In conclusion, the findings of the present study suggest that treatment with valsartan, even at a low dose, may ameliorate some glycation and oxidative stress markers independently of an effect on blood pressure in hypertensive type 2 diabetic subjects.

Disclosure statement: Fujirebio Inc., Tokyo (formerly Chugai Diagnostic Science Inc., Tokyo) and Dr. Hirose have a partial patent concerning the HMW-adiponectin measurement kit. Dr. Saito has received a research grant from Novartis Pharma K.K., Tokyo.

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Cardio-Ankle Vascular Index and Ankle Pulse Wave Velocity as a Marker of Arterial Fibrosis in Kidney Failure Treated by Hemodialysis

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Background: Patients with kidney failure treated with hemodialysis have a high incidence of cardiovascular diseases caused by accelerated arteriosclerosis. However, accurate evaluation of the extent of arteriosclerosis is difficult. This study sought to compare the strength of predictions of arterial fibrosis by using a new parameter, the cardio-ankle vascular index (CAVI), versus pulse wave velocity (PWV) in patients with kidney failure treated with hemodialysis.

Study Design: Diagnostic test study.

Setting & Participants: 103 patients with kidney failure undergoing surgical construction of an arteriovenous access for hemodialysis therapy.

Index Test: CAVI and PWV.

Reference Test: Arterial fibrosis, evaluated by using Masson trichrome stain on part of the brachial artery obtained during surgery, expressed as percentage of fibrosis of the layer of vascular smooth muscle cells.

Results: Median percentage of arterial stiffness was 52.85%. Mean PWV and CAVI were 18.3 ± 5.6 (SD) m/s and 9.9 ± 2.6, respectively. Multivariate regression analysis showed that arterial fibrosis was significantly associated with older age (0.247%/y; 95% confidence interval, 0.013 to 0.482) and CAVI (6.117%/unit; 95% confidence interval, 4.764 to 4.740), but not with systolic blood pressure (0.039%/mm Hg; 95% confidence interval, −0.076 to 0.153) or PWV (−0.044%/m/s; 95% confidence interval, −0.646 to 0.558). The area under the receiver operating characteristic curve to predict greater than median percentage of arterial stiffness was 0.892 for CAVI and 0.779 for PWV ($P = 0.006$).

Limitation: It is unclear whether arterial fibrosis of the brachial artery evaluated by using CAVI is applicable for arteriosclerosis of other arterial districts, and clinical outcomes were not evaluated in this study.

Conclusion: CAVI reflects the histological arterial fibrosis of hemodialysis patients and is a useful clinical marker for evaluating arterial stiffness in these patients.

Am J Kidney Dis 52:947-955.

INDEX WORDS: Arteriosclerosis; blood pressure; diabetes; lipid; Masson trichrome.

Accelerated atherogenesis is a serious problem in patients with chronic kidney disease (CKD) and significantly contributes to the increased cardiovascular mortality rate in patients with end-stage renal disease.[1,2] Recent studies have shown that pulse wave velocity (PWV) is a useful parameter for evaluating arterial stiffness in patients with hypertension,[3] dyslipidemia,[4] diabetes,[5] or end-stage renal disease.[6] However, accurate evaluations of arterial stiffness in hemodialysis patients with changeable blood pressures (BPs) may be difficult using PWV because PWV is essentially dependent on BP. Studies have shown a significant association between PWV and systolic BP in hemodialysis patients.[6,7] To overcome this disadvantage, the cardio-ankle vascular index (CAVI) recently was developed as a new parameter for evaluating arterial stiffness independently of BP.[8,9] Because the formula to calculate CAVI is derived from the Bramwell-Hill equation and the stiffness parameter β, CAVI may be less influenced by BP than PWV, and its measurement has been shown to be reproducible[9,10] and associated with intima-media thickness, but not plaque score, of the common carotid artery in patients with essential hypertension.[11]

The present study examines whether CAVI reflects histological fibrosis in the arteries of patients with end-stage renal disease, that is,

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Received December 3, 2007. Accepted in revised form June 1, 2008. Originally published online as doi: 10.1053/j.ajkd.2008.06.007 on September 2, 2008.

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doi:10.1053/j.ajkd.2008.06.007

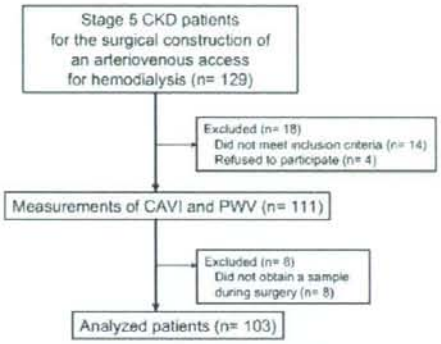

Figure 1. Patient inclusion and flow through the study. Abbreviations: CKD, chronic kidney disease; CAVI, cardio-ankle vascular index; PWV, pulse wave velocity.

stage 5 CKD according to KDOQI (Kidney Disease Outcomes Quality Initiative).

METHODS

Study Design and Patients

The present study was conducted in 129 patients with kidney failure, diagnosed stage 5 CKD according to KDOQI criteria, who attended the Yoyogi Yamashita Clinic, Tokyo, Japan, for surgical construction of an arteriovenous access for hemodialysis therapy. As shown in Fig 1, there were 18 patients with arrhythmias, previous cardiovascular events, peripheral arterial disease, secondary hyperparathyroidism, or adynamic bone disease and 4 patients who refused to participate excluded from the study. Baseline arterial stiffness measurements were obtained using CAVI and PWV in the remaining 111 patients, and arterial samples were obtained in 103 patients. We could not collect arterial samples of 8 patients during surgery. In the analyzed 103 patients, decreased estimated glomerular filtration rate, averaging 5.8 ± 3.2 mL/min/1.73 m^2 (0.10 ± 0.05 mL/s/1.73 m^2), was confirmed using the equation for Japanese.[12] Causes of kidney failure were diabetes for 44 patients, nephritis for 56 patients, hypertensive nephrosclerosis for 2 patients, and polycystic kidney disease for 1 patient, and 55 patients were treated with angiotensin-converting enzyme inhibitors or angiotensin II receptor blockers as BP medications. Clinical and biological parameters were measured in the morning on the day before surgery and immediately before the session on the day of hemodialysis. Clinic BP was measured in the patient's right arm while the patient was in a sitting position; a standard sphygmomanometer with an appropriately sized cuff was used to measure BP after the patient had been seated for at least 10 minutes. The study was approved by the review board of Keio University Medical School Hospital, Tokyo, Japan, and written informed consent was obtained from every participant.

Evaluation of Arterial Fibrosis

Arterial fragments obtained from patients were fixed in 4% paraformaldehyde in phosphate buffer (pH 7.4). Paraffin-embedded sections were stained with Masson trichrome for microscopic examination, as shown in Fig 2. Severity of arterial fibrosis was determined by using Biozero (BZ-8000; Keyence, Osaka, Japan) and expressed as percentage of fibrosis involving the layer of vascular smooth muscle cells.

Measurements of CAVI and PWV

CAVI and PWV were measured using a VaSera VS-1000 (Fukuda Denshi, Tokyo, Japan). Cuffs were applied to the 4 extremities, and electrocardiographic electrodes were attached to the upper extremities. A microphone was placed at the sternal angle to obtain a phonocardiogram. Patients rested in the supine position for at least 10 minutes before the start of monitoring. PWV from the heart to the ankle was obtained by measuring the length from the aortic valve to the ankle.[8]

Venous Blood Samples

Venous blood samples were obtained on the same days as CAVI measurements and were drawn in the morning on the day before surgery and immediately before the session on the day of hemodialysis, after an overnight fast. Creatinine, total cholesterol, triglycerides, calcium, and phosphate were measured using standard methods. High-density lipoprotein cholesterol, low-density lipoprotein cholesterol, plasma renin activity, and plasma aldosterone concentration were determined using commercially available kits.

Statistical Analyses

Differences in arterial fibrosis by sex and presence of diabetes were analyzed by using an unpaired *t*-test. Univariate and multivariate regression analyses were performed using StatView 5.0 software (SAS Institute Inc, Cary, NC). Univariate regression analyses of arterial fibrosis indicated significant associations with age, presence of diabetes, systolic BP, PWV, and CAVI, and univariate regression analyses of CAVI indicated significant associations with presence of diabetes, PWV, and arterial fibrosis. Multivariate regression analyses were performed using these response variables. *P* less than 0.05 is considered significant. Data are reported as mean ± SD.

Arterial fibrosis of 52.85% or greater (average value), 31.97% or greater (average value − SD), and 73.73% or greater (average value + SD) were used as the cutoff values to draw receiver operating characteristic (ROC) curves for both CAVI and PWV. The area under the ROC curve for predicting arterial stiffness was analyzed using Dr. SPSS II (SPSS Inc, Chicago, IL).

