mRNA expression of platelet activating factor receptor (PAFR) in peripheral blood mononuclear cells is associated with albuminuria and vascular dysfunction in patients with type 2 diabetes

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Abstract

Aims

Renal dysfunction in addition to diabetes is a serious risk factor for cardiovascular events. We hypothesized that some of the changes in gene expression in blood cells cause renal dysfunction and macrovascular disease through impaired endothelial function. This study aimed to define which changes in gene expression in peripheral blood mononuclear cells (PBMCs) are related to renal function parameters and endothelial function of large arteries in patients with type 2 diabetes mellitus (T2DM).

Methods

We recruited 95 patients with T2DM. After matching for gender, age, BMI and HbA1c levels, the patient cohort included 42 with normoalbuminuria, 28 with microalbuminuria, and 25 with macroalbuminuria. All patients in the three groups were assessed for urinary albumin to creatinine ratio (ACR), estimated glomerular filtration rate (eGFR), flow-mediated dilatation (FMD), and mRNA expression in PBMCs.

Results

The mRNA expression of platelet activating factor receptor (PAFR) differed most markedly between the three groups and was significantly higher in the macroalbuminuric group ($p < 0.001$ vs. normoalbuminuric group; $p < 0.05$ vs. microalbuminuric group). PAFR mRNA expression significantly correlated with log transformed ACR ($\rho = 0.424$, $p < 0.001$) but not eGFR. PAFR mRNA expression also had a significant negative correlation with FMD ($\rho = -0.379$, $p < 0.001$). Furthermore, the prevalence of macrovascular complications, particularly stroke, was significantly higher in patients with elevated PAFR mRNA expression in PBMCs.

Conclusions

PAFR overexpression in PBMCs may link diabetic nephropathy to macroangiopathy through impairment of endothelial function in patients with T2DM.

Keywords: Platelet activating factor receptor; Peripheral blood mononuclear cells; Flow-mediated dilatation; Impaired endothelial function; Macrovascular complication

1. Introduction

The incidence of cardiovascular disease is more than three times higher in patients with diabetes than in those without [1-3]. The East–West study has shown that the incidence of myocardial infarction (MI) in patients with diabetes and without a history of MI was similar to that of non-diabetic patients with a history of MI [4]. In addition, follow-up studies such as the subgroup analysis in the Action in Diabetes and Vascular disease: preterax and diamicron-MR Controlled Evaluation (ADVANCE) study revealed that albuminuria and a decrease in estimated glomerular filtration rate (eGFR) are further independent risk factors for cardiovascular events among patients with type 2 diabetes mellitus (T2DM) [5]. The increased risk factor for cardiovascular disease in patients with diabetes associated with renal disorder may be partially attributed to endothelial dysfunction of large vessels. Many studies have shown that flow-mediated dilatation (FMD) in patients with diabetes is lower than that in healthy controls; furthermore, FMD has been shown to be even lower in patients with diabetes associated with microalbuminuria, macroalbuminuria, or low eGFR [6-9].

Changes in gene expression in peripheral leukocytes such as lymphocytes and mononuclear cells have been observed in patients with diabetes, diabetic nephropathy, chronic kidney disease, metabolic syndrome, and advanced atherosclerosis [10-14].

Peripheral leukocytes from these patients exhibit altered mRNA expression of genes associated with inflammation, oxidative stress, and lipids metabolism as compared to controls [10-14], suggesting that these changes in gene expression might reflect pathophysiological changes in the associated diseases. Since peripheral leukocytes are in constant contact with the endothelial cells of arteries through systemic blood flow, these changes in gene expression may be influenced by alterations in endothelial function and, in turn, may affect endothelial function. To our knowledge, no studies have focused on the association between the changes in peripheral leukocyte gene expression and the endothelial function of arteries in patients with diabetes and renal dysfunction.

We hypothesized that the changes in peripheral leukocyte gene expression induced during the pathogenesis of diabetic nephropathy cause macrovascular complications as well as renal dysfunction by impairing endothelial function in patients with T2DM. This study aimed to define related gene expression in peripheral leukocytes to eGFR, urinary albumin to creatinine ratio (ACR), and FMD to clarify the molecular link between diabetic nephropathy and macroangiopathy in patients with T2DM. We selected peripheral blood mononuclear cells (PBMCs) for the gene investigation because these cells reflect the altered immunity and metabolism in systemic pathology [15-17] and play an actual role in the pathogenesis of diabetic nephropathy and atherosclerosis

[18-21]. We analyzed the expression of genes associated with inflammation, reactive oxygen species (ROS) generation, and adherence factors in PBMCs using real-time quantitative reverse transcription polymerase chain reaction (RT-PCR) and studied endothelial function using FMD in patients with T2DM in various stages of nephropathy.

2. Methods

2.1. Subjects and study design

Between July 2014 and October 2015, a total of 165 Japanese patients with T2DM undergoing regular urinary albumin and the ocular fundus check-ups were selected from the outpatient clinic of Kagoshima University hospital. Patients with the following conditions were excluded: allergic diseases (e.g. asthma and allergic rhinitis), connective tissue disease, acute infections, chronic infection with hepatitis B or C virus, other glomerular nephritis, hematuria, malignant disease in the previous three years, ongoing steroid or immunosuppressive therapy, undergoing artificial dialysis, kidney transplantation, or nephrectomy. Participants were classified into three groups based on urinary albumin level: normoalbuminuria, ACR < 30 mg/g Cr (n = 84); microalbuminuria, 30 ≤ ACR < 300 mg/g Cr (n = 56); macroalbuminuria, ACR ≥ 300 mg/g Cr (n = 25). We recruited all patients with macroalbuminuria and after matching for gender, average age,

body mass index (BMI), and HbA1c levels, only half of those with either normoalbuminuria or microalbuminuria were selected. Finally, 95 patients were recruited into this cross-sectional study as follows: normoalbuminuric group, 42; microalbuminuric group, 28; macroalbuminuric group, 25. All participants had blood samples drawn and underwent physiological tests in a fasting state on a single day. Urine samples were collected in the early morning for two consecutive days. For this study, macrovascular complication was defined as coronary heart disease, stroke, or peripheral artery disease. We defined the occurrence of these diseases as patients having a history of hospitalization for invasive or non-invasive treatment of said conditions. This definition did not include patients with asymptomatic disease or those treated as outpatients. The study was approved by Kagoshima University hospital ethics committee (#26-32, #27-178, #28-255), and all participants provided written informed consent before participation.

