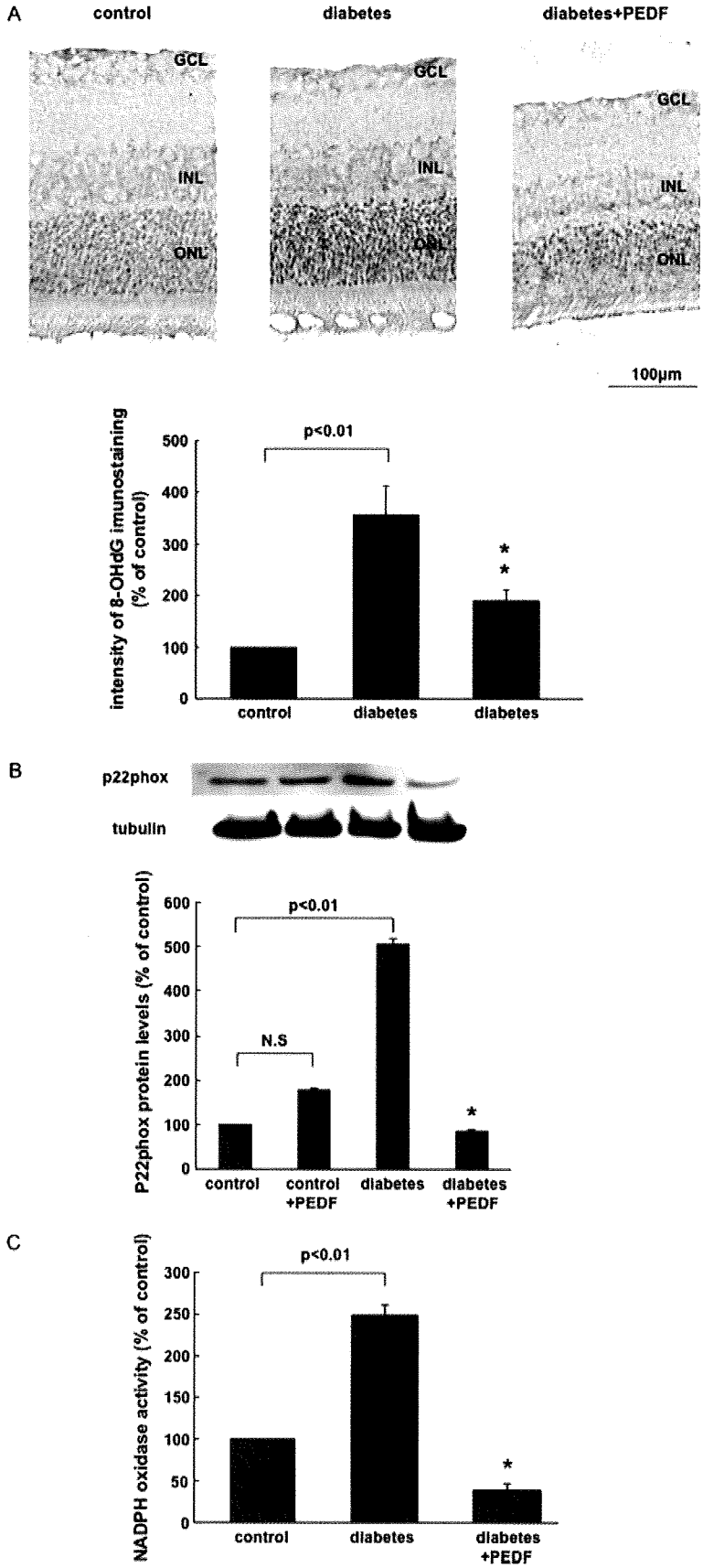

Figure 4. Effects of pigment epithelium-derived factor administration on oxidative stress generation in diabetic or non-diabetic control rats. (A) Upper panel shows a typical immunohistochemical staining of 8-hydroxydeoxyguanosine in rat retinas. Lower panel shows quantitative data. (B) p22phox levels in rat retinas. Upper panel shows typical bands. Lower panel shows quantitative data normalized by the intensity of tubulin bands. (C) NADPH oxidase activity. $^{*}p < 0.01$ compared with the value of diabetic rats. $^{**}p < 0.05$ compared with the value of diabetic rats. $N = 3–4$ per group. This figure is available in colour online at www.interscience.wiley.com/journal/dmrr