RESULTS

Patient Characteristics

Mean age of the 103 patients with stage 5 CKD treated with hemodialysis was 65 ± 11 years, 50 patients were men, 14 were smokers,

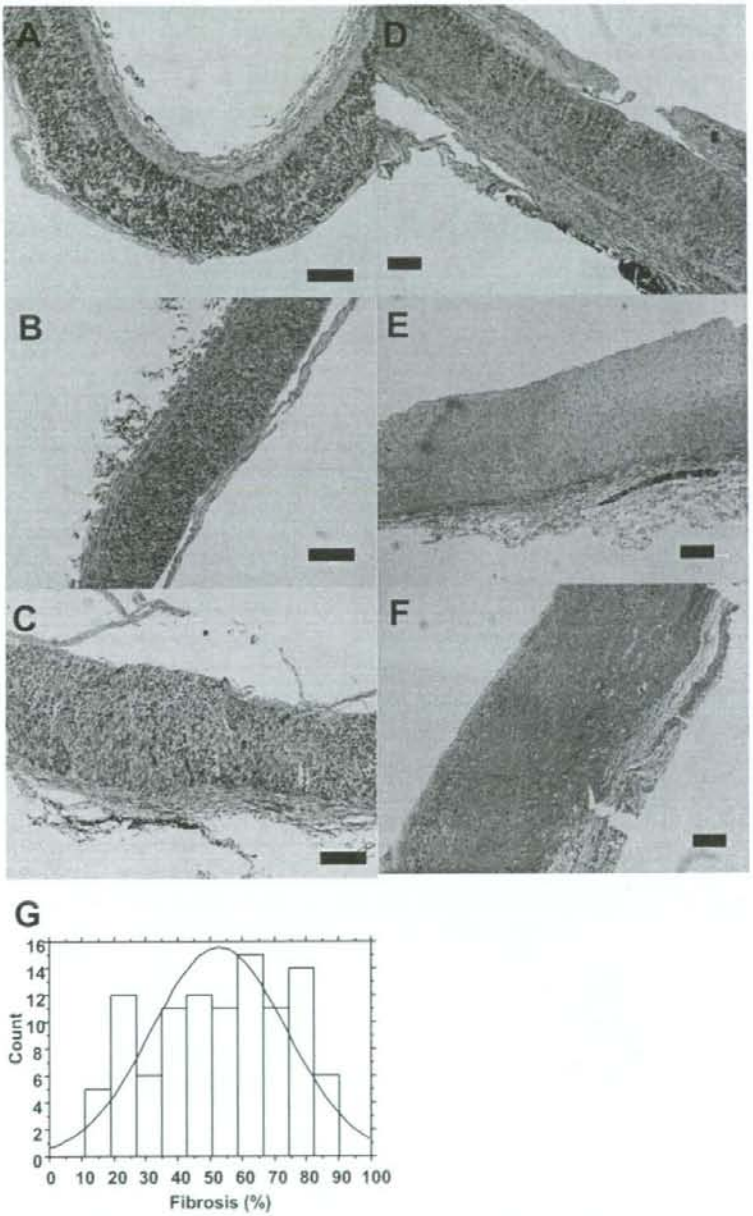

Figure 2. Representative arterial specimens: (A) fibrosis, 23.3%; cardio-ankle vascular index (CAVI), 5.0; (B) fibrosis, 39.7%; CAVI, 8.6; (C) fibrosis, 53.6%; CAVI, 10.6; (D) fibrosis, 60.3%; CAVI, 12.8; (E) fibrosis, 75.0%; CAVI, 14.0; (F) fibrosis, 90.2%; CAVI, 17.3; and (G) histogram of distribution of percentage of fibrosis in 103 patients. (Scale bars, 200 μm; Masson trichrome stain.)

and 44 had diabetes. They had been receiving dialysis therapy for 8.2 ± 8.3 years, and 55 were on angiotensin-converting enzyme inhibitor or angiotensin II receptor blocker therapy, whereas 22 were on statin therapy. Systolic and diastolic BPs averaged 151 ± 23 and 84 ± 13 mm Hg, respectively. All blood studies yielded the following average values: plasma total cholesterol, 169 ± 42 mg/dL (4.37 ± 1.09 mmol/L); plasma triglycerides, 121 ± 59 mg/dL (1.37 ± 0.67 mmol/L); plasma high-density lipoprotein cholesterol, 44 ± 12 mg/dL (1.14 ± 0.31 mmol/L); plasma low-density lipoprotein cholesterol, 102 ± 38 mg/dL (2.64 ± 0.98 mmol/L); plasma calcium-phosphorus product, 50 ± 17 mg^2/dL^2 (4.0 ± 1.4 $mmol^2/L^2$); plasma renin activity, 3.5 ± 5.9 ng/mL/h (1 ± 2 ng/L/s); and plasma aldosterone, 161 ± 319 ng/dL (4.47 ± 8.85 nmol/L). Mean ankle-brachial index was 1.03 ± 0.17 and similar in patients with and without diabetes (1.06 ± 0.10 *v* 1.01 ± 0.20, $P = 0.1$). Mean PWV and CAVI were 18.3 ± 5.6 m/s and 9.9 ± 2.6, respectively.

Arterial Fibrosis

Figure 2 shows representative arterial specimens and normal distribution of arterial fibrosis in 103 patients with kidney failure treated with hemodialysis. In these patients, mean arterial fibrosis was 52.8% ± 20.9%.

Univariate Regression Analyses of Arterial Fibrosis

As shown in Fig 3, systolic BP significantly correlated with PWV ($R^2 = 0.039$; $P = 0.04$), but not with CAVI ($R^2 = 0.035$; $P = 0.06$). Univariate regression analyses for arterial fibrosis and other variables showed significant associations of arterial fibrosis with age, presence of diabetes, systolic BP, PWV, and CAVI (Table 1; Fig 3). In addition, as shown in Fig 4, arterial fibrosis was significantly greater in patients with

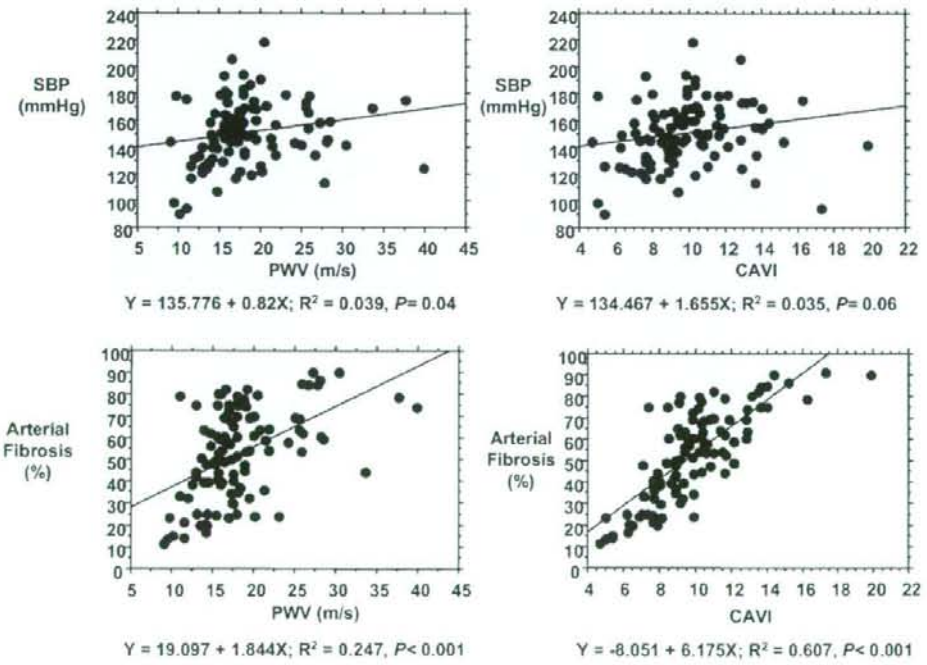

Figure 3. Significant associations of systolic blood pressure (SBP) and arterial fibrosis with pulse wave velocity (PWV) and cardio-ankle vascular index (CAVI) in patients with kidney failure treated with hemodialysis.

Table 1. Univariate Regression Analyses of Arterial Fibrosis Versus Other Variables

Covariate	Mean ± SD or No./Total No. of Patients	R^2	P
Age (y)	65 ± 11	0.039	0.05
Men/women	50/53	0.016	0.2
Body mass index (kg/m^2)	21.0 ± 3.5	0.007	0.4
Duration of dialysis (y)	8.2 ± 8.3	0.001	0.9
Presence of diabetes (yes/no)	44/59	0.070	0.004
Use of BP medications (yes/no)	55/48	0.004	0.7
Systolic BP (mm Hg)	151 ± 23	0.040	0.04
Diastolic BP (mm Hg)	84 ± 13	0.005	0.5
Plasma total cholesterol (mg/dL)	169 ± 42	0.014	0.3
Plasma triglycerides (mg/dL)	121 ± 59	0.008	0.4
Plasma HDL cholesterol (mg/dL)	44 ± 12	0.007	0.5
Plasma LDL cholesterol (mg/dL)	102 ± 38	0.006	0.5
Plasma calcium-phosphorus product (mg^2/dL^2)	50 ± 17	0.004	0.6
Ankle-brachial index	1.03 ± 0.17	0.008	0.4
PWV (m/s)	18.3 ± 5.6	0.247	<0.001
CAVI	9.9 ± 2.6	0.603	<0.001
Plasma renin activity (ng/mL/h)	3.5 ± 5.9	0.001	0.8
Plasma aldosterone (ng/dL)	161 ± 319	0.001	0.9

Note: To convert serum creatinine in mg/dL to μmol/L, multiply by 88.4; plasma total cholesterol, HDL cholesterol, and LDL cholesterol in mg/dL to mmol/L, multiply by 0.02586; plasma triglycerides in mg/dl to mmol/L, multiply by 0.01129; plasma calcium-phosphorus product in mg^2/dL^2 to mmol^2/L^2, multiply by 0.08056; plasma renin activity in ng/mL/h to ng/(Lxs), multiply by 0.2778; plasma aldosterone in ng/dL to nmol/L, multiply by 0.02774.