2.2. Blood and urine data analysis

Blood samples were collected into NaF-coated tubes for analysis of glucose and HbA1c, EDTA-coated tubes for analysis of monocyte chemotactic protein-1 (MCP-1) and PBMCs gene expression, and serum-separating tubes for analysis of LDL cholesterol (LDL-C), HDL cholesterol (HDL-C), triglycerides (TG), oxidized LDL (oxLDL),

creatinine (Cr), and high sensitivity C-reactive protein (hs-CRP). Glucose, HbA1c, LDL-C, HDL-C, TG, and Cr were immediately measured at the Kagoshima University Hospital clinical laboratory center. eGFR was calculated based on the serum Cr, age, and sex: eGFR = $194 \times (\mathrm{Cr})^{-1.094} \times (\mathrm{age})^{-0.287} \times$ (0.739 if female). Serum hs-CRP and ACR were determined by SRL Inc. (Kagoshima, Japan). The plasma and urine MCP-1 levels were determined using a human MCP-1 ELISA kit (R&D system Inc., Minneapolis, MN, USA). The urine MCP-1 level was adjusted for urine creatinine. Serum oxLDL was determined using a human ox-LDL/MDA Adduct ELISA Kit (Immundiagnostik).

2.3. PBMCs Preparation and RNA extraction

Blood samples (10 mL) collected in EDTA-coated tubes were diluted twice with an equal volume of PBS, layered over 10 mL Lymphoprep (Axis-Shield, Oslo, Norway), and centrifuged at 800 × g for 20 minutes. After centrifugation, the sample/medium interface containing mononuclear cells was obtained.

The mononuclear cell pellet was homogenized with 0.75 mL of TRIzol reagent (Life Technologies) and incubated for 5 min at room temperature (RT). Chloroform (0.2 mL) then was added to the homogenates followed by centrifugation at 12,000 × g in a microcentrifuge (Eppendorf5417R) for 15 minutes at 4°C. The colorless upper aqueous

phase containing RNA was carefully transferred to a new microcentrifuge tube. The RNA was precipitated by addition of 0.5 mL 100% isopropanol and then centrifuged at 12,000 × g for 10 minutes at 4°C. The RNA pellet was washed with 1 mL of 75% ethanol and centrifuged at 7,500 × g for 5 minutes at 4°C. The pellet was air-dried and then dissolved in 100 µL of RNAase-free water. RNA purity and quantity was measured spectrophotometrically using NanoDrop1000 (Thermo Fisher Scientific, USA). All samples had an A 260/A 280 ratio of 1.70–2.00, indicating high RNA purity.

2.4. Real-time quantitative RT-PCR

Single-stranded cDNA was synthesized from RNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Total RNA (1 µg) was combined with 20 µL reaction reagent and incubated at 37°C for 2 hours, followed by denaturation at 85°C for 5 min using Power BLOCK (ATTO, Japan). Quantitative RT-PCR was performed using a StepOnePlus thermocycler (Applied Biosystems) according to the manufacturer's protocol (Applied Biosystems). The reaction included 100 ng of cDNA and 20 µL PCR reaction mix containing TaqMan qPCR master mix (Applied Biosystems) and TaqMan gene expression assays (Applied Biosystems; Supplementary table1). The reactions were performed in triplicate in 96-well plates under the following conditions:

holding stage of 20 sec at 95°C, then 40 cycles consisting of 1 sec at 95°C and 20 sec at 60°C. On the basis of previous researches, we selected the particular set of the following genes related to pathology of diabetes [10, 13, 15], atherosclerosis [14, 22-26], or renal dysfunction [11, 27-29] for analysis; tumor necrosis factor α (TNF-α), interleukin 1β (IL-1β), interleukin 6 (IL-6), C-C motif chemokine ligand 2 (CCL2, MCP-1), Cluster differentiation 36 (CD36), TNF receptor (TNFR), toll-like receptor 4 (TLR4), C-C motif chemokine receptor 2 (CCR2), CD11b/CD18 (Mac-1), CD11a/CD18 (LFA-1), NADH oxidase 2 (NOX2), 67 KDa neutrophil oxidase factor 2 (p67phox), platelet activating factor receptor (PAFR), cyclooxygenase 1 (COX1), cyclooxygenase 2 (COX2), thrombospondin 1 (THBS1), nuclear factor-kappa B (NFκB). Those mRNA expressions were normalized by Hypoxanthine phosphoribosyltransferase 1 (HPRT1).

2.5. Western Blot analysis

The protein expression of PAFR was assessed in 10 patients, those with the 5 highest and 5 lowest mRNA expressions of PAFR, by Western Blot analysis. Isolated PBMCs from venous blood (10 mL) drawn again from those patients were divided into two aliquots: one was used for mRNA extraction, and the other was lysed in 0.5 mL of RIPA buffer (ATTO, Tokyo, Japan) to extract protein. The lysates were centrifuged at 10,000 × g at

4°C for 15 minutes, and the supernatants collected. Protein concentrations were determined using the Bradford protein assay kit (Bio-Rad, USA) with BSA as the standard. Lysate protein (20 μg) was subjected to 4–12% SDS-PAGE (Life technologies, USA) and electrotransferred onto PVDF membrane using the iBlot 2 Dry Blotting System (Life technologies, USA). The blot was incubated with blocking solution (Nacalai tesque, Japan) for 1 hour at RT. The membrane was subsequently incubated overnight at 4°C with rabbit polyclonal anti-PAFR antibody (Cayman) diluted 1:200. The membrane was washed twice and incubated for 1 hour at RT with polyclonal HRP-conjugated goat anti-rabbit IgG (Life technologies). The same membrane was stripped and immunoblotted again with rabbit polyclonal anti-HPRT1 antibody (Abcam) diluted 1:1500, followed by polyclonal HRP-conjugated goat anti-rabbit IgG (Life technologies). The bound antibodies were visualized using an enhanced chemiluminescence system (Nacarai tesque, Japan). Imaging was performed using FlourChem FC2 (Alpha Innotec), and the protein expression levels were quantified using Image J 1.48 (http://imagej.nih.gov/ij). The ratio of PAFR protein expression to that of HPRT1 was calculated and statistically analyzed.

2.6. Physiological tests

Anthropometric measures and physiological tests were performed at the beginning of the study. Blood pressure, brachial-ankle pulse wave velocity (baPWV), and FMD of the brachial artery were measured after resting for 15 min in a lying down position. The baPWV was evaluated using a volume-plethysmographic apparatus (Form PWV/ABI, Omron Colin Co. Ltd., Japan). FMD was evaluated by means of A- and B-mode using UNEX EF38G (UNEX Corp, Japan) according to the following procedure. The baseline diameter of the right brachial artery was measured at 5 cm proximal to the elbow joint. A sphygmomanometer cuff placed around the forearm was inflated to the systolic blood pressure + 50 mmHg for 5 minutes. After the cuff was deflated, the maximum diameter of the brachial artery after hyperemia was measured for 120 seconds. The rate of change (%) in diameter compared with the baseline was defined as FMD.

