Pathological role of receptor-associated prorenin system and tumor necrosis factor-α for the development of proliferative retinopathy in type II diabetes mellitus

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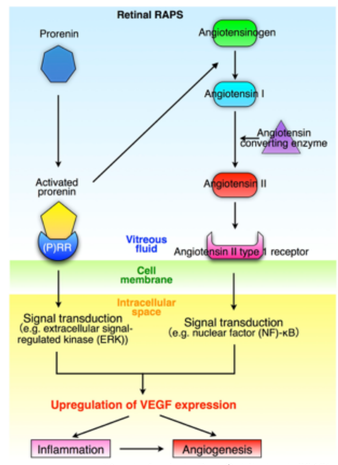


(Kanda A, et al., Br J Ophthalmol. 2013)

Background:

The renin-angiotensin system (RAS) is an important controller of systemic blood pressure, called circulatory RAS. It also plays distinct roles in inflammation and pathological vascular conditions in various organs including the eye [1,2]. This type of RAS is referred to as the tissue RAS. Binding of prorenin to (pro)renin receptor [(P)RR] triggers dual activation of RAS and RAS-independent signaling pathways, called as the receptor-associated prorenin system (RAPS), involved in inflammation and angiogenesis which are the pathogenesis of proliferative diabetic retinopathy (PDR) [2,3]. In this study, we explored correlations between protein levels of RAPS components including (P)RR, prorenin, and inflammatory and angiogenic molecules in the plasma obtained from patients with PDR.

Materials & Methods:

Human Plasma Samples
Plasma samples were collected from 20 patients with PDR and 20 patients with non-DM such as idiopathic epiretinal membrane (ERM) or macular hole (MH). All the plasma samples were collected preoperatively. Characteristics of the subjects enrolled in this study are shown in Table 1.

Enzyme-linked immunosorbent assays (ELISA)
The plasma protein levels of prorenin, soluble (P)RR [s(P)RR] and LRG1 in the plasma were determined with ELISA kits. Activated prorenin corresponds to prorenin bound with s(P)RR, and the dissociation constant (KD) for the binding of prorenin with s(P)RR was calculated in a previous report [3] as follows: KD (4.0 nmol/l) = [prorenin] x [s(P)RR] / [activated prorenin].
The plasma levels of inflammatory and angiogenic molecules [TNF-α, CFD, VAP-1, MCP-1/CCL2, IFN-γ, IL-6, VCAM-1, ICAM-1, FGF-1, FGF-2, PlGF, endostatin, VEGF and Insulin] were determined by magnetic multiplex bead-based quantitative immunoassay using the MAGPIX and Luminex assay kit.

Cell Culture and Chemicals
Human retinal microvascular endothelial cells (HRMECs) were cultured in CS-C complete medium containing 5 mM glucose, which corresponds to blood glucose concentration under normal conditions. For high-glucose stimulation, HRMECs were cultured in CS-C complete medium containing 30 mM glucose, which corresponds to blood glucose concentration under hyperglycemic conditions.

After serum starvation, HRMECs were treated with recombinant human LRG1, TNF-α, and CFD proteins and processed for analysis to detect mRNA expression levels. For the TNF-α neutralization bioassay, recombinant human TNF-α was pre-incubated with rabbit anti-TNF-α neutralizing antibody. After pre-incubation, the cells were treated and processed for analysis to detect gene expression levels. Normal rabbit IgG was used as the control for the anti-TNF-α neutralizing antibody.

To cover the handle region of the prorenin molecule, which is the binding site of (P)RR, we synthesised decoy peptides NH_2-RIFLKRMPSI-COOH as a human (P)RR blocker (PRRB), and purified them by high-pressure liquid chromatography. After the cells were serum-deprived, HRMECs were pretreated with 1 μM PRRB or 10 μM AT1R blocker losartan for 1 hour. Prorenin or angiotensin II (Ang II) was then added at final concentration of 10 nM or 1 μM, respectively. Cells were incubated and processed for analysis to detect RNA expression levels.

Real-Time Quantitative PCR (qPCR)
Total RNA isolation and reverse transcription were performed from cells using SuperPrep Cell Lysis & RT Kit for qPCR with oligo dT and random primers following the manufacturers' protocols. Real-time qPCR was performed using the GoTaq qPCR Master mix, THUNDERBIRD Probe qPCR Mix, and StepOne plus Systems.