Abbreviations: BP, blood pressure; CAVI, cardio-ankle vascular index; HDL, high-density lipoprotein; LDL, low-density lipoprotein; PWV, pulse wave velocity.

diabetes mellitus (59.6% ± 20.6% *v* 47.8% ± 19.8%) and was similar in men and women. However, no other continuous or explanatory variable was associated with arterial fibrosis.

Multivariate Regression Analysis of Arterial Fibrosis

Table 2 lists the multivariate regression analysis for arterial fibrosis and the 5 parameters that showed significant associations in univariate regression analyses. Arterial fibrosis was significantly associated with age and CAVI, but did not correlate with any other parameters, including PWV. Thus, arterial fibrosis of patients with kidney failure treated with hemodialysis can be assessed by using CAVI independently of systolic BP, presence of diabetes, or PWV.

Univariate and Multivariate Regression Analyses of CAVI

Table 3 lists univariate regression analyses between CAVI and other variables. The presence of diabetes, PWV, and arterial fibrosis were significantly associated with CAVI, but other variables were not associated with CAVI. As listed in Table 4, a multivariate regression analysis of CAVI indicated that CAVI was significantly associated with the presence of diabetes, PWV, and arterial fibrosis, but did not correlate with age or systolic BP. In addition, as shown in Fig 3, CAVI was significantly greater in patients with diabetes (11.0 ± 3.0) than in those without diabetes (9.0 ± 2.0) and was similar in men and women.

ROC Curves for CAVI and PWV for the Diagnosis of Arterial Stiffness in Patients With Kidney Failure

As shown in Fig 5, ROC curves were drawn at the 3 cutoff values for arterial fibrosis; 52.85% (average value of arterial fibrosis), 31.97% (average value of arterial fibrosis − SD), and 73.73% (average value of arterial fibrosis + SD). Using the average value as a cutoff, areas under the ROC curve for CAVI and PWV were 0.892 and 0.779, respectively. At the average minus-SD value, areas under the ROC curve for CAVI and PWV were 0.935 and 0.773, respectively, and at the average plus-SD value, areas under the ROC curve for CAVI and PWV were 0.833 and 0.684, respectively. At each cutoff value, the area under the ROC curve for CAVI was significantly greater than the area under the ROC curve for PWV (P = 0.006, P = 0.001, and P = 0.01 for the

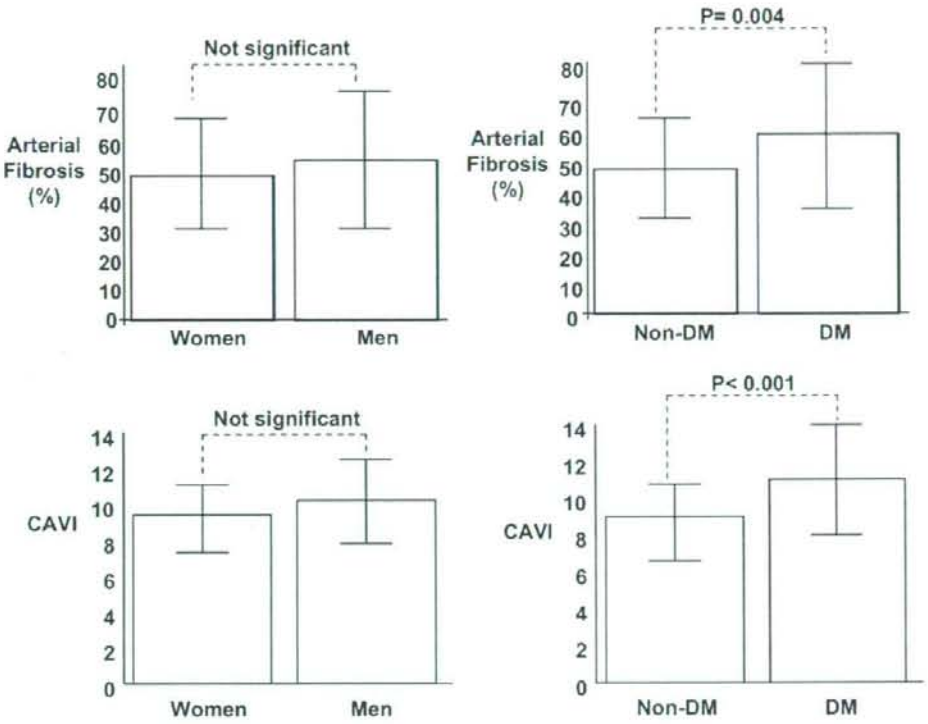

Figure 4. Arterial fibrosis and cardio-ankle vascular index (CAVI) by sex or in the presence and absence of diabetes (DM).

cutoff value of average, average − SD, and average + SD, respectively).

DISCUSSION

Previous studies showed that mean CAVI was 8.3 in a general population of 1,033 participants[10] and was 8.1 in 229 hemodialysis patients without signs suggesting atherosclerotic disease.[9] In the present study, mean CAVI of 103 hemodialysis patients was 9.9 and tended to be greater than that of 28 hemodialysis patients with signs suggesting atherosclerotic disease.[9] Al-

Table 2. Multivariate Regression Analysis of Arterial Fibrosis Versus Age, Systolic BP, Presence of Diabetes, PWV, and CAVI

Independent Factor	Coefficient	95% Confidence Interval	*P*
Intercept	−28.524	−50.793 to −6.255	0.01
Age (y)	0.247	0.013 to 0.482	0.04
Systolic BP (mm Hg)	0.039	−0.076 to 0.153	0.5
Diabetes (yes/no)	−0.446	−6.084 to 5.192	0.9
PWV (m/s)	−0.044	−0.646 to 0.558	0.9
CAVI	6.117	4.764 to 7.470	<0.001

Note: $R^2 = 0.627$; $F = 32.6$; $P < 0.001$.
Abbreviations: BP, blood pressure; CAVI, cardio-ankle vascular index; PWV, pulse wave velocity.

Table 3. Univariate Regression Analyses of CAVI Versus Other Variables

Covariate	Mean ± SD or No./Total No. of Patients	R^2	P
Age (y)	65 ± 11	0.007	0.4
Men/women	50/53	0.025	0.1
Body mass index (kg/m^2)	21.0 ± 3.5	0.008	0.4
Duration of dialysis (y)	8.2 ± 8.3	0.08	0.03
Diabetes (yes/no)	44/59	0.130	<0.001
Use of BP medications (yes/no)	55/48	0.026	0.1
Systolic BP (mm Hg)	151 ± 23	0.035	0.06
Diastolic BP (mm Hg)	84 ± 13	0.007	0.4
Serum creatinine (mg/dL)	10.3 ± 2.4	0.011	0.3
Plasma total cholesterol (mg/dL)	169 ± 42	0.002	0.7
Plasma triglycerides (mg/dL)	121 ± 59	0.001	0.8
Plasma HDL cholesterol (mg/dL)	44 ± 12	0.003	0.7
Plasma LDL cholesterol (mg/dL)	102 ± 38	0.002	0.7
Plasma calcium-phosphorus product (mg^2/dL^2)	50 ± 17	0.001	0.7
Ankle-brachial index	1.03 ± 0.17	0.001	0.8
PWV (m/s)	18.3 ± 5.6	0.418	<0.001
Arterial fibrosis (%)	52.8 ± 20.9	0.603	<0.001
Plasma renin activity (ng/mL/h)	3.5 ± 5.9	0.001	0.9
Plasma aldosterone (ng/dL)	161 ± 319	0.044	0.1

Note: To convert serum creatinine in mg/dL to μmol/L, multiply by 88.4; plasma total, HDL, and LDL cholesterol in mg/dL to mmol/L, multiply by 0.02586; plasma triglycerides in mg/dL to mmol/L, multiply by 0.01129; plasma calcium-phosphorus product in mg^2/dL^2 to mmol^2/L^2, multiply by 0.08056; plasma renin activity in ng/mL/h to ng/(Lxs), multiply by 0.2778; plasma aldosterone in ng/dL to nmol/L, multiply by 0.02774.