2.7. Statistical analysis

Data are expressed as the mean ± SD, median (interquartile range), or number (%). Comparison of means of variables with normal distribution between the three groups was performed using one-way ANOVA followed by Tukey's post hoc test. Comparison of medians of variables with skewed distribution between the groups was performed using the Kruskal–Wallis test followed by the Steel–Dwass test. The proportion variables were

analyzed using the Chi-square free test. The correlation between two variables was determined using the Spearman rank order correlation test. Stepwise multiple linear regression analysis (*p* value for entry was <0.05 and for exit was >0.10) was performed to evaluate the contribution of each covariate for FMD and mRNA expression of PAFR. Age, sex and other variables associated with the dependent variables with a *p* value <0.20 in univariate analysis were included in the stepwise model. All variables with skewed distribution were logarithmically transformed. Stepwise multiple logistic regression model was used to analyze the association of the prevalence of macrovascular complications with gene expression (mRNA of PAFR), renal function (eGFR, ACR), endothelial function (FMD) and duration of diabetes. Statistical analysis was performed using SPSS version 22.0 (SPSS Inc, USA) and R-software version 3.1.2 (https://www.r-project.org/). $p < 0.05$ was considered statistically significant.

3. Results

3.1. Baseline characteristics

Table 1 shows the baseline characteristics of 95 participants. No significant differences were observed between groups with respect to age, sex, duration of diabetes, BMI, fasting plasma glucose (FPG), HbA1c, TG, oxLDL, or hs-CRP. Systolic blood pressure (SBP)

was significantly higher in the macroalbuminuric group than the normoalbuminuric group ($p < 0.01$). The HDL-C was significantly lower in the microalbuminuric group than that of the other two groups ($p < 0.05$ vs. normoalbuminuric group; $p < 0.05$ vs. macroalbuminuric group). The urinary MCP-1 level in the macroalbuminuric group was significantly higher than that of the other two groups ($p < 0.001$ vs. normoalbuminuric group; $p < 0.05$ vs. microalbuminuric group). The plasma MCP-1 level was also significantly higher in the macroalbuminuric group than that of the other two groups ($p < 0.001$ vs. normoalbuminuric group; $p < 0.05$ vs. microalbuminuric group). The FMD was significantly lower in the macroalbuminuric group than in the normoalbuminuric group ($p < 0.001$), while baPWV was highest in the macroalbuminuric group ($p = 0.047$). According to the progression of proteinuria, more patients were treated with calcium channel blockers (CCB) and Angiotensin II Receptor Blocker (ARB)/ACE inhibitor (ACEI). As for the usage of insulin or oral hypoglycemic agents, there were no significant differences between the groups. Antiplatelet therapy with at least one antiplatelet agent (aspirin, clopidogrel or cilostazol) was significantly different among the groups and it was highest in the macroalbuminuric group ($p = 0.012$). No subject regularly took nonsteroidal anti-inflammatory drugs. The prevalence of having at least one macrovascular complication was significantly different among the three groups and it was

highest in the macroalbuminuric group (macroalbuminuric group, 12; microalbuminuric group, 6; normoalbuminuric group, 4; $p = 0.015$). The prevalence of stroke was significantly higher in the macroalbuminuric group ($p = 0.003$), although those of coronary heart disease and peripheral artery disease did not differ significantly among the groups. The occurrence of retinopathy or neuropathy was highest albeit not significantly in macroalbuminuric group.

3.2. Screening for PAFR gene expression in PBMCs isolated from patients with diabetes

mRNA expression in PBMCs was compared between three groups classified by urinary albumin level (supplementary Table 2). The mRNA expression of PAFR, p67phox, TNFR, and CD36 differed significantly between the three groups. The mRNA expression of PAFR revealed the most variation between the groups, with significantly higher expression in the macroalbuminuric group than the other two groups ($p < 0.001$ vs. normoalbuminuric group; $p < 0.05$ vs. microalbuminuric group; Fig. 1a). Furthermore, Spearman correlation analysis revealed that the mRNA expression of PAFR significantly correlated with the log transformed ACR ($\rho = 0.424$, $p < 0.001$; Fig. 1b) but not eGFR ($\rho = -0.140$, $p = 0.177$; Fig. 1c).

3.3. Association between PAFR mRNA expression and FMD in PBMCs

Only mRNA expression of PAFR had a significant negative correlation with FMD ($\rho = -0.379$, $p < 0.001$; Fig. 1d). Stepwise multiple linear regression analysis showed that log-transformed mRNA expression of PAFR and eGFR were independent covariates associated with log-transformed FMD (eGFR: $\beta = 0.282$, $p = 0.004$; $\log_{10}$ PAFR: $\beta = -0.271$, $p = 0.006$; R^2 for the model: 0.18, $p < 0.001$; Supplementary Table 3).

3.4. PAFR protein expression in PBMCs

Fig. 2a shows the protein expression of PAFR in PBMCs from 10 patients who had the lowest and highest PAFR mRNA expression in the cohort. Relative PAFR protein expression in PBMCs was significantly higher in the 5 patients with the highest PAFR mRNA expression than in those with the lowest PAFR mRNA expression (0.597 ± 0.050 vs. 0.428 ± 0.027; $p = 0.019$; Fig. 2b). Thus, the results of PAFR protein and mRNA expression were consistent ($r = 0.711$, $p = 0.021$).

3.5. Comparison of clinical characteristics between patients with different PAFR mRNA expression

Table 2 shows the clinical characteristics of the patients classified into three groups based on the mRNA expression level of PAFR in PBMCs. The ACR, FMD, and oxLDL differed significantly between the three groups (ACR, $p = 0.002$; FMD, $p < 0.001$; oxLDL, $p = 0.034$). Stepwise multiple linear regression analysis showed that only log-transformed ACR and log-transformed FMD were independent covariates associated with log-transformed mRNA expression of PAFR ($\log_{10}$ ACR: $\beta = 0.368$, $p < 0.001$; $\log_{10}$ FMD: $\beta = -0.226$, $p = 0.022$; R^2 for the model: 0.23, $p < 0.001$; Supplementary Table 3).