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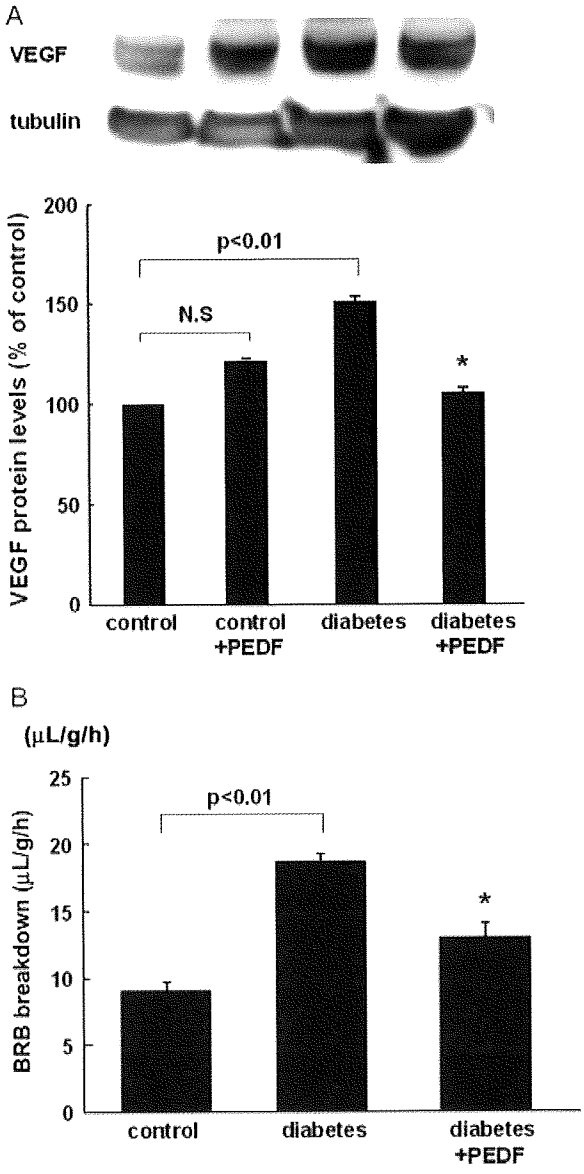

Figure 5. Effects of pigment epithelium-derived factor administration on vascular endothelial growth factor (VEGF) expression (A) and blood retinal barrier (BRB) dysfunction (B) in diabetic or non-diabetic control rats. (A) VEGF levels in rat retinas. Upper panel shows typical bands. Lower panel shows quantitative data normalized by the intensity of tubulin bands. $N = 3$ per group. (B) BRB function. *$p < 0.01$ compared with the value of diabetic rats. $N = 6$ per group

the endfeet of the Müller cells, a cellular marker of retinal damage [3] in diabetic rats. Further, we found here that PEDF treatment inhibited retinal vascular hyperpermeability in diabetic rats. Since PEDF expression was decreased in our animal models of early diabetic retinopathy and that PEDF administration reduced retinal 8-OHdG immunostaining, substitution of PEDF may offer a promising strategy for halting the development of diabetic retinopathy through its anti-oxidative properties. In support of the clinical relevance of PEDF substitution in early diabetic retinopathy, low content of the natural ocular PEDF in aqueous humour has been reported to predict the progression of diabetic retinopathy in humans [2].

In the present study, we found that PEDF reduced expression levels of p22phox, an essential membrane component of NADPH oxidase [6] and its enzymatic activity in diabetic retinas, thus suggesting that the inhibition of NADPH oxidase was a molecular target for the anti-oxidative and protective properties of PEDF in early diabetic retinopathy. The findings have extended our previous observations showing that PEDF not only inhibits AGE-induced endothelial cell damage *in vitro* but also prevents AGE-elicited retinal vascular hyperpermeability in rats by suppressing NADPH oxidase activity [6,18]. NADPH oxidase has been found to be essential in the pathophysiological response of the cells, including growth, migration and gene expression of adhesion molecules and cytokines [19]. Indeed, NADPH oxidase plays an essential role in the vascular inflammation and breakdown of BRB in experimental diabetic retinopathy [8]. Further, AGE could exert pleiotropic actions, including neuronal cell damage, glial reaction and vascular injury by inducing NADPH oxidase-driven reactive oxygen species generation [20,21]. In addition, we have previously shown that PEDF inhibits retinal leukostasis in diabetic or AGE-injected non-diabetic rats by reducing intercellular adhesion molecule-1 expression via suppressing oxidative stress generation [22]. Taken together, these findings suggest that PEDF could have salutary effects on early diabetic retinopathy by blocking the deleterious actions of AGE via inhibition of NADPH oxidase-induced oxidative stress generation.