Abbreviations: BP, blood pressure; CAVI, cardio-ankle vascular index; HDL, high-density lipoprotein; LDL, low-density lipoprotein; PWV, pulse wave velocity.

though the patients analyzed in the present study were free of previous cardiovascular events, peripheral arterial disease, or secondary hyperparathyroidism, they included 44 patients with diabetes, were older (65 *v* 60 years), and had a longer duration of dialysis therapy (8.2 *v* 7.6 years) compared with patients reported previously.[9]

Although both CAVI and PWV are parameters for evaluating arterial stiffness, PWV is dependent on BP, but the dependency of CAVI on BP is undetermined. In the present study, univariate regression analyses showed a weak and insignificant correlation between CAVI and systolic BP ($R^2 = 0.035$; $P = 0.06$), but multivariate regression analysis showed no significant relationship between CAVI and systolic BP ($P = 0.7$). These results suggest that CAVI is a parameter for evaluating arterial stiffness less influenced by BP than PWV in patients with kidney failure.

Multiple regression analyses clearly showed a significant association of arterial fibrosis with CAVI, but not with PWV. Furthermore, the area under the ROC curve for CAVI was significantly greater than that for PWV with any cutoff values. These results verified that CAVI is a better test for predicting arterial stiffness in hemodialysis

Table 4. Multivariate Regression Analysis of CAVI Versus Age, Systolic BP, Presence of Diabetes, PWV, and Arterial Fibrosis

Independent Factor	Coefficient	95% Confidence Interval	P
Intercept	3.553	1.124 to 5.982	0.005
Age (y)	−0.016	−0.042 to 0.011	0.2
Systolic BP (mm Hg)	0.002	−0.010 to 0.015	0.7
Diabetes (yes/no)	0.740	0.137 to 1.342	0.02
PWV (m/s)	0.151	0.093 to 0.210	<0.001
Arterial fibrosis (%)	0.074	0.058 to 0.091	<0.001

Note: $R^2 = 0.716$; $F = 48.849$; $P < 0.001$.

Abbreviations: BP, blood pressure; CAVI, cardio-ankle vascular index; PWV, pulse wave velocity.

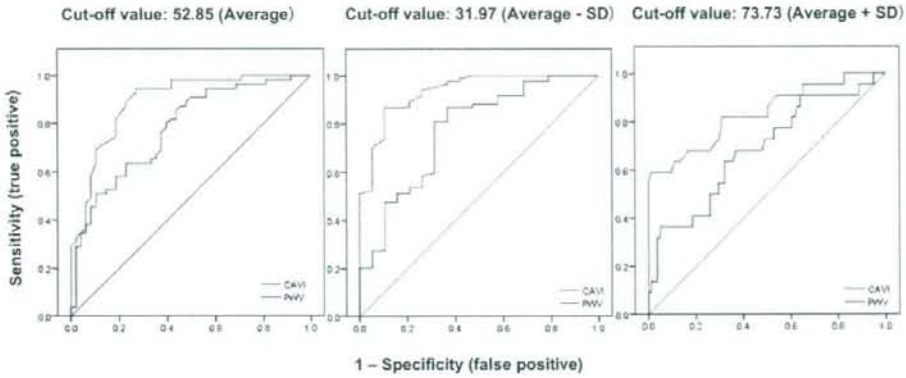

Figure 5. Receiver operating characteristic (ROC) curves for cardio-ankle vascular index (CAVI; blue lines) and pulse wave velocity (PWV; red lines) for the diagnosis of arterial stiffness in patients with kidney failure at the cutoff values based on the average, average minus SD, and average plus SD of arterial fibrosis.

patients. In addition, the costs of CAVI and PWV are similar and approximately $10,000/device. The time needed to obtain the measurements was within 5 minutes and also similar. Therefore, CAVI is a more accurate and useful parameter for evaluating arterial stiffness in hemodialysis patients with changeable BP.

Patients with CKD have a high incidence of cardiovascular diseases as a result of accelerated arteriosclerosis through a dyslipidemia-independent mechanism.[13] In particular, patients with diabetes with CKD have a greater incidence of cardiovascular diseases than patients with CKD without diabetes.[14] In the present study, both mean arterial fibrosis and mean CAVI of hemodialysis patients with diabetes were significantly greater than those of hemodialysis patients without diabetes. These results suggest that CAVI may predict the development of microvascular complications in patients with diabetes. However, prospective studies based on clinical outcomes will be needed to confirm this hypothesis. Although previous studies showed that CAVI was greater in men than women in the general population,[10] both arterial fibrosis and CAVI were similar in men and women treated with hemodialysis. Decreased kidney function may cancel the advantage in the arteries of women.

The present study has several limitations. First, although arterial fibrosis of the brachial artery was evaluated by using CAVI, it is unclear whether CAVI also reflects arterial fibrosis of other arterial districts, such as coronary artery and aorta. Second, in the present study, 55 patients had been treated with angiotensin-converting enzyme inhibitors or angiotensin II receptor blockers as BP medications. Because both BP and the renin-angiotensin system contribute to the development and progression of arteriosclerosis, the BP medications might affect the relationship of CAVI and PWV to BP and the renin-angiotensin system. However, use of BP medications was not associated with arterial fibrosis or CAVI. Thus, we believe that use of BP medications did not influence the relationship of CAVI to arterial fibrosis. Finally, clinical outcomes were not evaluated in the present study. Additional validation studies based on clinical outcomes are needed.

In conclusion, the novel parameter CAVI was strongly associated with histological arterial fibrosis in hemodialysis patients with a changeable BP. CAVI may serve as an accurate and useful clinical marker of arterial stiffness in these patients.

ACKNOWLEDGEMENTS

We appreciate the skillful secretarial work of Chika Miki.

Support: This work was supported in part by a grant from the Ministry of Education, Science and Culture of Japan (17390249).

Financial Disclosure: None.

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The American Journal of Pathology, Vol. 173, No. 6, December 2008
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DOI: 10.2353/ajpath.2008.080457

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Vascular Biology, Atherosclerosis and Endothelium Biology

(Pro)renin Receptor Promotes Choroidal Neovascularization by Activating Its Signal Transduction and Tissue Renin-Angiotensin System

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The receptor-associated prorenin system (RAPS) refers to pathogenic mechanisms whereby prorenin binding to its receptor activates both the tissue renin-angiotensin system (RAS) and RAS-independent intracellular signaling pathways. Although we found significant involvement of angiotensin II type 1 receptor (AT1-R)-mediated inflammation in choroidal neovascularization (CNV), a central abnormality of vision-threatening age-related macular degeneration, the association of RAPS with CNV has not been defined. Here, (pro)renin receptor blockade in a murine model of laser-induced CNV led to the significant suppression of CNV together with macrophage infiltration and the up-regulation of intercellular adhesion molecule (ICAM-1), monocyte chemotactic protein (MCP-1), vascular endothelial growth factor (VEGF), VEGF receptor (VEGFR)-1, and VEGFR-2. To clarify the role of signal transduction via the (pro)renin receptor in CNV, we used mice in which renin-angiotensin system was deactivated by either the pharmacological blockade of AT1-R with losartan or the genetic ablation of AT1-R or angiotensinogen. Compared with wild-type controls, these mice exhibited significant reduction of CNV and macrophage infiltration, both of which were further suppressed by (pro)renin receptor blockade. The (pro)renin receptor and phosphorylated extracellular signal-regulated kinases (ERK) were co-localized in vascular endothelial cells and macrophages in CNV. (Pro)renin receptor blockade suppressed ERK activation and the production of MCP-1 and VEGF, but not ICAM-1, VEGFR-1, or VEGFR-2, in AT1-R-deficient mice with CNV and in losartan-treated microvascular endothelial cells and macrophages. These results indicate the significant contribution of RAPS to CNV pathogenesis.
(Am J Pathol 2008, 173:1911–1918; DOI: 10.2353/ajpath.2008.080457)

Although several types of organ damage are known to result from the activation of tissue renin-angiotensin system (RAS), the precise mechanism for activating tissue RAS is not fully understood. (Pro)renin receptor, a recently identified transmembrane protein consisting of 350 amino acids, interacts with prorenin to exert renin activity through the conformational change of the prorenin molecule instead of the conventional proteolysis of the prorenin prosegment achieved by processing enzymes such as cathepsin B. Since the membrane-bound (pro)renin receptor is reported to exist in the major organs but not in the circulation,[1] the nonproteolytic activation of prorenin is hypothesized to play a critical role in the activation of tissue, but not circulatory, RAS. In addition, prorenin binding to its receptor is shown to cause RAS-independent signal transduction via phosphorylation of extracellular signal-regulated kinase (ERK) 1/2 in cells bearing (pro)renin receptor.[1–4] Thus, we proposed the nomenclature "receptor-associated prorenin system (RAPS)" for the dual activation of tissue RAS and RAS-independent signaling pathway. In streptozo-

Supported by Grant-in-Aid for Scientific Research of Japanese Ministry of Education, Culture, Sports, Science and Technology (No. 18791296 to S.S.).

Accepted for publication August 28, 2008.