3.6. Association of PAFR mRNA expression in PBMCs with complications

Table 2 also shows the prevalence of vascular complications with respect to the level of PAFR mRNA expression in PBMCs. While the 3 groups defined by PAFR mRNA expression level did not differ in the occurrence of all stages of retinopathy, they differed significantly in the occurrence of neuropathy with impaired autonomic function ($p = 0.011$). The prevalence of stroke differed significantly among the three groups ($p < 0.001$), although those of coronary heart disease and peripheral artery disease did not. The prevalence of having at least one macrovascular complication was significantly different among the three groups and it was highest in the PAFR mRNA high-expression group (high-expression group, 13; middle-expression group, 5; low-expression group, 4, $p =$

0.015). Moreover, the prevalence of having two or more macrovascular complications was highest albeit not significantly in the PAFR mRNA high-expression group (high-expression group, 5; middle-expression group, 2; low-expression group, 1). Stepwise multiple logistic regression analysis showed that the duration of diabetes and log-transformed ACR were independently associated with the prevalence of macrovascular complications (duration: OR = 1.142, p = 0.001; $\log_{10}$ ACR: OR = 1.724, p = 0.001), while the duration of diabetes and mRNA expression of PAFR were independent explanatory variables for the prevalence of stroke (duration: OR = 1.114, p = 0.014; PAFR mRNA: OR = 2.594, p = 0.003) (Table 3).

4. Discussion

In the present study, we observed a significant association between the level of PAFR mRNA expression in PBMCs and the degree of albuminuria and endothelial function in patients with diabetes.

PAF is a proinflammatory phospholipid produced by a variety of cells in response to inflammation or injury [30-34]. The activation of phospholipase A2 by inflammatory stimuli leads to the release of free arachidonic acid from phosphatidylcholine in the plasma membrane, producing lysophosphatidylcholine

(lysoPC), a precursor of PAF. LysoPC is acetylated by acetyltransferase, producing bioactive PAF [35, 36]. The receptor for PAF (PAFR) is a 7-transmenbrane G-protein–coupled protein widely expressed in blood cells such as platelets, neutrophils, monocytes/macrophages, and eosinophils, as well as a variety of organs [37]. The relationship between PAFR signaling and endothelial dysfunction in renal pathophysiology has been reviewed in several articles [38-40]. PAFR activation is assumed to affect glomerular permeability by altering the glomerular size selectivity and anionic charge barrier [41, 42]. Administration of a PAFR antagonist was reported to prevent proteinuria and glomerular injury in several renal disease models [43, 44]. Importantly, we observed that elevated PAFR mRNA expression in PBMCs was associated with an increase in albuminuria but not with a decrease in eGFR in patients with T2DM, indicating that the increase in PAFR mRNA expression was not caused by a deterioration of kidney function. Furthermore, kidney biopsies of patients with diabetic nephropathy showed stronger PAFR staining than those of healthy controls [27], suggesting that PAFR signaling plays a pathophysiological role in diabetic nephropathy.

This report is the first to demonstrate an increase in PAFR mRNA expression in PBMCs from T2DM patients with albuminuria. Interestingly, we found that the patients with higher PAFR expression in PBMCs demonstrated not only an increase in urinary

albumin excretion but also impaired endothelial function, as assessed by FMD. Multivariate regression analysis revealed that the increase in PAFR mRNA expression and decrease in eGFR are independent predictive factors for the decrease in FMD, suggesting that an increase in PAFR signaling in PBMCs affects vascular endothelial function. Brocheriou et al. have demonstrated that monocytes adjacent to endothelial cells strongly expressed PAFR in human carotid atherosclerotic plaque [22]. PAFR signaling promotes the generation of superoxide anions and hydrogen peroxide in human monocyte-derived macrophages [45] and increases CD36 expression and oxLDL uptake in human monocytes [46]. In the present study, CD36 gene expression was found to be associated with PAFR gene expression (data not shown) and ACR in univariate analysis. The binding ligand to CD36 was reported to progress diabetic nephropathy via activating PKC-MAPK signaling cascade [28]. CD36 also plays significant roles in atherogenic processes, including foam cell formation, release of inflammatory mediators, macrophage trapping in atherosclerotic plaque and thrombosis [23]. Rios *et al* showed co-localization of PAFR and CD36 in lipid rafts is required for oxLDL uptake and IL-10 production [47]. Thus, the activation of PAFR in cooperation with such other molecules may impair endothelial function, leading to the development of renal dysfunction and atherosclerosis.

In fact, we found that the prevalence of macrovascular complications was significantly higher in the group of patients with elevated PAFR mRNA expression in PBMCs.

Our results also showed that PAFR protein expression in PBMCs is elevated in parallel with its mRNA expression, indicating that the increase in PAFR mRNA expression in PBMCs is not only a molecular marker but may play an actual role in the pathogenesis of diabetes complications. Previous studies have shown that the PAFR gene is regulated through two promoters that include the binding site for NF-kB [48], which is known to be up-regulated in diabetes [49]. In addition, Balestrieri et al. reported that poor glycemic control causes PAFR activation through negative regulation of SIRT1 in endothelial progenitor cells of PBMCs in patients with T2DM [50]. However, in the present study, we observed no relationship between PAFR mRNA expression in PBMCs and markers of glycemic control such as FPG and HbA1c. Further investigation is needed to clarify the mechanism of PAFR regulation in the PBMCs of patients with diabetes.

There are several limitations to this study. First, we could not elucidate the cause of the relationship between elevated PAFR mRNA expression in PBMCs and albuminuria and endothelial dysfunction, as this investigation was cross-sectional. Follow-up study will be needed to confirm the role of elevated PAFR expression in PBMCs in the pathogenesis of diabetes complications. Second, we cannot confirm the role of the

diabetic state in mRNA expression in PBMCs because non-diabetic control subjects were not recruited in this study. Third, we could not confirm whether the PAFR signal in PBMCs actually affects the kidney or vascular function.

In conclusion, elevated PAFR mRNA expression in PBMCs is associated with albuminuria in patients with T2DM, indicating a connection between PAFR signaling and the development of albuminuria in these patients. The increase in PAFR mRNA expression in PBMCs also independently correlated with a decrease in FMD of the brachial artery. Thus, PAFR overexpression in PBMCs may link diabetic nephropathy to macroangiopathy through impairment of endothelial function in patients with T2DM. PAFR in PBMCs may serve as a molecular target for treating diabetes complications and for selecting patients suitable for such treatment.

Conflict of interest

No potential conflicts of interest relevant to this article were reported.