In this study, we found that PEDF administration reduced retinal VEGF expression in early diabetic retinopathy. Since VEGF plays a pivotal role in vascular alterations such as inflammation, thrombosis, hyperpermeability and angiogenesis in diabetic retinopathy [7], PEDF may be a novel therapeutic target for preventing the progression of various stages of diabetic retinopathy. Further, we have previously shown that AGE is one of the initial stimulants of VEGF expression in early diabetic retinopathy [23]. The observations further support the concept that PEDF could inhibit the development of diabetic retinopathy by blocking the harmful actions of AGE on diabetic retinas.

Limitations

In this study, PEDF administration restored the decrease in retinal PEDF levels in diabetic rats. Further, in early diabetic retinopathy, BRB is reported to be disturbed [8]. These findings suggest that intravenously injected PEDF, a large polypeptide, could have accessed to the retina in the therapeutic dose, probably due to the breakdown of BRB in diabetes. However, since oxidative stress decreases PEDF levels in both cell culture and animal models [6,15,24], we cannot totally exclude the possibility that PEDF injection induces its own expression in the retina via anti-oxidative properties. So, it would be interesting to examine whether labelled PEDF protein, when injected intravenously, can indeed be found in diabetic retina.

Serum PEDF levels were significantly higher in type 1 diabetic patients with retinopathy compared with the patients without it (10.4 μg/mL *versus* 7.7 μg/mL) [25]. These findings suggest that the beneficial effects of PEDF on diabetic retinopathy are already present in these patients and no further therapeutic effects could be obtained. However, our recent study suggests that most of circulating PEDF in the serum may exist as a protein-bound form [26]. Therefore, serum PEDF levels may not necessarily reflect its biological activity *in vivo*, and the increase of serum PEDF in diabetic patients with retinopathy may not be enough to protect the disease progression. This is one possible reason why small dose of free PEDF injected in this study (5 μg PEDF/100 g body weight) exerted beneficial effects on experimental diabetic retinopathy. Owing to the lack of specific enzyme-linked immunosorbent assay system for rat PEDF, we cannot accurately measure serum concentration of PEDF in diabetic or non-diabetic rats. However, we have found that single PEDF injection (10 μg PEDF/100 g body weight) increases serum concentration of PEDF to about 2-fold of the basal level in Sprague–Dawley rats [27] and that repeated PEDF injections (2.5 μg PEDF/100 g body weight, three times a week) increase its serum level to about 2-folds in obese insulin resistant rats (unpublished data). Development of specific enzyme-linked immunosorbent assay system for rat-free PEDF would be helpful to elucidate the role of endogenous PEDF in experimental diabetic retinopathy.

Although insulin itself can reduce complications that result in the progression of diabetic retinopathy [28], it is unlikely that insulin itself could affect the beneficial effects of PEDF in our models because for both PEDF-injected and non-injected diabetic rats insulin was given (subcutaneous injections of 2 U insulin, two times a week).

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Conflict of interest

None declared.