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tocin-induced diabetes, blockade of prorenin interaction with its receptor led to complete suppression of proteinuria, glomerulosclerosis and renal production of angiotensin I and II without affecting circulatory RAS, indicating a critical contribution of RAPS to the pathogenesis of diabetic nephropathy.[3,5–7]

Age-related macular degeneration (AMD) is the most common cause of blindness in developed countries. AMD is complicated by choroidal neovascularization (CNV), leading to severe vision loss due to hemorrhage and exudation from the immature new vessels.[8,9] Epidemiological risk factors for AMD were reported to include hypertension,[10] dyslipidemia,[10] and atherosclerosis,[11] all of which are related to the metabolic syndrome. Recently, angiotensin II type 1 receptor (AT1-R) signaling has been shown to play a significant role in various pathological processes complicating the metabolic syndrome such as angiogenesis and inflammation.[12–15] CNV has proven to be an inflammatory disorder depending on intercellular adhesion molecule (ICAM)-1,[16] monocyte chemotactic protein (MCP)-1[17] and vascular endothelial growth factor (VEGF).[18] We have recently shown that AT1-R-mediated up-regulation of these inflammatory and angiogenic molecules is required for the development of CNV[19]; however, the role of (pro)renin receptor as a trigger to activate tissue RAS in CNV has not been defined. Although we have further shown that tissue RAS promoting retinal inflammation[20] and neovascularization[21] is activated by nonproteolytic activation of prorenin, it has not been determined whether (pro)renin receptor-mediated intracellular signaling, the other pathway of RAPS, is pathogenic in the eye.

We therefore hypothesize that prorenin binding to its receptor promotes CNV by dually activating tissue RAS and RAS-independent ERK pathway via the receptor. In the present paper, we report the first evidence of significant relationship between RAPS and CNV together with underlying molecular and cellular mechanisms related to inflammation.

Materials and Methods

Animals

Male C57BL/6J mice (CLEA, Tokyo, Japan) at the age of 6 to 9 weeks, age- and sex-matched AT1-R-deficient mice[22] (based on the C57BL/6J strain and donated by Tanabe Seiyaku Co., Ltd., Osaka, Japan), angiotensinogen (AGT)-deficient mice[23] (based on the C57BL/6J strain and purchased from YS Institute Inc, Tochigi, Japan) and Long-Evans rats (SLC, Shizuoka, Japan) were used. All animal experiments were conducted in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Preparation of (Pro)renin Receptor Blocker

To cover the handle region (positions 11–15) of the prorenin molecule, which is the binding site of (pro)renin receptor,[24] we designed a decoy peptide, NH_2-IPLKK-MPS-COOH (positions 11 to 18), as murine (pro)renin receptor blocker (PRRB) and purified it by high performance liquid chromatography, as described previously.[3] The specific inhibitory action of PRRB against RAPS was confirmed in our recent *in vivo* data.[3,5] As a negative control for PRRB, we also prepared a control peptide (CP), NH_2-MTRLSAE-COOH (positions 30 to 36) with an amino acid sequence outside the handle region.

Induction of Laser-Induced CNV

Laser-induced CNV is widely used as an animal model for neovascular AMD and reflects the pathogenesis of CNV-related inflammation seen in AMD. In this model, new vessels from the choroid invade the subretinal space after photocoagulation. Laser photocoagulation was performed around the optic nerve with the wavelength of 532 nm, the power of 200 mW, the duration of 100 ms and the spot size of 75 μm for mice or 100 μm for rats using a slit lamp delivery system (Novus Spectra; Lumenis, Tokyo, Japan), as described previously.[17]

Treatment with PRRB, CP, and Losartan

Mice received intraperitoneal injections of vehicle (PBS), CP (1.0 mg/kg), PRRB (1.0 mg/kg), or losartan (2, 20, or 50 mg/kg; Cayman Chemical, Ann Arbor, MI) 1 day before photocoagulation and the treatments were continued daily until the end of the study. The present dose of PRRB is equivalent to that applied to significantly reduce retinal neovascularization in mice.[21] As for losartan, the dose of 20 mg/kg was the most potent in inhibiting CNV (data not shown) and used as the maximal-effect dose in the present data.

Quantification of Laser-Induced CNV

One week after laser injury, eyes were enucleated and fixed with 4% paraformaldehyde. Eye cups obtained by removing anterior segments were incubated with 0.5% fluorescein-isothiocyanate-isolectin B4 (Vector Laboratories, Burlingame, CA). CNV was visualized with blue argon laser on a confocal microscope (FV1000; Olympus, Tokyo, Japan). Horizontal optical sections of CNV were obtained at every 1-μm step from the surface to the deepest focal plane. The area of CNV-related fluorescence was measured by National Institutes of Health ImageJ (Bethesda, MD). The summation of the whole fluorescent area was used as the volume of CNV, as described previously.[17]

Quantification of Infiltrating Macrophages

Three days after laser injury, whole-mount retinal pigment epithelium (RPE)-choroid complex was incubated with a goat polyclonal antibody against platelet-endothelial cell adhesion molecule-1 (PECAM-1/CD31) and a rat poly-

clonal antibody against F4/80 (Serotec, Oxford, UK). Alexa 488- and Alexa 546-tagged secondary antibodies (Molecular Probes, Eugene, OR) were then applied. PECAM-1-stained area of CNV and F4/80-positive macrophages were evaluated, and the volume-adjusted number of macrophages was calculated.

Quantitative Reverse Transcription-Polymerase Chain Reaction Analyses

We isolated total RNA from the RPE-choroid and performed quantitative reverse transcription (RT)-PCR with an ABI Prism 7700 HT Detection System (Applied Biosystems, Foster City, CA); and probes and primers for the rat genes that encode prorenin, (pro)renin receptor, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH), as described previously.[5,7,25]

Western Blot Analyses

Three days after laser injury, the RPE-choroid complex was carefully isolated and placed into the lysis buffer. After blocking nonspecific binding with 5% skim milk, polyvinylidene fluoride membranes were incubated with a goat polyclonal antibody against angiotensin II (Santa Cruz Biotechnology, Santa Cruz, CA) or a mouse monoclonal antibody against phosphorylated ERK1/2 (Cell Signaling Technology, Beverly, MA), total ERK1/2 (Cell Signaling Technology) or α-tubulin (Sigma, St. Louis, MO). Membranes were then incubated with biotin-conjugated secondary antibody (Jackson Immuno Research Laboratories, West Grove, PA) followed by avidin-biotin complex (Vectastain ABC Elite Kit; Vector Laboratories). Finally the signals were detected through enhanced chemiluminescence (ECL Blotting Analysis System; GE Health Care).

Enzyme-Linked Immunosorbent Assay

Protein extracts were obtained from the RPE-choroid complex 3 days after photocoagulation. The protein levels of ICAM-1, MCP-1, VEGF, VEGF receptor (VEGFR)-1 and VEGFR-2 were determined with the enzyme-linked immunosorbent assay (ELISA; R&D Systems).

Immunohistochemistry

Three days after photocoagulation, rat eye cups were incubated with a goat anti-rat (pro)renin receptor antibody together with fluorescein-isothiocyanate-isolectin B4 (Vector Laboratories) or a rabbit anti-EMR 1 (corresponding to murine F4/80) antibody (Santa Cruz Biotechnology). The anti-(pro)renin receptor antibody was raised by using the previously established COS-7 cells producing rat (pro)renin receptor protein.[5] Alexa 488- and Alexa 546-tagged secondary antibodies (Molecular Probes) were then applied. For immunohistochemical staining of phosphorylated ERK1/2, a goat polyclonal antibody against rat phosphorylated ERK1/2 (Santa Cruz Biotechnology) was applied as the primary antibody.

In Vitro *Assays*

Murine brain-derived capillary endothelial cells (b-End3) were cultured with Dulbecco's modified Eagle's medium (Sigma). After 6-hour incubation with tumor necrosis factor(TNF)-α (Sigma, 1 ng/ml) plus losartan (10 μmol/L), or TNF-α plus PRRB (100 μmol/L) and losartan (10 μmol/L), the supernatant and cell lysate were collected for protein analyses, and then the concentration of MCP-1 in the supernatant and ICAM-1, VEGFR-1 and VEGFR-2 in the cell lysate were measured by the ELISA kits (R&D Systems). Murine macrophages (RAW264.7) were treated with Dulbecco's modified Eagle's medium containing lipopolysaccharide (100 ng/ml) plus losartan (10 μmol/L) or lipopolysaccharide plus PRRB (100 μmol/L) and losartan (10 μmol/L). After 6-hour incubation, supernatant was processed for ELISA analyses for VEGF (R&D Systems).

Statistical Analyses

All results were expressed as mean ± SEM. The values were processed for statistical analyses (Mann-Whitney test). Differences were considered statistically significant when the P values were <0.05.

Results

Prorenin Expression Is Up-Regulated in CNV and Blockade of Prorenin Binding to Its Receptor Inhibits CNV

To elucidate the involvement of prorenin and (pro)renin receptor in the pathogenesis of CNV, we first performed quantitative RT-PCR analyses for prorenin and (pro)renin receptor in the RPE-choroid complex. Prorenin mRNA levels (ratio to GAPDH mRNA) were up-regulated ($P < 0.05$) in the RPE-choroid of laser-treated rats, compared with age-matched normal controls (Figure 1A). In contrast, mRNA levels of (pro)renin receptor showed no significant difference ($P > 0.05$) between laser-treated rats and normal controls (Figure 1B). The CNV volume was measured to evaluate the effects of PRRB on the development of CNV. CP treatment did not significantly ($P > 0.05$) change the CNV volume (501,810 ± 66,820 μm^3), compared with vehicle-treated animals (504,411 ± 49,791 μm^3) (Figure 1, C and D). However, PRRB-treated mice showed a significant ($P < 0.01$) decrease in the CNV volume (208,355 ± 29,388 μm^3), compared with vehicle-treated mice (504,411 ± 49,791 μm^3) or CP-treated mice (501,810 ± 66,820 μm^3) (Figure 1, C and D).

