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Plasmin reduces fibronectin deposition by mesangial cells in a protease-activated receptor-1 independent manner

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ABSTRACT

Background: Protease-activated receptor-1 (PAR-1) potentiates diabetic nephropathy (DN) as evident from reduced kidney injury in diabetic PAR-1 deficient mice. Although thrombin is the prototypical PAR-1 agonist, anticoagulant treatment does not limit DN in experimental animal models suggesting that thrombin is not the endogenous PAR-1 agonist driving DN.

Objectives: To identify the endogenous PAR-1 agonist potentiating diabetes-induced nephropathy.

Methods: Unbiased protease expression profiling in glomeruli from human kidneys with DN was performed using publically available microarray data. The identified prime candidate PAR-1 agonist was subsequently analysed for PAR-1-dependent induction of fibrosis *in vitro*.

Results: Of the 553 proteases expressed in the human genome, 247 qualified as potential PAR-1 agonists of which 71 were significantly expressed above background in diabetic glomeruli. The recently identified PAR-1 agonist plasmin(ogen), together with its physiological activator tissue plasminogen activator, were among the highest expressed proteases. Plasmin did however not induce mesangial proliferation and/or fibronectin deposition *in vitro*. In a PAR-1 independent manner, plasmin even reduced fibronectin deposition.

Conclusion: Expression profiling identified plasmin as potential endogenous PAR-1 agonist driving DN. Instead of inducing fibronectin expression, plasmin however reduced mesangial fibronectin deposition *in vitro*. Therefore we conclude that plasmin may not be the endogenous PAR-1 agonist potentiating DN.

1. Introduction

The World Health Organization approximates that over 300 million people will suffer from diabetes in 2025 [1]. The health implications of this endemic disease are expected to be larger as diabetic patients frequently develop complications like (among others) diabetic nephropathy leading to end-stage renal disease (ESRD) [2]. Diabetic nephropathy actually emerged as the major causative pathology in patients entering ESRD worldwide and it is responsible for 30–40% of all ESRD cases. In individuals with diabetes, the presence and severity of nephropathy adversely affects their well-being, significantly contributes to disease morbidity and increases their risk of a premature death [3,4]. Although the progression of diabetic nephropathy can be delayed by strict control of plasma glucose levels and/or by lowering blood pressure, the majority of patients eventually need renal replacement therapy. The large impact of this latter therapy, both on the social and economic level [5,6], urges the need for alternative treatment options.

In the search for alternative targets to pursue in combatting diabetic nephropathy, we recently identified protease-activated receptor (PAR) − 1 as an attractive candidate. Indeed, PAR-1 deficient mice showed reduced diabetes-induced albuminuria, plasma cystatin C levels, mesangial expansion and tubular atrophy as compared to wild type diabetic controls [7]. Subsequent mechanistic experiments showed that PAR-1 activation induces proliferation and fibronectin production by MES13 mesangial cells *in vitro*.

PAR-1 is a seven transmembrane domain receptor that is activated by proteolytic cleavage rather than by ligand binding [8–10]. Generally, PAR-1 is recognized as a blood coagulation factor receptor and thrombin is considered the prototypical PAR-1 agonist. Importantly however, anticoagulation with low-molecular-weight heparin did not protect against diabetic nephropathy in diabetic wild type mice despite the fact that it normalized markers of coagulation (*i.e.* thrombin-antithrombin, D-dimer and renal fibrin deposition). Indeed, albuminuria, kidney weight, histological PAS scores and glomerular size were

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similar in saline and low-molecular-weight heparin treated diabetic mice [11]. Similarly, the direct thrombin inhibitor hirudin had also no significant effect on key parameters of nephropathy in diabetic wild type mice [12]. In the setting of diabetic nephropathy, thrombin may therefore not be the endogenous PAR-1 agonist potentiating kidney injury.

Next to thrombin, activated protein C (APC) is a well-recognized PAR-1 agonist [13]. APC-dependent PAR-1 activation, however, prevents hyperglycemia-induced apoptosis of endothelial cells and podocytes *in vitro*, whereas APC overexpression reduces diabetes-induced kidney injury *in vivo* [11]. These data indicate that APC is not the endogenous PAR-1 agonist potentiating diabetic nephropathy but also imply that targeting the PAR-1 agonist driving diabetic nephropathy might be a more effective treatment strategy than targeting the receptor itself. Indeed, preventing PAR-1-dependent mesangial expansion and tubular atrophy without blocking the beneficial PAR-1 effects on endothelial cells and podocytes may even further decrease diabetes-induced nephropathy.

To fully appreciate the importance and potential clinical relevance of the PAR-1 pathway in diabetic nephropathy, it is important to identify the endogenous PAR-1 agonist that potentiates kidney injury during diabetes. In the current manuscript, we therefore aimed to pinpoint candidate proteases as endogenous PAR-1 agonists potentiating diabetic nephropathy. To this end, we employed an unbiased approach in which expression levels of all proteases expressed in the human genome were assessed in diabetic glomeruli.

2. Materials and methods

2.1. Mining of publically available RNA microarray dataset

The GSE10009 [14] dataset was downloaded from Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/gds). This dataset reports whole-genome gene expression of glomeruli isolated from patients with diabetes mellitus. Background expression was determined for individual chips using Affymetrix negative control probes and protease expression levels above the mean + 2SD were considered significantly expressed and were used for further analysis. Subcellular localisation of proteases was assessed using the UniProtKB/Swiss-Prot and COMPARTMENTS databases [15].