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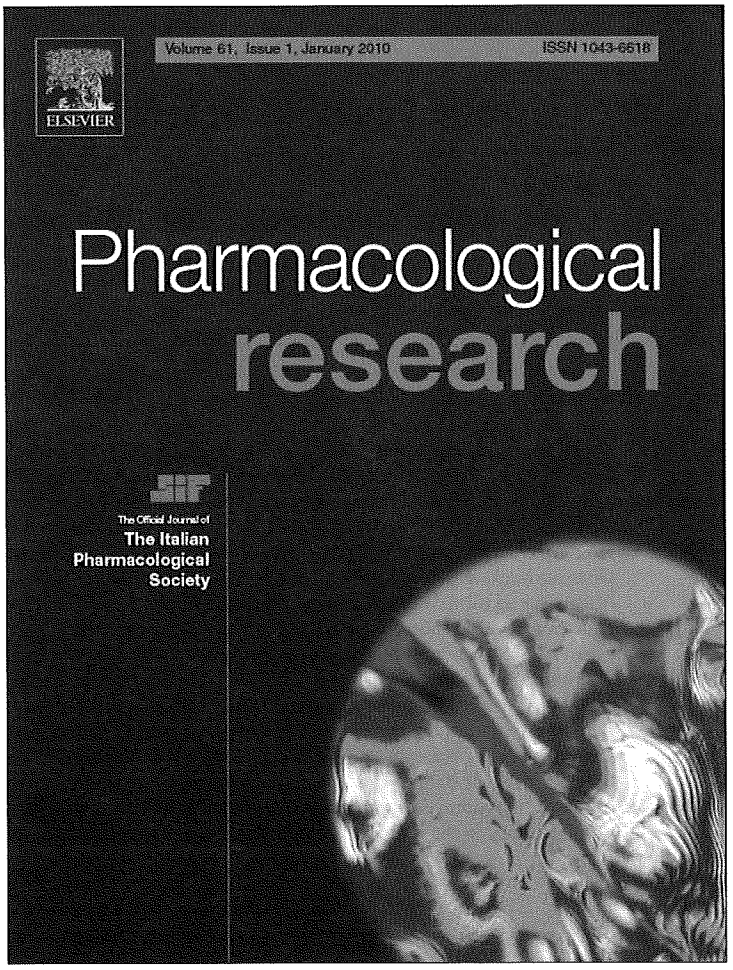

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Irbesartan inhibits advanced glycation end product (AGE)-induced proximal tubular cell injury *in vitro* by suppressing receptor for AGEs (RAGE) expression

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ABSTRACT

Renin-angiotensin system (RAS) plays a central role in the development and progression of diabetic nephropathy. Further, there is a growing body of evidence that advanced glycation end products (AGEs) and their receptor (RAGE) axis also contributes to diabetic nephropathy. However, the pathophysiological crosstalk between the RAS and AGE-RAGE system in tubular cell injury, which is more important than glomerulopathy in terms of renal prognosis in diabetic nephropathy, remains unknown. In this study, we examined whether and how irbesartan, an angiotensin II type 1 receptor blocker (ARB), inhibited the AGE-induced tubular cell apotptosis and damage *in vitro*. Gene expression was analyzed by quantitative real-time reverse transcription-polymerase chain reactions. Intracellular formation of reactive oxygen species (ROS) was measured with dihydroethidium staining. Apoptosis levels were evaluated for DNA fragments with an enzyme-linked immunosorbent assay kit and for caspase-3 activity. Irbesartan inhibited the AGE-induced up-regulation of RAGE mRNA levels and subsequently reduced ROS generation in human proximal tubular cells. AGEs induced apoptosis and increased inflammatory, thrombogenic and fibrogenic gene expressions in tubular cells, which were also blocked by the treatment with irbesartan. Our present data suggest that there exists a crosstalk between the RAS and AGE-RAGE system in 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.

1. Introduction

Diabetic nephropathy is a leading cause of end-stage renal disease, and accounts for disabilities and the high mortality rates in patients with diabetes [1]. Diabetic nephropathy is characterized by functional and structural changes in the glomerulus, such as glomerular hyperfiltration, thickening of glomerular basement membranes and an expansion of extracellular matrix in mesangial areas [1]. However, it has recently been recognized that changes within tubulointerstitium are more important than glomerulopathy in terms of renal prognosis in diabetic nephropathy [2,3].