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Table 1. Baseline characteristics of participants

	Group			
	Normoalbuminuria	Microalbuminuria	Macroalbuminuria	
Characteristic	n = 42	n = 28	n = 25	*p*
Age (years)	64.2 ± 9.2	61.5 ± 10.7	64.4 ± 14.2	0.543
Sex (male/female)	29/13	19/9	16/9	0.911
Duration (years)	15.1 ± 7.4	13.4 ± 8.7	17.1 ± 9.5	0.297
BMI (kg/m^2)	25.2 ± 4.2	26.8 ± 6.1	24.7 ± 5.1	0.327
SBP (mmHg)	127.2 ± 17.9	131.4 ± 13.0	141.8 ± 15.0[b]	0.002
FPG (mg/dL)	140.3 ± 27.4	146.4 ± 39.6	133.3 ± 32.3	0.115
HbA1c (%)	7.0 ± 0.6	7.1 ± 1.1	7.0 ± 1.0	0.729
TG (mg/dL)	123.6 ± 69.3	166.5 ± 100.2	118.5 ± 52.9	0.071
LDLC (mg/dL)	111.6 ± 27.1	106.9 ± 34.5	96.0± 27.9[c]	0.045
HDLC (mg/dL)	59.3 ± 13.6	50.2 ± 12.7[c]	63.1 ± 19.0[f]	0.006
oxLDL (ng/mL)	195.0 ± 236.6	223.4 ± 188.6	259.4 ± 392.1	0.107
Cr (mg/dL)	0.85 ± 0.22	1.02 ± 0.66	1.86 ± 1.93[ae]	<0.001
eGFR ($mL/min/1.73m^2$)	70.1 ± 16.9	67.6 ± 25.7	42.8 ± 22.1[ae]	<0.001
ACR (mg/g Cr)	11.4 ± 7.8	125.7 ± 85.4[a]	1546.7 ± 1074.2[ad]	<0.001
hs-CRP (ng/L)	677 ± 668	1369 ± 1841	1670 ± 4918	0.315
u-MCP-1/Cr (mg/g Cr)	99.5 ± 99.2	152.5 ± 143.5	238.1 ± 152.3[af]	<0.001
p-MCP-1 (mg/dL)	152.8 ± 47.6	189.6 ± 132.6	238.1 ± 156.1[af]	<0.001
baPWV (cm/s)	1772 ± 371	1727 ± 210	1982 ± 560	0.047
FMD (%)	5.4 ± 2.3	4.4 ± 2.6	3.5 ± 2.0[a]	0.002
Current smoking (%)	9 (21)	4 (14)	4 (16)	0.717

Past smoking (%)	18 (43)	17(61)	15 (60)	0.236
Drug usage				
HMG-CoA RIs (%)	21 (50)	16 (57)	15 (60)	0.696
ACE-I or ARB (%)	17 (40)	13 (46)	22 (88)	<0.001
CCB (%)	10 (24)	15 (54)	17 (68)	0.001
Insulin (%)	12 (29)	8 (29)	12 (48)	0.221
Metformin (%)	24 (57)	13 (46)	7 (28)	0.069
DPP4-I (%)	20 (48)	12 (43)	13 (52)	0.989
Antiplatelet therapy (%)	5 (12)	7 (25)	11(44)	0.012
Microvascular Complications				
Neuropathy (%)	27 (64)	15 (54)	20(80)	0.129
Retinopathy (%)	19(45)	13 (46)	18 (72)	0.078
Macrovascular Complications				
Coronary heart disease (%)	4 (10)	5 (18)	6 (24)	0.273
Stroke (%)	1 (2)	2 (7)	7 (28)	0.003
Peripheral artery disease (%)	1 (2)	1 (4)	3 (12)	0.208

Data are presented as the means ± SD and number (%). BMI, body mass index; SBP, systolic blood pressure; FPG, fasting plasma glucose level; TG, triglyceride level; LDLC, low density lipoprotein cholesterol; HDLC, high density lipoprotein cholesterol; Cr, creatinine; eGFR, estimated glomerular filtration rate; ACR, albumin to creatinine ratio; hs-CRP, high-sensitive C-reactive protein; u-MCP-1, urinary monocyte chemoattractant protein-1 to creatinine ratio; p-MCP-1, plasma monocyte chemoattractant protein-1 level; baPWV, brachial-ankle pulse wave velocity; FMD, flow mediated dilation; HMG-CoA RIs, hydroxyl methylglutaryl coenzyme A reductase inhibitors; ACE-I, angiotensin converting enzyme inhibitor; ARB, angiotensin II receptor blocker; CCB, calcium channel blocker; DPP4-I, dipeptidyl peptidase-4 inhibitor. Differences among groups were analyzed by one-way ANOVA followed by Tukey post hoc test or Kruskal-Wallis test followed by Steel-Dwass test or Chi-square free test. [a]$p < 0.001$; [b]$p < 0.01$; [c]$p < 0.05$ vs normoalbuminuric group. [d]$p < 0.001$; [e]$p < 0.01$; [f]$p < 0.05$ vs microalbuminuric group.

Table 2. Comparison of clinical variables and vascular complications between patients with differing PAFR mRNA expression levels

	PAFR mRNA expression in PBMCs			
	low	middle	high	
	N = 32	N = 31	N = 32	*p*
PAFR/HPRT1 mRNA	1.27 ± 0.33	2.37 ± 0.37[a]	3.79 ± 0.88[ad]	<0.001
Age (years)	62.7 ± 9.2	62.9 ± 10.8	64.7 ± 13.2	0.768
Duration (years)	15.6 ± 8.8	12.7 ± 7.3	17.0 ± 8.7	0.116
Current smoking (%)	5 (16)	3 (10)	9 (29)	0.148
BMI (kg/m^2)	24.7 ± 3.9	27.1 ± 6.2	24.7 ± 4.7	0.097
SBP (mmHg)	130.2 ± 15.2	135.7 ± 18.3	131.1 ± 16.6	0.360
ACR (mg/g･Cr)	122.3 ± 420.6	474.6 ± 1035.9	543.0 ± 689.5[a]	0.002
eGFR (mL/min/1.73m^2)	66.4 ± 19.2	57.7 ± 23.2	62.1 ± 28.6	0.362
u-MCP1/Cr (mg/g･Cr)	122.9 ± 130.4	165.8 ± 153.1	166.4 ± 132.8	0.130
p-MCP1 (mg/dL)	160.5 ± 71.5	198.2 ± 118.3	199.9 ± 145.5	0.194
LDLC (mg/dL)	110.7 ± 28.7	110.0 ± 30.2	97.7 ± 30.3	0.173
HDLC (mg/dL)	56.4 ± 13.7	55.3 ± 15.7	61.5 ± 17.2	0.348
TG (mg/dL)	138.5 ± 95.9	152.6 ± 77.8	114.1 ± 51.9	0.144
oxLDL (ng/mL)	130.3 ± 68.2	275.8 ± 370.6[c]	256.3 ± 270.4	0.034
FPG (mg/dL)	140.4 ± 38.8	138.3 ± 26.4	142.0 ± 32.4	0.776
HbA1c (%)	7.1 ± 0.9	6.8 ± 0.8	7.2 ± 0.9	0.074
hs-CRP (ng/L)	986.5 ± 1319.9	943.9 ± 1458.3	1393.7 ± 4336.8	0.488
FMD (%)	5.9 ± 2.0	3.6 ± 2.4[a]	4.2 ± 2.2[b]	<0.001
baPWV (cm/s)	1749 ± 315	1811 ± 326	1879 ± 532	0.498
Antiplatelet therapy (%)	6 (19)	5 (16)	12 (38)	0.095
Microvascular complication				
Neuropathy				
peripheral type (%)	22 (69)	18 (53)	22 (69)	0.591
autonomic type (%)	0 (0)	0 (0)	4 (13)	0.011
Retinopathy				
simple type (%)	4 (13)	4 (13)	6 (19)	0.300
proliferating type (%)	12 (38)	11 (35)	13 (41)	0.914
Macrovascular complication				
Coronary heart disease (%)	4 (13)	4 (13)	7 (22)	0.510
Stroke (%)	0 (0)	1(3)	9 (28)	<0.001
Peripheral artery disease (%)	1 (3)	2 (6)	2 (6)	0.393