2.2. Cell culture and stimulation

Mouse mesangial cells (SV40 MES13; CRL-1927 ATCC) were cultured according to the recommended protocol (https://www.lgcstandards-atcc.org/Products/Cells_and_Microorganisms/By_Tissue/Kidney/CRL-1927.aspx?geo_country=nl#culturemethod) using a 3:1 mixture of Dulbecco's Modified Eagle's Medium containing 1 g/L glucose with Ham's-F12 medium, supplemented with 5% heat inactivated fetal calf serum, 100 U/ml penicillin, 100 μg/ml streptomycin and 2 mM L-glutamine. Cells were cultured at 37 °C in an atmosphere of 5% CO_2. Cells were serum starved overnight in low glucose (1 g/L) medium before stimulation with 100 μM PAR-1 agonist peptide (PAR-1-AP; H-SFLLRN-NH2; Biochem, Shanghai, China) or 2 or 8 μg/ml plasmin (Biopur, Switzerland) in high glucose (4 g/L) medium (concentrations based on [16,17] for PAR-1-AP and plasmin, respectively). If indicated, cells were pretreated with 10 μM PAR-1 pepducin (P1pal12; palmitate-RCLSSSAVANRS-NH2; Biochem, Shanghai, China) or 100 nM vorapaxar (AdooQ BioScience, Irvine, CA; concentration based on [18,19] for P1pal12 and vorapaxar, respectively).

2.3. MTT assay

Cells were seeded at a density of 5000 cells/well in 96 well plates. After stimulation with plasmin or PAR-1 agonist peptide for 24 h, MTT (St. Louis, MO, USA) was added to the culture medium. After 2 h incubation at 37 °C, cells were lysed with DMSO and OD_{570} was measured using a microplate reader (Synergy HT, BioTek).

2.4. Western Blot

Cells were seeded at a density of 50000 cells/well in 24 well plates. After stimulation for 24 h, cells were lysed with RIPA lysis buffer (50 mM Tris HCl (pH 7.4), 150 mM NaCl, 0.5% deoxycholate, 1.0% Triton X-100, 0.1% SDS and 1 mM EGTA) supplemented with 1x Halt Protease and Phosphatase Inhibitor Cocktail (Thermo Scientific, #78445). Cell lysates were subsequently diluted (1:1) in Laemmli buffer and separated on 10% SDS-PAGE gel and transferred onto Immobulin-PL membranes (Millipore) as described before [20]. Membranes were blocked for 1 h at room temperature in 5% bovine serum albumin (BSA) in TBS + 0,1% tween-20 (TBS-T) and subsequently incubated with the following primary antibodies, diluted in TBS-T: mouse-anti-tubulin 1:2500 (Santa Cruz; sc-23948) or goat-anti-fibronectin 1:1000 (Santa Cruz; sc-6953). After overnight incubation, the membranes were washed 3 times with TBS-T and incubated 1 h at room temperature with horseradish peroxidase (HRP)-conjugated (1:1000, DakoCytomation, Glostrup, Denmark) secondary antibodies diluted 1:5000 in TBS-T. Membranes were washed 3 times with TBS-T and imaged using Luminata Forte western blot substrate (Merck Millipore, Billerica, Massachusetts, USA) on an ImageQuant LAS 4000 biomolecular imager (GE Healthcare, Zeist, the Netherlands).

2.5. RNA isolation and RT-qPCR

Cells were seeded at a density of 50000 cells/well in 24 well plates. After stimulation for the indicated time points, mRNA was isolated using TriReagent isolation reagent (#11667165001; Roche Diagnostics) according to the manufacturers recommendations. All mRNA samples were quantified by spectrophotometry and stored at −80 °C until further analysis. 0.75 μg of mRNA was treated with DNAse using the RQ1 DNAse kit (M6101, Promega, Madison, WI, USA) and subsequently converted to cDNA using M-MLV reverse transcriptase (M1705, Promega, Madison, WI, USA) and random hexamer primers (#SO142, Fisher scientific, Landsmeer, the Netherlands) according to the manufacturers recommendations. qPCR and subsequent analysis were performed using a Roche lightcycler with SYBR green PCR master mix (#04707516001; Roche, Almere, the Netherlands) on a Lightcycler 480 machine and corresponding software (Software release 1.5.0 (1.5.0.39), Roche, Almere, the Netherlands). Expression levels were normalized using the average expression levels of HPRT and TBP. The following primer sequences were used: Fibronectin forward 5′-CCATGTAGGAGAACAGTGGCA-3′ and reverse 5′-GAAGCACTCAATGGGGCA-3′; TBP forward: 5′-GGAGAATCATGGACCAGAACA-3′ and reverse: 5′-GATGGGAATTCCAGGAGTCA-3′; HPRT forward: 5′-TCCTCCTCAGACCGCTTTT-3′ and reverse: 5′-CCTGGTTCATCATCGCTAATC-3′.

2.6. Statistics

All values are expressed as mean ± SEM. Differences between groups were analysed using a Mann-Whitney *U*-test for non-parametric data. All analyses were performed using GraphPad Prism version 5.01.

3. Results

The proteolytic activation mechanism of PAR-1 dictates the endogenous PAR-1 agonist inducing glomerular expansion and subsequent diabetic nephropathy to be a protease. Consequently, we assessed protease mRNA expression levels in glomeruli obtained from patients with diabetic nephropathy using the publically available GSE1009 gene expression omnibus dataset [14]. From the total of 553 genes that have been annotated to encode proteases or protease homologues in the