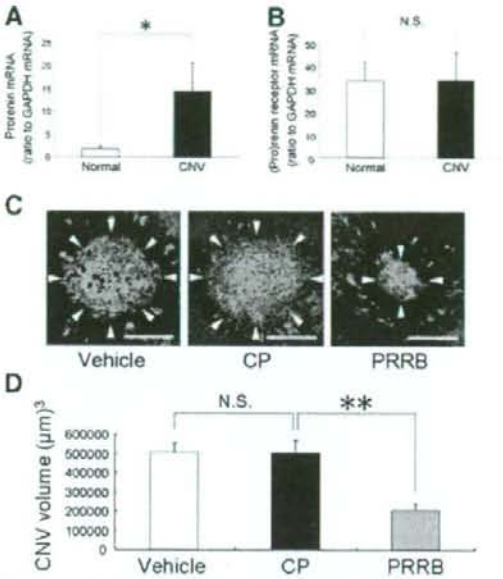

Figure 1. Prorenin expression is up-regulated in CNV and PRRB inhibits CNV. **A:** Up-regulation of prorenin mRNA levels in the RPE-choroid complex by inducing CNV (n = 6 to 7). **B:** (Pro)renin receptor mRNA levels were unchanged following CNV induction (n = 6 to 7). **C:** Flatmounted choroids from vehicle-, CP-, and PRRB-treated mice. **D:** The graph shows the CNV volume. PRRB application led to significant suppression of CNV, compared with vehicle or CP treatment. **Arrowheads** in (**C**) indicate lectin-stained CNV tissues (n = 33 to 37. $^{**}P < 0.01$, $^{*}P < 0.05$). Scale bars = 100 μm.

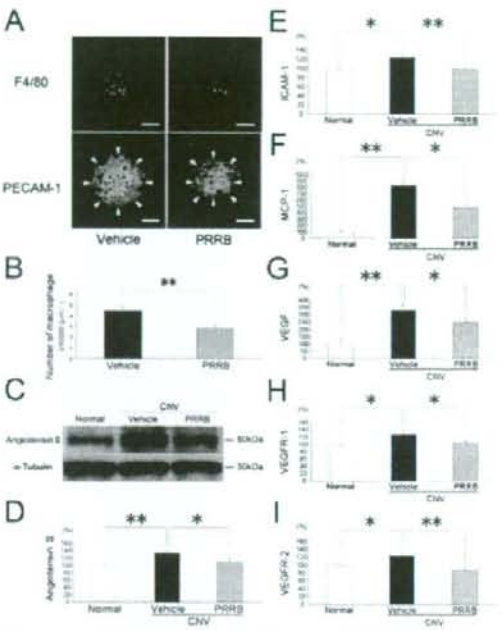

Figure 2. PRRB inhibits macrophage infiltration and RPE-choroidal production of angiotensin II and CNV-related inflammatory molecules. F4/80-positive macrophages (**A, top**) and PECAM-1-stained CNV (**arrowheads** in **A, bottom**) were evaluated and the volume-adjusted number of macrophages is shown in the graph (**B**). PRRB led to significant suppression of macrophage infiltration into CNV (n = 20). Scale bars = 50 μm. **C–D:** RPE-choroidal generation of angiotensin II was significantly reduced by treatment with PRRB. PRRB significantly suppressed the protein levels of ICAM-1 (**E**), MCP-1 (**F**), VEGF (**G**), VEGFR-1 (**H**), and VEGFR-2 (**I**) in the RPE-choroid (n = 4 to 9. $^{**}P < 0.01$, $^{*}P < 0.05$).

PRRB Inhibits CNV-Associated Macrophage Infiltration, Angiotensin II Generation and the Expression of Angiogenic and Inflammatory Molecules

As the cellular mechanism in the pathogenesis of CNV, infiltration of inflammatory cells including macrophages plays a critical role. We compared the number of macrophages, which was adjusted by the area of CNV, between mice treated with PRRB versus vehicle. PRRB-treated mice showed a significant decrease in the number of F4/80-positive macrophages, compared with vehicle-treated animals ($P < 0.01$, 2.84 ± 0.31/10,000 μm^3 versus 4.39 ± 0.41/10,000 μm^3) (Figure 2, A and B). To investigate the effect of PRRB on angiotensin II generation during CNV, we analyzed angiotensin II levels in the RPE-choroid complex. RPE-choroidal levels of angiotensin II were higher ($P < 0.01$) in animals with CNV than in age-matched normal controls (Figure 2, C and D). Application of PRRB significantly suppressed protein levels of angiotensin II in the RPE-choroid ($P < 0.05$) (Figure 2, C and D). To determine whether PRRB affects angiogenic and inflammatory molecules related to the pathogenesis of CNV, protein levels of ICAM-1, MCP-1, VEGF, VEGFR-1, and VEGFR-2 in the RPE-choroid complex were analyzed by ELISA. RPE-choroidal protein levels of ICAM-1, MCP-1, VEGF, VEGFR-1, and VEGFR-2 were up-regulated by inducing CNV. Systemic administration of PRRB significantly suppressed protein levels of ICAM-1 ($P < 0.01$), MCP-1 ($P < 0.05$), VEGF ($P < 0.05$), VEGFR-1 ($P < 0.05$), and VEGFR-2 ($P < 0.01$) (Figure 2, E–I).

RAS-Independent (Pro)renin Receptor-Mediated Intracellular Signaling Contributes to CNV Development and Macrophage Infiltration

To clarify the role of RAS-independent intracellular signaling via (pro)renin receptor, we used mice in which RAS was deactivated by pharmacological blockade of AT1-R with losartan or genetic ablation of AT1-R or AGT. Compared with vehicle-treated wild-type animals (504,411 ± 49,791 μm^3), these mice exhibited a significant ($P < 0.01$) reduction of CNV (305,244 ± 37,883 μm^3 for losartan treatment, 314,120 ± 34,023 μm^3 for AT1-R deficiency, 278,811 ± 30,462 μm^3 for AGT deficiency), which was further suppressed by additional PRRB application (212,643 ± 38,779 μm^3 for losartan treatment [$P < 0.01$], 198,206 ± 15,536 μm^3 for AT1-R deficiency [$P < 0.05$], 163,457 ± 23,767 μm^3 for AGT deficiency [$P < 0.01$]) (Figure 3, A and B). We further examined the role of RAS-independent intracellular signaling via (pro)renin receptor in macrophage infiltration into CNV. Compared with wild-type

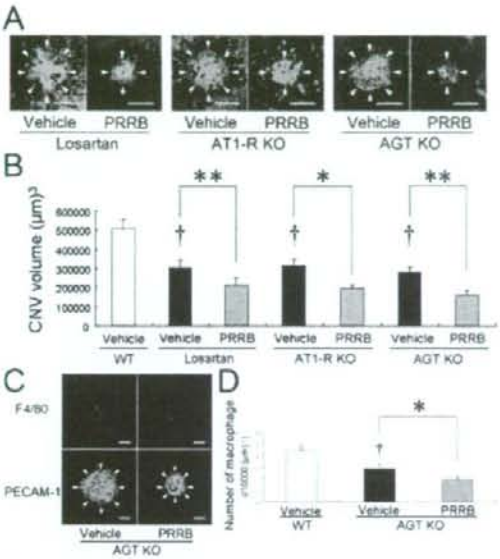

Figure 3. RAS-independent (pro)renin receptor-mediated intracellular signaling contributes to CNV development and macrophage infiltration. The graph shows the choroidal flatmounts (**A**) and the CNV volume (**B**). PRRB treatment further induced a significant decrease in the CNV volume in losartan-treated, AT1-R-deficient and AGT-deficient mice. **Arrowheads** in (**A**) indicate lectin-stained CNV tissues (n = 23 to 40). Scale bars = 100 μm. F4/80-positive macrophages (**C**, **top**) and PECAM-1-stained CNV (**arrowheads** in **C, bottom**) were evaluated in AGT-deficient mice, and the volume-adjusted number of macrophages is shown in the graph (**D**). PRRB further caused significant suppression of macrophage infiltration (n = 14 to 17. †P < 0.01, **P < 0.01, *P < 0.05). Scale bars = 50 μm.

animals (4.39 ± 0.41/10,000 μm^3), AGT-deficient mice exhibited a significant ($P < 0.05$) decrease in the number of F4/80-positive macrophages (2.85 ± 0.38/10,000 μm^3), which was further attenuated by PRRB treatment (1.89 ± 0.31/10,000 μm^3, $P < 0.05$) (Figure 3, C and D).