Data are presented as the mean ± SD and number (%). Differences between groups were analyzed by one-way ANOVA followed by Tukey's post hoc test or Kruskal–Wallis test followed by Steel-Dwass test or Chi-square free test. [a]$p < 0.001$; [b]$p < 0.01$; [c]$p < 0.05$ vs low expression group. [d]$p < 0.001$ vs middle expression group.

Table 3. Stepwise multiple logistic regression analysis for macrovascular complications (upper) and stroke (lower)

Variable	B	SE	OR	95% CI	*p*
intercept	−5.834	1.201			
duration	0.124	0.038	1.142	1.051 to 1.218	0.001
Log_{10} ACR	0.544	0.163	1.724	1.251 to 2.374	0.001
PCP for the model: 84.0% ($p < 0.001$)					

Variable	B	SE	OR	95% CI	*p*
intercept	−7.030	1.665			
duration	0.108	0.044	1.114	1.022 to 1.204	0.014
PAFR mRNA	0.953	0.325	2.594	1.371 to 4.909	0.003
PCP for the model: 91.5% ($p < 0.001$)					

Variables included in the stepwise model: duration of diabetes, log_{10} ACR, eGFR, FMD, and mRNA expression of PAFR. B, regression coefficient; SE, standard error; β, standardized regression coefficients; 95% CI, 95% confidence interval; OR, odds ratio; PCP, percent correctly predicted

Figure 1.

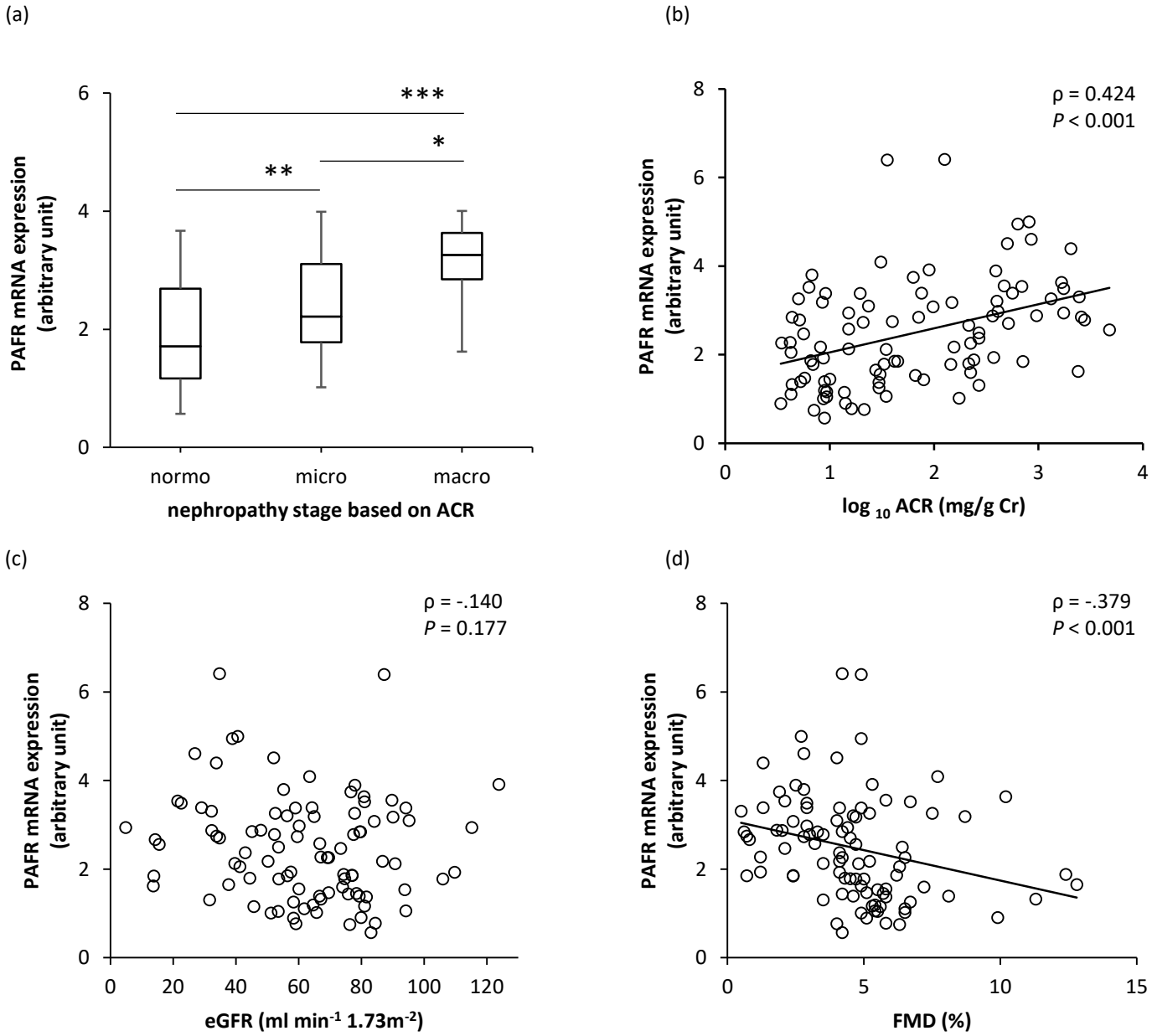
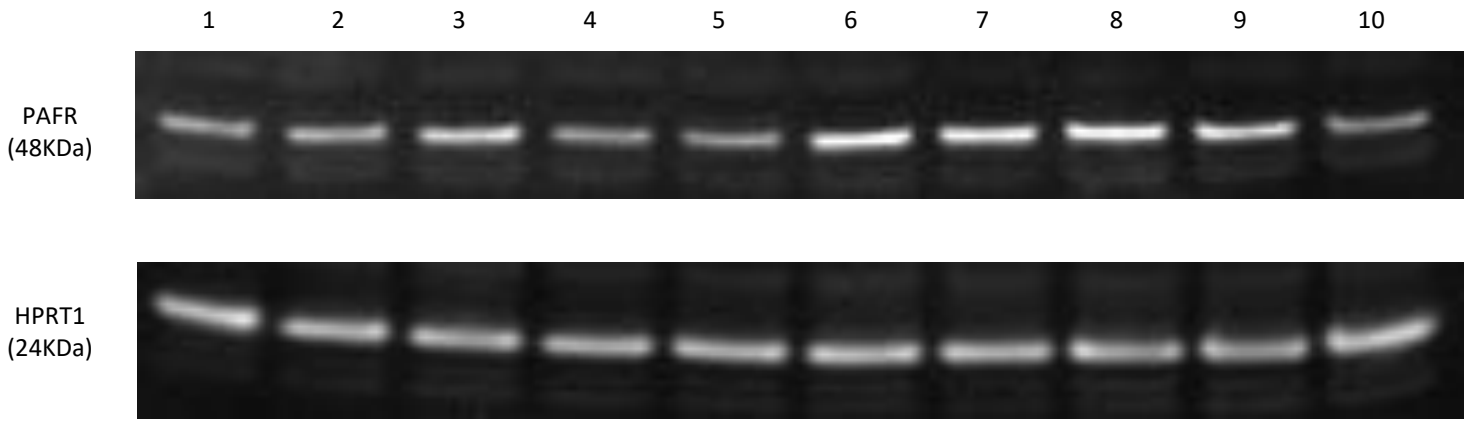