Statistical Analysis
All results were expressed as mean ± standard error of the mean (SEM) with n-numbers as indicated. Student's t-test was used for statistical comparison between the groups. Differences between the means were considered statistically significant when the probability values were less than 0.05.

Table 1. Basal characteristics of participating patients

Patients	Non-DM	PDR	p value
n	20	20	-
Age (years)	63.35 ± 0.96	62.40 ± 1.95	0.665
Gender (%male)	50	35	0.523
RBS (mg/dl)	105.90 ± 2.95	159.40 ± 12.02	2.934E-04
HbA1c (%)	Not tested	7.12 ± 0.28	-
Duration (years)	Not applicable	12.94 ±1.89	-
HT (%)	45	55	0.752
BMI	23.60 ± 0.73	24.28 ± 0.96	0.580
SBP (mmHg)	128.50 ± 3.82	132.00 ± 4.49	0.556
DBP (mmHg)	71.70 ± 2.04	73.35 ± 3.20	0.666
sCr (mg/dl)	0.71 ± 0.03	1.00 ± 0.11	0.024
eGFR (ml/min/1.73m^2)	76.45 ± 3.49	64.02 ± 7.34	0.138
Log MAR	0.71 ± 0.09	1.28 ± 0.16	0.003
IOP (mmHg)	14.75 ± 0.79	15.00 ± 0.83	0.829
CRT (μm)	222.30 ± 63.38	349.80 ± 42.20	0.103
ARB use (%)	35	45	0.748

RBS, random blood sugar; HT, hypertension; Duration, duration of diabetes mellitus; BMI, body mass index; SBP,systolic blood pressure; DBP, diastolic blood pressure; sCr, serum creatinine; eGFR, estimated glomerular filtration rate; MAR, minimum angle of resolution; IOP, intraocular pressure; CRT, central retinal thickness; ARB, angiotensin II receptor blocker.

There were no significant differences between PDR and non-DM groups except for RBS levels, sCr levels and visual acuity.

Results:

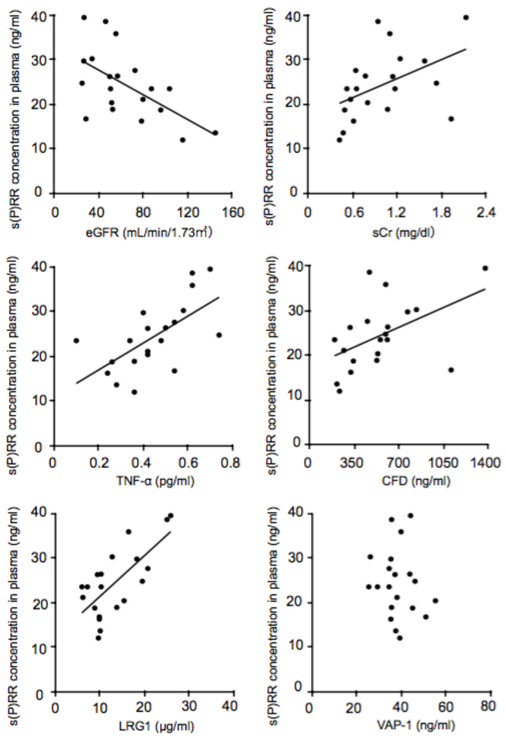
Table 2. Plasma concentrations of inflammatory and angiogenic molecules in patients with non-DM and PDR