(Pro)renin Receptor and Phosphorylated ERK1/2 Are Present in CNV-Associated Macrophages and Vascular Endothelial Cells and PRRB Inhibits ERK1/2 Activation Following CNV Induction

To examine the expression and tissue localization of (pro)renin receptor and phosphorylated ERK1/2, a known downstream pathway via (pro)renin receptor, rat CNV tissues were immunostained with antibodies against (pro)renin receptor and phosphorylated ERK1/2 together with isolectin B4 or an anti-EMR 1 antibody, markers for vascular endothelial cells or macrophages, respectively. The immunohistochemical analyses of rat CNV tissues showed (pro)renin receptor immunoreactivity in isolectin B4-positive endothelial cells (Figure 4A) and EMR1-positive macrophages (Figure 4B), both of which were positive for phosphorylated ERK1/2. We examined the effect of PRRB on the activation of ERK1/2 in the RPE-choroid excised from mice with CNV. The relative ratio of phosphorylated to total ERK1/2 in the RPE-choroid, increased by inducing CNV, was significantly ($P < 0.01$) suppressed by PRRB treatment (Figure 4, C and D), while no significant ($P > 0.05$) difference was detected in total ERK1/2 protein levels.

RAS-Independent (Pro)renin Receptor-Mediated Intracellular Signaling Contributes to CNV-Related ERK1/2 Activation and the Expression of Inflammatory and Angiogenic Molecules In Vivo *and* In Vitro

To further determine whether RAS-independent intracellular signaling via (pro)renin receptor contributes to the activation of ERK1/2 in CNV, phosphorylated ERK1/2 was examined in the RPE-choroid from AT1-R-deficient mice

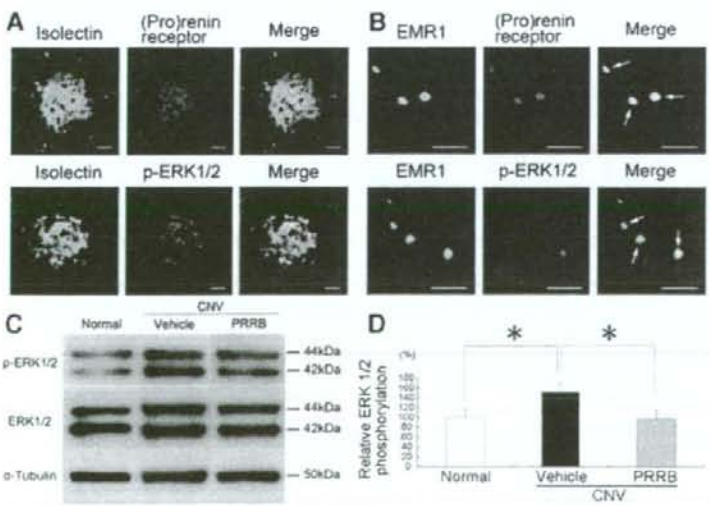

Figure 4. (Pro)renin receptor and phosphorylated ERK1/2 are present in CNV-associated macrophages and vascular endothelial cells, and PRRB inhibits ERK1/2 activation following CNV induction. The immunohistochemical analyses show (pro)renin receptor and phosphorylated ERK1/2 in isolectin B4-positive endothelial cells (**A**) and EMR1-positive macrophages (**B**). **A:** Green fluorescence from isolectin B4 (left) and red fluorescence from an anti-(pro)renin receptor (middle, top) or an anti-phosphorylated ERK1/2 antibody (middle, bottom) identified the isolectin B4-positive endothelial cells as having (pro)renin receptor or phosphorylated ERK1/2, respectively, when the images were superimposed (right). Scale bars = 50 μm. **B:** Green fluorescence from an anti-EMR1 antibody (left) and red fluorescence from an anti-(pro)renin receptor (middle, top) or an anti-phosphorylated ERK1/2 antibody (middle, bottom) identified the EMR1-positive macrophages as having (pro)renin receptor or phosphorylated ERK1/2, respectively, when the images were superimposed (**arrows**, right). Scale bars = 50 μm. **C-D:** Western blotting for phosphorylated and total levels of ERK1/2 in the RPE-choroid after photocoagulation. Relative phosphorylation of ERK1/2 in the RPE-choroid complex, increased by inducing CNV, was suppressed by PRRB treatment (n = 11 to 13. *P < 0.05).

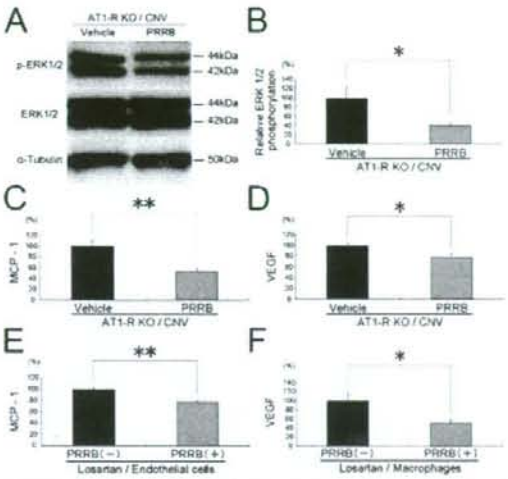

Figure 5. RAS-independent (pro)renin receptor-mediated intracellular signaling contributes to CNV-related activation of ERK1/2 and expression of inflammatory molecules *in vivo* and *in vitro*. **A,B:** Western blotting for phosphorylated and total levels of ERK1/2 in AT1-R-deficient mice with CNV. PRRB suppressed relative phosphorylation of ERK1/2. **C–F:** *In vivo* (**C,D**) and *in vitro* (**E,F**) effects by blocking intracellular signaling via (pro)renin receptor on protein levels of CNV-related molecules. MCP-1 (**C,E**), and VEGF (**D,F**) levels were significantly suppressed with PRRB ($n = 9$ to 11). $^{**}P < 0.01$, $^{*}P < 0.05$.

with CNV. PRRB treatment significantly ($P < 0.05$) suppressed phosphorylated but not total ERK1/2 (Figure 5A). Relative phosphorylation of ERK1/2 was significantly ($P < 0.05$) suppressed by PRRB application, compared with vehicle-treated AT1-R-deficient mice (Figure 5B). To examine whether RAS-independent intracellular signaling via (pro)renin receptor contributes to the up-regulation of the inflammatory and angiogenic molecules responsible for CNV (Figure 2, E–I), CNV was induced in AT1-R-deficient mice to measure protein levels of ICAM-1, MCP-1, VEGF, VEGFR-1, and VEGFR-2 in the RPE-choroid. PRRB application to AT1-R-deficient mice with CNV led to significant suppression of MCP-1 ($P < 0.01$) (Figure 5C) and VEGF ($P < 0.05$) (Figure 5D), but not ICAM-1, VEGFR-1, or VEGFR-2 ($P > 0.05$, data not shown), compared with vehicle treatment to AT1-R-deficient mice with CNV. To confirm the *in vivo* molecular mechanisms mediated by RAS-independent pathway via (pro)renin receptor (Figure 5, C and D), we further performed *in vitro* analyses, using murine cell lines including b-End3 microvascular endothelial cells (Figure 5E) and RAW264.7 macrophages (Figure 5F), both of which were treated by losartan. We analyzed protein levels of ICAM-1, MCP-1, VEGFR-1, and VEGFR-2 inTNF-α-stimulated endothelial cells and of VEGF in lipopolysaccharide-stimulated macrophages. In losartan-treated endothelial cells, protein levels of MCP-1 were significantly ($P < 0.01$) suppressed by the treatment with PRRB (Figure 5E). In contrast, no significant difference was detected in protein levels of ICAM-1, VEGFR-1, or VEGFR-2 following PRRB treatment (data not shown). In losartan-treated macrophages, VEGF protein levels were significantly ($P < 0.05$) suppressed by the treatment with PRRB (Figure 5F).

Discussion

The present study reveals, for the first time to our knowledge, several important findings concerning the role of (pro)renin receptor in CNV generation. First, CNV development was associated with up-regulation of prorenin expression in the RPE-choroid complex and PRRB treatment showed a significant decrease in the CNV volume, indicating that prorenin binding with its receptor contributes to CNV (Figure 1). Second, the cellular and molecular mechanisms in the PRRB-induced suppression of CNV included the inhibitory effects on macrophage infiltration into CNV, angiotensin II generation and the up-regulated expression of inflammatory and angiogenic molecules such as ICAM-1, MCP-1, VEGF, VEGFR-1, and VEGFR-2, all of which were downstream molecules of angiotensin II[19] (Figure 2). Although the detailed molecular and cellular mechanisms underlying CNV are not fully clarified, ICAM-1 expression[16,26] and macrophage infiltration[18,19] were observed in CNV tissues from human eyes with AMD and the laser-induced murine model, suggesting the close association of inflammation with the progression of CNV. Pharmacological depletion of macrophages[17,27] or genetic ablation of CCR2,[28] a receptor for MCP-1, was shown to result in the reduction of CNV, suggesting that macrophages, recruited by MCP-1 released from RPE or vascular endothelial cells, facilitate the development of CNV by producing VEGF. In concert with our previous data,[19] the currently observed PRRB-induced suppression of CNV indicates that tissue RAS is activated during CNV by (pro)renin receptor-mediated nonproteolytic activation of prorenin, leading to AT1-R signaling-mediated up-regulation of CNV-related inflammatory molecules.