(a)
***
*
**
PAFR mRNA expression (arbitrary unit)
normo
micro
macro
nephropathy stage based on ACR
(b)
ρ = 0.424
P < 0.001
PAFR mRNA expression (arbitrary unit)
log 10 ACR (mg/g Cr)
(c)
ρ = -.140
P = 0.177
PAFR mRNA expression (arbitrary unit)
eGFR (ml min-1 1.73m-2)
(d)
ρ = -.379
P < 0.001
PAFR mRNA expression (arbitrary unit)
FMD (%)

Figure 2.

(a)


1
2
3
4
5
6
7
8
9
10
PAFR
(48KDa)
HPRT1
(24KDa)

(b)

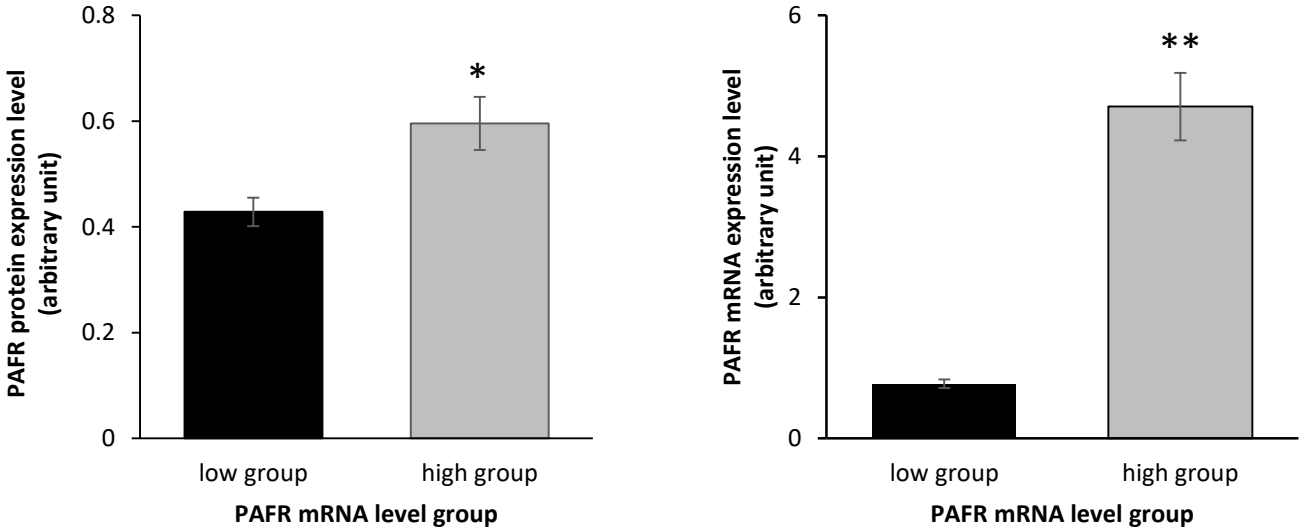

PAFR protein expression level
(arbitrary unit)
0.8
0.6
0.4
0.2
0
*
low group
high group
PAFR mRNA level group
PAFR mRNA expression level
(arbitrary unit)
6
4
2
0
**
low group
high group
PAFR mRNA level group

Figure legends

Figure 1. (**a**) mRNA expression of PAFR in PBMCs in the study group. PAFR mRNA expression was adjusted to that of the housekeeping gene HPRT1. Differences were analyzed by the Kruskal–Wallis test followed by the Steel–Dwass test (***$p < 0.001$; **$p < 0.01$; *$p < 0.05$) (**b**) Correlation between PAFR mRNA expression and log-transformed ACR. (**c**) Correlation between PAFR mRNA expression and eGFR. (**d**) Correlation between PAFR mRNA expression and FMD.

Figure 2. PAFR expression in PBMCs. (**a**) Western blot analysis of PAFR (above) and HPRT1 (below) in low PAFR mRNA expression cases (lanes 1–5) and high PAFR mRNA expression cases (lanes 6–10). (**b**) Comparison of PAFR protein (left) and mRNA expression (right) between low PAFR mRNA expression group (black bars) and high PAFR mRNA expression group (grey bars). The column charts represent the mean ± SEM. Differences were analyzed using the unpaired t-test. **$p < 0.001$; *$p < 0.05$

Supplementary Table 1.

TaqMan Primers and probe (TaqMan Gene Expression Assays, Applied Biosystems) and Assay ID

Target gene	Assay ID	Accession #	Amplicon length (bp)
HPRT1	Hs02800695_m1	NM_000194	82
TNF-α	Hs01113624_g1	NM_000594	143
IL1-β	Hs00174097_m1	NM_000576	94
IL-6	Hs00985639_m1	NM_000600	66
CCL2	Hs00234140_m1	NM_002982	101
CD36	Hs01567185_g1	NM_000072	116
TNFR	Hs01042313_m1	NM_001065	150
TLR4	Hs00152939_m1	NM_003266	89
CCR2	Hs00704702_s1	NM_001123396	61
Mac-1	Hs00355885_m1	NM_000632	60
LFA-1	Hs00158218_m1	NM_001114380	90
NOX2	Hs00166163_m1	NM_000397	78
p67phox	Hs01084940_m1	NM_000433	106
PAFR	Hs00265399_s1	NM_000952	98
COX1	Hs00377726_m1	NM_000962	60
COX2	Hs00153133_m1	NM_000963	75
THBS1	Hs00962908_m1	NM_003246	59
NFkB	Hs00765730_m1	NM_001165412	66

HPRT1, Hypoxanthine phosphoribosyltransferase 1; TNF-α, tumor necrosis factor receptor; IL-1β, interleukin 1β; IL-6, interleukin 6; CCL2, C-C motif chemokine ligand 2; CD36, Cluster differentiation 36 molecule; TNFR, tumor necrosis factor receptor; TLR4, toll-like receptor 4; CCR2, C-C motif chemokine receptor 2; Mac-1, CD11b/CD18; LFA-1, CD11a/CD18; NOX2, NADH oxidase 2; p67phox, 67 KDa neutrophil oxidase factor 2; PAFR, platelet activating factor receptor; COX1, cyclooxygenase 1; COX2, cyclooxygenase 2; THBS1, thrombospondin 1; NFkB, nuclear factor-kappa B.