	Non-DM	PDR	p value
s(P)RR (ng/ml)	16.60 ± 0.85	24.34 ± 1.72	3.97E-04
Prorenin (ng/ml)	0.77 ± 0.14	2.33 ± 0.40	0.001
Activated prorenin (pmol/ml)	2.48 ± 0.57	11.04 ± 2.40	0.002
LRG1 (μg/ml)	7.68 ± 0.93	13.28 ± 1.33	0.001
TNF-α (pg/ml)	0.34 ± 0.02	0.45 ± 0.04	0.015
CFD (μg/ml)	0.36 ± 0.02	0.54 ± 0.07	0.018
VAP-1 (ng/ml)	22.25 ± 1.04	38.41 ± 1.69	2.865E-09
MCP-1/CCL2 (pg/ml)	15.00 ± 2.40	15.00 ± 1.32	0.945
IFN-γ (pg/ml)	1.26 ± 0.01	1.28 ± 0.01	0.097
IL-6 (pg/ml)	0.22 ± 0.03	0.25 ± 0.04	0.455
VCAM-1 (μg/ml)	0.14 ± 0.02	0.38 ± 0.12	0.067
ICAM-1 (ng/ml)	3.00 ± 0.96	8.29 ± 4.15	0.229
FGF-1 (pg/ml)	1.99 ± 0.38	1.62 ± 0.12	0.370
FGF-2 (pg/ml)	1.20 ± 0.06	1.13 ± 0.05	0.363
PlGF (pg/ml)	0.04 ± 0.02	0.06 ± 0.02	0.419
Endostatin (ng/ml)	3.62 ± 0.32	4.31 ± 0.54	0.286
VEGF (pg/ml)	0.41 ± 0.02	0.44 ± 0.04	0.605
Insulin (pg/ml)	37.00 ± 5.00	52.00 ± 8.00	0.137

s(P)RR, soluble (pro)renin receptor; LRG, leucine-rich alpha-2-glycoprotein; TNF, tumor necrosis factor; CFD, complement factor D; VAP, vascular adhesion protein; MCP, monocyte chemoattractant protein; CCL, C-C motif chemokine; IFN, interferon; IL, interleukin; VCAM, vascular cell adhesion molecule; ICAM, intercellular adhesion molecule; FGF, fibroblast growth factors; PlGF, placental growth factor; VEGF, vascular endothelial growth factor.

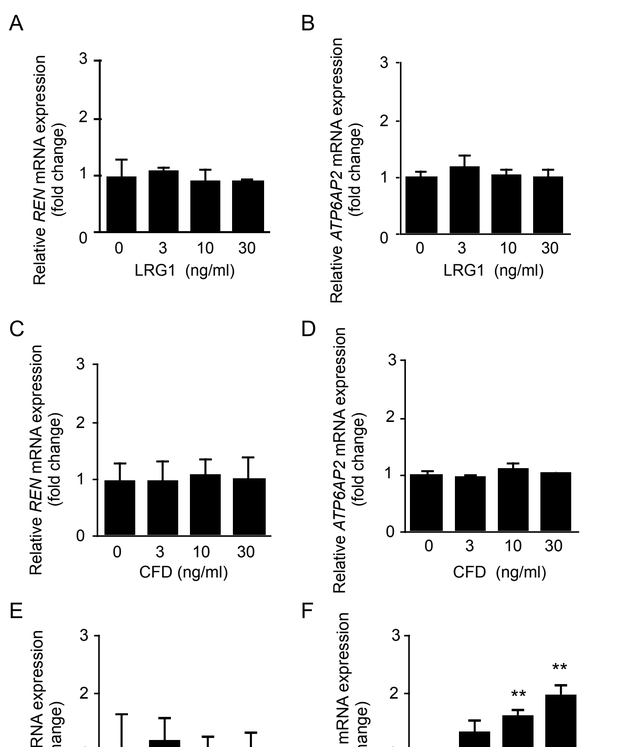
Plasma concentrations of RAPS components [s(P)RR, prorenin and activated prorenin] and inflammatory and angiogenic molecules (LRG1, TNF-α, CFD and VAP-1) in PDR were significantly higher than those of non-DM.

Figure 1. Correlation between s(P)RR and renal dysfunction parameters, and inflammatory and angiogenic molecules in PDR patients



There was correlation between s(P)RR and renal dysfunction parameters. Soluble (P)RR correlated with TNF-α, CFD and LRG1 but not VAP-1.