The present study further revealed the role of RAS-independent (pro)renin receptor signaling in CNV generation (Figure 3). This study is the first to show the involvement of RAPS, (pro)renin receptor-mediated signal transduction and tissue RAS activation, in *in vivo* angiogenesis as well as in ocular pathogenesis. We recently showed the contribution of (pro)renin receptor signaling to diabetic nephropathy using AT1-R-deficient mice.[3] AT1-R-deficient mice with streptozotocin-induced diabetes exhibited reduced proteinuria and glomerulosclerosis in the early phase as compared to wild-type diabetes, indicating a significant role of tissue RAS in diabetic nephropathy. Surprisingly, these renal events in AT1-R-deficient diabetes later progressed to the equivalent levels seen in wild-type diabetic mice. The glomerulosclerosis observed in AT1-R-deficient diabetic mice was associated with ERK activation, which was completely blocked together with the phenotype by sustained application of PRRB, suggesting that the redundant pathways of RAPS were involved in the pathogenesis of diabetic nephropathy. In the present study, we administered PRRB to CNV mice receiving the AT1-R blocker losartan or genetically deficient in AT1-R or AGT, and these three

different methods for deactivating RAS confirmed the significant role of intracellular signaling via (pro)renin receptor in the development of CNV (Figure 3, A and B). The data are compatible with the result of parallel experiments showing that macrophage infiltration into CNV was also suppressed by PRRB in AGT-deficient mice (Figure 3, C and D). Importantly, (pro)renin receptor was present in macrophages and vascular endothelial cells, the major cellular components in CNV tissues, together with the activation of ERK 1/2, a known intracellular signaling via (pro)renin receptor (Figure 4, A and B). Indeed, *in vivo* quantitative analyses for ERK 1/2 revealed PRRB-induced suppression of the phosphorylation of ERK1/2, which was enhanced following CNV induction (Figure 4, C and D). However, because AT1-R has been shown to mediate CNV generation,[19] the data (Figure 4) could not exclude the possibility of ERK activation via AT1-R as well as (pro)renin receptor, leading us to further perform the following *in vivo* and *in vitro* experiments (Figure 5) to confirm that (pro)renin receptor signaling per se caused the activation of ERK and up-regulation of inflammatory molecules responsible for CNV formation. Importantly, PRRB application to AT1-R-deficient mice with CNV led to significant suppression of ERK activation (Figure 5, A and B). Out of the CNV-related molecules, the expression of which was inhibited by PRRB (Figure 2, E–I), our *in vivo* (Figure 5, C and D) and *in vitro* (Figure 5, E and F) data showed that MCP-1 and VEGF were also regulated by (pro)renin receptor signaling per se. These new findings (Figures 3–5) clarified molecular and cellular mechanisms mediated by RAS-independent intracellular signaling via (pro)renin receptor in CNV generation. In addition to our recent reports[3,5,6,25] showing that RAPS contributes to glomerulosclerosis in the kidney and fibrosis in the heart, the present data are the first to show the association of RAPS with inflammation and angiogenesis in the eye.

Although hypertension is a known risk factor predisposing to AMD, there are indeed a large number of normotensive patients with CNV who have the potential risk of hypotension caused by the use of antihypertensive agents including AT1-R blockers and angiotensin-converting enzyme inhibitors. In contrast, since (pro)renin receptor is present in the major organs but not in the circulation, PRRB does not affect circulatory RAS or systemic blood pressure.[5,7] Interestingly, PRRB treatment to CNV was shown to cause not only tissue RAS deactivation but also additional suppression of (pro)renin receptor signaling-mediated expression of MCP-1 and VEGF, the major pathogenic factors responsible for CNV formation. Collectively, inhibition of RAPS with PRRB may prove more useful as a novel therapeutic strategy for CNV than RAS suppression with conventional AT1-R blockers or angiotensin-converting enzyme inhibitors.

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ORIGINAL ARTICLE

Angiotensin II Reduces Mitochondrial Content in Skeletal Muscle and Affects Glycemic Control

Masanori Mitsuishi, Kazutoshi Miyashita, Ayako Muraki, and Hiroshi Itoh

OBJECTIVE—Blockade of angiotensin (Ang) II has been shown to prevent new-onset type 2 diabetes. We focused on the effects of AngII on muscle mitochondria, especially on their biogenesis, as an underlining mechanism of type 2 diabetes.

RESEARCH DESIGN AND METHODS—C2C12 cells and C57bl/6 mice were used to examine roles for AngII in the regulation of muscle mitochondria and to explore whether the effect was mediated by type 1 AngII receptor (AT1R) or type 2 receptor (AT2R).

RESULTS—C2C12 cells treated with 10^{-8}–10^{-6} mol/l AngII reduced the mitochondrial content associated with downregulation of the genes involved in mitochondrial biogenesis. The action of AngII was diminished by blockade of AT2R but not AT1R, whereas overexpression of AT2R augmented the effect. AngII increased mitochondrial ROS and decreased mitochondrial membrane potential, and these effects of AngII were significantly suppressed by blockade of either AT1R or AT2R. Chronic AngII infusion in mice also reduced muscle mitochondrial content in association with increased intramuscular triglyceride and deteriorated glycemic control. The AngII-induced reduction in muscle mitochondria in mice was partially, but significantly, reversed by blockade of either AT1R or AT2R, associated with increased fat oxidation, decreased muscle triglyceride, and improved glucose tolerance. Genes involved in mitochondrial biogenesis were decreased via AT2R but not AT1R under these in vivo conditions.

CONCLUSIONS—Taken together, these findings imply the novel roles for AngII in the regulation of muscle mitochondria and lipid metabolism. AngII reduces mitochondrial content possibly through AT1R-dependent augmentation of their degradation and AT2R-dependent direct suppression of their biogenesis. ***Diabetes* 58:710–717, 2009**

Recent studies have shown that mitochondrial content and function are significantly reduced in the skeletal muscle of patients with type 2 diabetes (1,2). Percutaneous biopsy of vastus lateralis muscle has revealed that subsarcolemmal mitochondria, which are believed to be crucial for glucose transport and fatty acid oxidation, were decreased in type 2 diabetic patients, compared with body weight–matched nondiabetic patients (2). Moreover, reduced mitochondrial content and function in muscle have been also observed in pre-diabetic subjects with a family history of type 2 diabetes (3). Recent microarray analyses have revealed that expression of genes involved in mitochondrial biogenesis and oxidative phosphorylation is coordinately decreased in the skeletal muscle of patients with type 2 diabetes (4,5), for example, peroxisome proliferator–activator receptor γ co-activator 1α (PGC1α), a representative transcriptional cofactor for the determination of mitochondrial content and function, and nuclear respiratory factor 1 (NRF1). Furthermore, it has been demonstrated that mitochondrial function evaluated by the rate of ATP synthesis is diminished in the skeletal muscle of diabetic patients and family history–positive pre-diabetic patients (6,7). These findings imply that reduced mitochondrial content in the skeletal muscle is likely to contribute to the development of insulin resistance and type 2 diabetes (8,9).

Angiotensin (Ang) II, which is composed of eight amino acids, is one of the most important molecules in the renin-angiotensin system. It provokes sodium reabsorption, vasoconstriction, and elevation of blood pressure and also plays a critical role in the physiological regulation of electrolytes and water homeostasis. However, an excess of AngII may lead to tissue damage, such as atherosclerosis, cardiomegaly, and heart and renal failure. AngII is known to exert its biological effects via two functional receptors, type 1 and type 2 angiotensin II receptors (AT1R and AT2R, respectively). To date, most of the known cardiovascular effects of AngII are believed to be attributable to AT1R (10). Recent large-scale clinical trials, including HOPE (Heart Outcomes Prevention Evaluation), LIFE (Losartan Intervention for Endpoint), CHARM (Candesartan in Heart Failure—Assessment of Mortality and Morbidity), and VALUE (Valsartan Antihypertensive Long-term Use Evaluation), have demonstrated that ACE inhibitors or angiotensin II receptor blockers (ARB) prevent new onset of type 2 diabetes via their ability to attenuate AngII signaling (11). As a result of these findings, the significance of AngII for the development of insulin resistance and regulation of energy metabolism has been attracting considerable attention (12–14).

It has been demonstrated that AngII provokes insulin resistance in the skeletal muscle through multiple mechanisms. AngII treatment was found to augment reactive oxygen species (ROS) production by stimulating NADPH oxidase in cultured skeletal muscle cells, thus activating multiple redox-sensitive signaling including nuclear factor-κB (NF-κB) and increasing proinflammatory cytokines such as tumor necrosis factor-α (TNF-α), which impair insulin action (15,16). Inhibition of insulin signaling by AngII at multiple levels including insulin receptor, insulin receptor substrate 1, and phosphatidylinositol 3-kinase has been demonstrated in aortic smooth muscle cells (17). Stimulation of primary cultured human preadipocytes by AngII was found to inhibit differentiation to mature adipocytes, suggesting that the effect of AngII on adipose tissue

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Received 15 July 2008 and accepted 2 December 2008.
Published ahead of print at http://diabetes.diabetesjournals.org on 15 December 2008. DOI: 10.2337/db08-0949.