＝Supplementary Table 2.

Relative mRNA expression of target genes and correlation with ACR and eGFR in three clinical stages of diabetic nephropathy

	mRNA expression				Correlation with ACR		Correlation with eGFR	
Target gene	Normoalbuminuric group N = 42	Microalbuminuric group N = 28	Macroalbuminuric group N = 25	*p*	ρ	*p*	ρ	*p*
TNF-α	0.24 (0.19–0.37)	0.26 (0.17–0.34)	0.26 (0.19–3.78)	0.870	−0.02	0.983	-0.172	0.095
IL-1β	1.16 (0.65–2.73)	1.27 (0.83–2.10)	1.32 (0.62–2.17)	0.983	-0.103	0.904	-0.004	0.973
IL-6	0.06 (0.04–0.10)	0.05 (0.03–0.11)	0.06 (0.04–0.10)	0.904	0.009	0.934	0.097	0.352
CCL2	0.15 (0.07–0.32)	0.19 (0.07–0.33)	0.17 (0.09–0.55)	0.949	0.043	0.68	-0.107	0.300
CD36	6.88 (4.85–9.10)	9.98 (7.29–11.77)[b]	9.00 (7.00–12.08)	0.010	0.210	0.041	0.085	0.412
TNFR	2.91 (2.40–4.06)	3.49 (2.31–4.06)	4.13 (3.14–4.89)[b]	0.039	0.206	0.046	−0.186	0.071
TLR4	2.02 (1.37–2.44)	2.18 (1.48–2.43)	2.35 (1.77–2.86)	0.058	0.177	0.087	−0.073	0.481
CCR2	14.21 (5.88–30.18)	14.70 (6.91–19.22)	13.00 (10.82–21.02)	0.840	0.038	0.711	−0.017	0.868
NOX2	48.04 (37.62–62.86)	46.25 (34.83–57.77)	49.87 (43.17–68.47)	0.250	0.036	0.730	0.038	0.712
p67phox	13.91 (9.87–16.84)	15.19 (11.11–17.95)	17.60 (13.5–21.71)[b]	0.025	0.181	0.079	−0.048	0.642
Mac-1	5.41 (4.47–6.34)	6.03 (4.49–7.58)	6.44 (4.93–7.47)	0.195	0.129	0.214	−0.041	0.693
LFA-1	16.76 (12.62–21.99)	14.66 (11.31–20.78)	16.60 (13.62–21.66)	0.542	0.014	0.890	−0.089	0.392
PAFR	1.71 (1.16–2.74)	2.21 (1.78–3.15)[b]	3.25 (2.81–3.76)[a c]	<0.001	0.424	<0.001	–0.14	0.177
COX1	3.88 (3.20–5.90)	4.65 (3.09–7.63)	4.02 (2.91–5.37)	0.699	0.007	0.946	0.185	0.072
COX2	0.47 (0.30–1.27)	0.81 (0.33–1.49)	0.59 (0.26–1.12)	0.475	0.007	0.947	−0.032	0.759
THBS1	7.19 (5.01–12.06)	10.54 (6.68–15.19)	8.50 (4.77–14.21)	0.162	0.011	0.918	0.200	0.052
NFkB	2.28 (1.97–2.57)	2.14 (1.91–2.58)	2.22 (1.96–2.62)	0.927	−0.07	0.503	0.081	0.437

Data are median (25th to 75th percentile range) of each mRNA expression after adjusting for that of housekeeping gene HPRT1. HPRT1, Hypoxanthine phosphoribosyltransferase 1; TNF-α, tumor necrosis factor α; IL-1β, interleukin 1β; IL-6, interleukin 6; CCL2, C-C motif chemokine ligand 2; CD36, CD36 molecule; TNFR, tumor necrosis factor receptor; TLR4, toll-like receptor 4; CCR2, C-C motif chemokine receptor 2; Mac-1, CD11b/CD18; LFA-1, CD11a/CD18; NOX2, NADH oxidase 2; p67phox, 67 KDa neutrophil oxidase factor 2; PAFR, platelet activating factor receptor; COX1, cyclooxygenase 1; COX2, cyclooxygenase 2; THBS1, thrombospondin 1; NFkB, nuclear factor-kappa B. Differences among groups were analyzed by Kruskal-Wallis test followed by Steel-Dwass test. [a]$p < 0.001$; [b]$p < 0.05$ vs. normoalbuminuric group. [c]$p < 0.05$ vs. microalbuminuric group.

Supplementary Table 3. Stepwise multiple linear regression analysis for log-transformed FMD (upper) and log-transformed PAFR mRNA expression in PBMCs (lower)

Variable	B	SE	β	95% CI	*p*
intercept	0.518				
eGFR	0.003	0.001	0.282	0.001 to 0.006	0.004
Log_{10} PAFR	−0.340	0.121	−0.271	−0.579 to −0.100	0.006
R^2 for the model: 0.18 ($p < 0.001$)					

Variables included in the stepwise model: age, sex, BMI, SBP, log_{10} ACR, eGFR, log_{10} hs-CRP, log_{10} p-MCP1, log_{10} u-MCP1, and log_{10} PAFR. B, regression coefficient; SE, standard error; β, standardized regression coefficients; R^2, coefficient of determination; 95% CI, 95% confidence interval

Variable	B	SE	β	95% CI	*p*
intercept	0.273				
Log_{10} ACR	0.040	0.011	0.368	0.019 to 0.061	<0.001
Log_{10} FMD	−0.178	0.076	-0.226	-0.330 to -0.027	0.022
R^2 for the model: 0.23 ($p < 0.001$)					

Variable included in the stepwise model: age, sex, HDLC, ox-LDL, log_{10} ACR, eGFR, log_{10} p-MCP1, log_{10} u-MCP1, log_{10} baPWV, and log_{10} FMD. B, regression coefficient; SE, standard error; β, standardized regression coefficients; R^2, coefficient of determination; 95% CI, 95% confidence interval