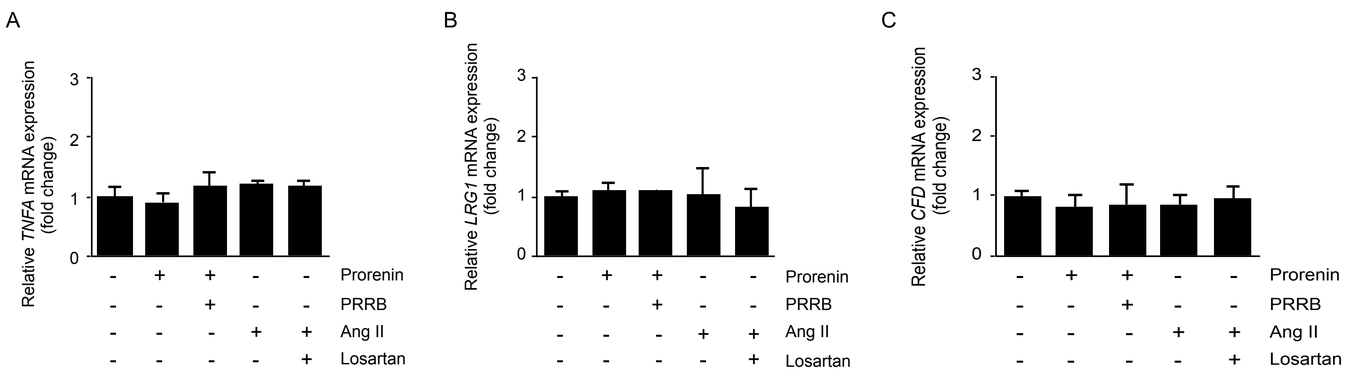
Figure 2. Prorenin/*REN* and (P)RR/*ATP6AP2* mRNA expressions in HRMECs stimulated by inflammatory and angiogenic molecules in PDR patients



(P)RR/*ATP6AP2* mRNA levels significantly increased solely after TNF-α stimulation to human retinal microvascular endothelial cells (HRMECs).

A-H: After serum starvation, HRMECs were treated with recombinant human LRG1 (A, B) CFD (C, D) or TNF-α (E, F) (0, 3, 10 and 30 ng/ml) for 24 h and processed for analysis to detect mRNA expression levels of *REN* and *ATP6AP2*. Phosphate-buffered saline (PBS) was added to the serum-free medium for the controls. n = 6, **p < 0.01. G: For TNF-α neutralization bioassay, recombinant human TNF-α (10 ng/ml) was pre-incubated with rabbit anti-TNF-α neutralizing antibody (200 ng/ml). n = 6, **p < 0.01.

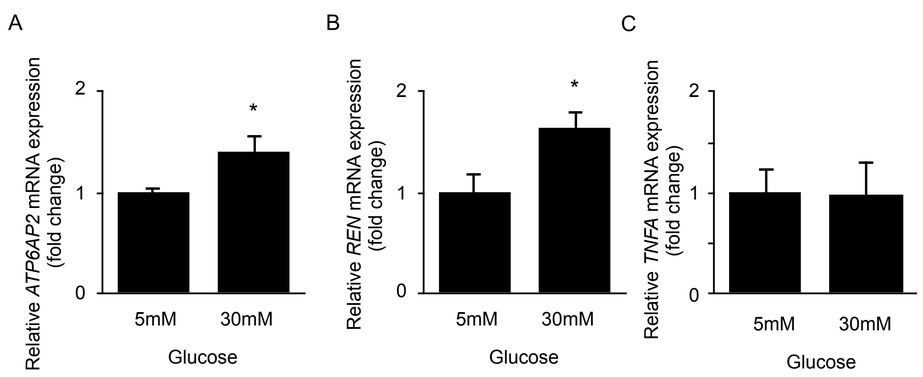
Figure 3. *TNFA, LRG1 and CFD* mRNA expression levels by prorenin and Ang II in HRMECs



A-C: Relative RNA expression levels of *TNFA, LRG1, and CFD* in HRMECs stimulated by prorenin or Ang II with or without those receptor antagonists. n = 6.

Administration of prorenin or Ang II to HRMECs didn't change the expression levels of *TNFA, LRG1* and *CFD*.

Figure 4. *ATP6AP2, REN* and *TNFA* mRNA expression levels in HRMECs under hyperglycemic condition



(P)RR/*ATP6AP2* and prorenin/*REN* levels were upregulated under high glucose condition, but no change in TNF-α/*TNFA* levels.

A-C: HEMECs were incubated with the medium containing 30-mM glucose for 72 h, and *ATP6AP2* (A), *REN* (B) and *TNFA* (C) gene expression levels were analyzed. n = 6, *p < 0.05.

Conclusions:

1. Our present data demonstrated that chronic inflammation, renal dysfunction and hyperglycemia enhanced s(P)RR levels in plasma, which triggered activation of RAS in the microvascular environment associated with the pathogenesis of DR.
2. Plasma s(P)RR may be used as an early marker of diabetic microvascular disease.

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