Numerous studies have demonstrated the active participation of the renin-angiotensin system (RAS) in the pathogenesis of diabetic nephropathy [1,4,5]. The renoprotective effects of the inhibitors of the RAS are largely ascribed to its blood pressure (BP)-lowering properties [1,4,5]. However, a recent clinical study suggests the pleiotropic effects of the RAS inhibitors, *that is*, beyond BP-lowering effects, on diabetic nephropathy [6]. Indeed, it has been shown that irbesartan, an angiotensin II type 1 receptor blocker (ARB), significantly prevents the progression of nephropathy in patients with type 2 diabetes, compared with calcium channel blocker, amlodipine with an equipotent BP-lowering property [6]. These observations suggest that the inhibition of the RAS itself is a therapeutic target for diabetic nephropathy.

Non-enzymatic modification of proteins by reducing sugars, a process that is also known as Maillard reaction, progress at an extremely accelerated rate under diabetes, leading to the formation of advanced glycation end products (AGEs) *in vivo* [7,8]. Recent understandings of this process have revealed that the AGE-their receptor (RAGE) axis also plays a role in the pathogenesis of diabetic nephropathy [7–10]. Indeed, engagement of RAGE by AGEs activates its down-stream signaling and subsequently evokes oxidative

Abbreviations: RAS, renin-angiotensin system; BP, blood pressure; ARB, angiotensin II type 1 receptor blocker; AGEs, advanced glycation end products; RAGE, receptor for AGEs; BSA, bovine serum albumin; RT-PCR, real-time reverse transcription-polymerase chain reactions; MCP-1, monocyte chemoattractant protein-1; PAI-1, plasminogen activator inhibitor-1; TGF-, βtransforming growth factor-β; DHE, dihydroethidium; ELISA, enzyme-linked immunosorbent assay; S.E.M., standard error; ROS, reactive oxygen species.

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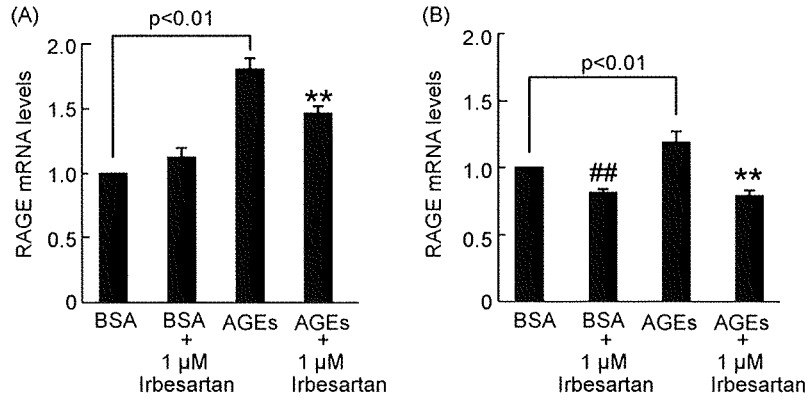

Fig. 1. Effects of irbesartan on RAGE gene expression in tubular cells. Cells were treated with 100 μg/ml AGE-BSA or non-glycated BSA in the presence or absence of 1 μM irbesartan for 4 h (A) or 24 h (B). Then total RNAs were transcribed and amplified by real-time PCR. Data were normalized by the intensity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

stress and inflammatory and fibrogenic responses in renal cells, thus contributing to the development and progression of diabetic nephropathy [7–10]. Blockade of AGE formation or accumulation by RAS inhibitors was reported both *in vitro* and *in vivo* [11,12]. However, the pathophysiological crosstalk between the RAS and the AGE-RAGE axis in tubular cell injury, one of the characteristic features in diabetic nephropathy [2,3], remains unknown. In this study, we examined whether and how irbesartan inhibited

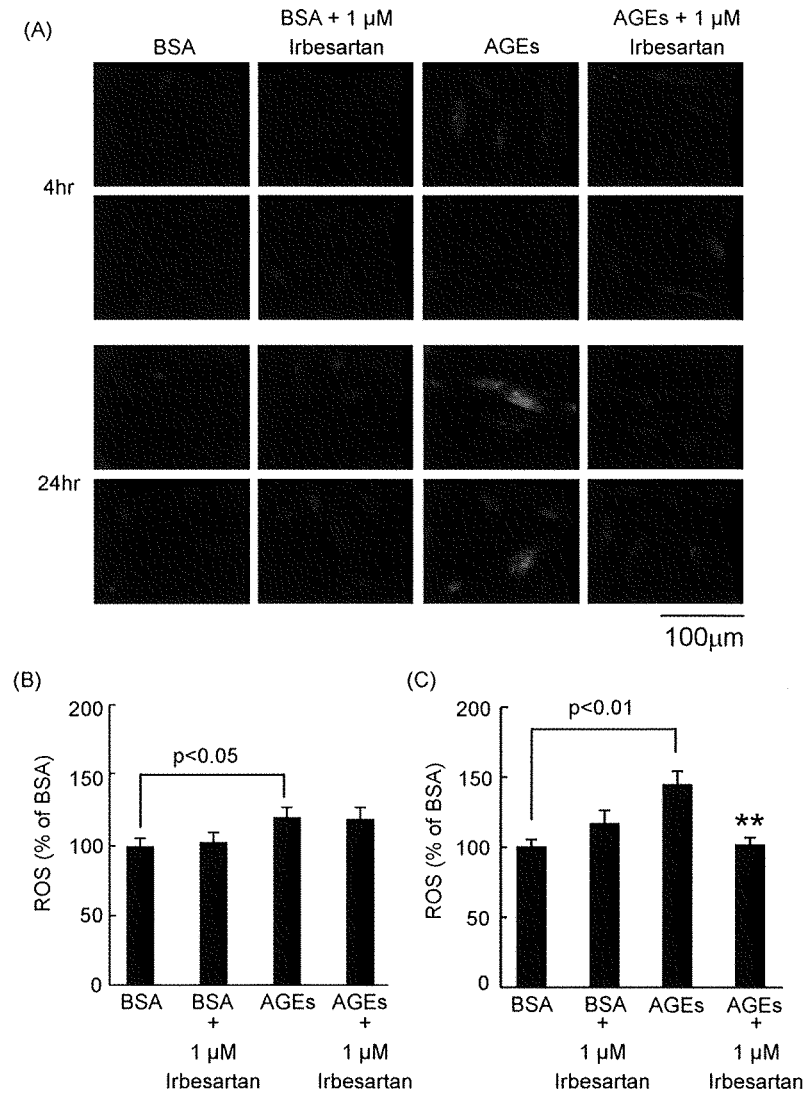

Fig. 2. Effects of irbesartan on ROS generation in tubular cells. Cells were treated with 100 μg/ml AGE-BSA or non-glycated BSA in the presence or absence of 1 μM irbesartan for 4 h (A) or 24 h (B and C). Then the cells were incubated with DHE. Upper panel shows typical microphotographs of the cells. Lower panel shows quantitative data of ROS generation evaluated by fluorescent intensity. $^{**}p < 0.01$ compared to the value with AGEs alone. $N = 5$ per group.

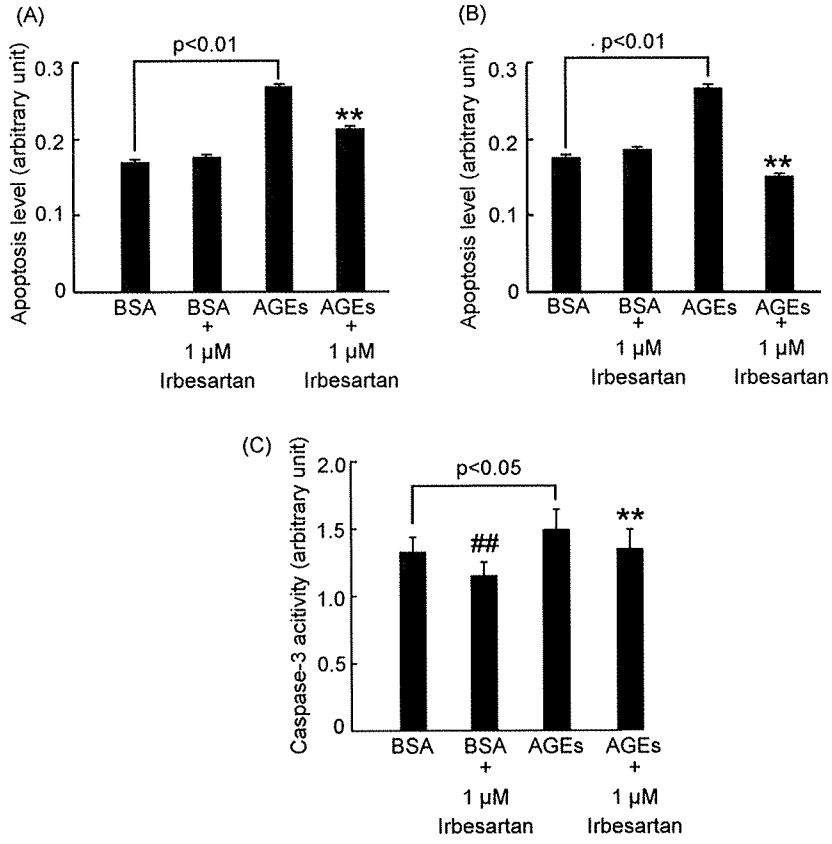

Fig. 3. Effects of irbesartan on apoptotic cell death in tubular cells. Cells were treated with 100 μg/ml AGE-BSA or non-glycated BSA in the presence or absence of 1 μM irbesartan for 12 h (A) or 24 h (B and C). Then the cells were lysed and the supernatant was analyzed for DNA fragments in an ELISA (A and B) and for caspase-3 activity (C). Apoptosis level is shown as absorbance at 405 nm (arbitrary units). Caspase-3 activity is shown as absorbance at 505 nm (arbitrary units). $^{\#\#}p < 0.01$ compared to the value with non-glycated BSA alone. $^{**}p < 0.01$ compared to the value with AGEs alone. $N = 4$ per group.

the AGE-induced tubular cell apoptotic cell death and damage *in vitro*.

2. Materials and methods

2.1. Materials

Irbesartan was generously gifted from Dainippon Sumitomo Pharma (Tokyo, Japan). Bovine serum albumin (BSA) (essentially fatty acid free and essentially globulin free, lyophilized powder) was purchased from Sigma (St. Louis, MO, USA). D-Glyceraldehyde from Nakalai Tesque (Kyoto, Japan).

2.2. Preparation of AGE-BSA

AGE-BSA was prepared as described previously [13]. Briefly, BSA (25 mg/ml) was incubated under sterile conditions with 0.1 M glyceraldehyde in 0.2 M $NaPO_4$ buffer (pH 7.4) for 7 days. Then unincorporated sugars were removed by PD-10 column chromatography and dialysis against phosphate-buffered saline. Control non-glycated BSA was incubated in the same conditions except for the absence of reducing sugars. Preparations were tested for endotoxin using Endospecy ES-20S system (Seikagaku Co., Tokyo, Japan); no endotoxin was detectable. The extent of chemical modification was determined as described with 2,4,6-trinitrobenzenesulfonic acid as a difference in lysine residues of modified and unmodified protein preparations [14]. The extent of lysine modification (%) of modified BSA preparations was 65% for AGE-BSA.

2.3. Cells

Proximal tubular epithelial cells from human kidney were maintained in basal medium supplemented with 5% fetal bovine serum, 0.5 μg/ml hydrocortisone, 10 ng/ml human epidermal growth factor, 0.5 μg/ml epinephrine, 6.5 ng/ml triiodo-L-thyronine, 10 μg/ml transferrin, 5 μg/ml insulin, and GA-1000 according to the supplier's instructions (Clonetics Corp., San Diego, CA, USA) [15]. Cells at 3–5 passages were used for the experiments. AGE treatments were carried out in a serum-free basal medium containing 10 μg/ml transferrin and GA-1000.

2.4. Real-time reverse transcription-polymerase chain reactions (RT-PCR)

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. Then total RNA was extracted with RNAqueous-4PCR kit (Ambion Inc., Austin, TX, USA) according to the manufacturer's instructions. Quantitative real-time RT-PCR was performed using Assay-on-Demand and TaqMan 5 fluorogenic nuclease chemistry (Applied Biosystems, Foster city, CA, USA) according to the suplier's recommendation. IDs of primers for human RAGE, monocyte chemoattractant protein-1 (MCP-1), plasminogen activator inhibitor-1 (PAI-1), transforming growth factor-β (TGF-β) and β-actin gene were Hs00153957_m1, Hs00234140_m1, Hs00171257_m1, Hs00171257_m1 and Hs99999903_m1, respectively.