

Original Article

Protease activated receptor 2 in diabetic nephropathy: a double edged sword

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Abstract: Diabetic nephropathy is a major microvascular complication of diabetes mellitus, and the leading cause of end stage renal disease worldwide. The pathogenesis of diabetic nephropathy is complex, making the development of novel treatments that stop or reverse the progression of microalbuminuria into end stage renal disease a challenge. Protease activated receptor (PAR)-2 has recently been shown to aggravate disease progression in diabetic nephropathy based upon which it was suggested that PAR-2 would be a potential target for the treatment of diabetic nephropathy. To fully appreciate the translational potential of PAR-2 in diabetic nephropathy, we evaluated the effect of PAR-2 deficiency on the development of diabetic nephropathy in a streptozotocin-induced diabetes model characteristic of type 1 diabetes. Although diabetic PAR-2 deficient mice showed reduced albuminuria compared to diabetic wild type mice, an increase in mesangial expansion was evident in the PAR-2 deficient mice. No differences were observed in blood glucose levels, podocyte numbers or in glomerular vascular density. These results show that PAR-2 plays a dual role in the development of streptozotocin-induced diabetic nephropathy and may thus not be the eagerly awaited novel target to combat diabetic nephropathy. Targeting PAR-2 should consequently only be pursued with great care in a clinical setting.

Keywords: Protease-activated receptor 2, diabetic nephropathy, mesangial expansion, albuminuria, streptozotocin, diabetes mellitus

Introduction

Diabetic nephropathy is a major microvascular complication of diabetes mellitus, and the leading cause of end stage renal disease worldwide [1]. Progression towards end stage renal disease can be limited by strict glycemic control and angiotensin-converting-enzyme inhibition [2]. However, due to the lack of knowledge on the complex pathogenesis of diabetic nephropathy, novel treatment modalities that ultimately stop or reverse this progressive pathology remain beyond reach.

Intriguingly, protease-activated receptor (PAR)-2, a cellular receptor widely expressed in the kidney and well-known to drive fibroproliferative disorders [3], was recently shown to potentiate diabetic nephropathy suggesting that

PAR-2 may be a novel target in the setting of diabetic nephropathy to pursue. Indeed, the lack of PAR-2 reduced the urinary albumin/creatinine ratio in diabetic endothelial NO synthase (eNOS)^{+/-} mice without affecting blood glucose levels. PAR-2 deficiency also inhibited glomerular mesangial expansion and resulted in a reduction in foot process effacement in this specific eNOS^{+/-} Akita type 1 diabetes model [4]. In line, PAR-2 inhibition limited glomerular endothelial injury, prevented glomerular sclerosis and attenuated albumin leakage into the retina of type 2 diabetic db/db mice [5]. Based on these data, it was suggested that PAR-2 inhibition would be a novel strategy to prevent diabetic nephropathy.

To fully appreciate the translational relevance of PAR-2 inhibition in the setting of diabetic

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nephropathy, and before extrapolating these findings to the increasingly heterogeneous population of diabetic nephropathy patients, it is however of utmost important to confirm the general nature of PAR-2 as driving factor in diabetic nephropathy. Consequently, we subjected wild type and PAR-2 deficient mice to a streptozotocin-induced diabetic nephropathy model.

Materials and methods

Mice

Homozygous PAR-2 deficient mice, generated on a C57Bl/6 background were originally purchased from The Jackson Laboratory (ME, USA) and bred in the Animal Research Institute Amsterdam. Wild type C57BL/6 mice were purchased from Charles River (Maastricht, Netherlands). All experiments were approved by the Institutional Animal Care and Use Committee of the University of Amsterdam. All mice were maintained according to institutional guidelines. Animal procedures were carried out in compliance with the Institutional Standards for Humane Care and Use of Laboratory Animals of the Academic Medical Center.

Experimental diabetic nephropathy model

Eight to twelve week-old male wild type and PAR-2 deficient mice (8 per group) were injected with streptozotocin (50 mg/kg body weight) for 5 consecutive days to induce diabetes. Six months after streptozotocin injections, mice were sacrificed, and blood, urine and kidneys were harvested for further analysis. Blood glucose levels were measured from tail vein blood using a Bayer Contour glucose meter. Urine albumin levels were determined by ELISA (Bethyl laboratories, AL, USA) according to the manufacturer's instructions. Urine creatinine levels were determined using an enzymatic mouse creatinine assay kit (CrystalChem, Zandam, Netherlands), according to the manufacturer's instructions.

(Immuno)histochemistry

Formalin-fixed, paraffin embedded, kidney sections were subjected to periodic acid-Schiff-diastase (PAS-D) and Masson's Trichrome staining, following routine procedures. The extent of glomerular injury was determined by two independent observers in a blinded fashion.

To quantify glomerular injury, per mouse we scored 50 glomeruli as either normal or deviated. Glomeruli were scored as deviated when mesangial expansion was apparent as clusters of >3 mesangial cells. The percentage of collagen per glomerulus was determined using imageJ software (U.S. National Institutes of Health, MD, USA) in 25 glomeruli per mouse. Podocytes and glomerular endothelial cells were detected using rabbit- α -WT-1 (sc-192, Santa Cruz Biotechnology, TX, USA) and rabbit- α -CD-31 (sc-1506-R, Santa Cruz Biotechnology) antibodies using routine procedures. In short, paraffin embedded slides were deparaffinized, and endogenous peroxidases were inhibited by 15 minutes incubation in 0.3% H₂O₂ at room temperature. After antibody retrieval was performed using citrate buffer (pH6.0) for 10 min at 96°C, slides were blocked with 5% normal goat serum in PBS or Ultra V block (Thermo Scientific, Runcorn, UK) for 30 min, and incubated overnight with the primary antibody. Next, slides were incubated with Powervision PolyHRP- α -rabbit IgG (DPVR-55HRP; Immunologic, Duiven, Netherlands) for 30 min at room temperature, visualized with DAB (BS04-999; Immunologic) and counterstained using haematoxylin. Slides incubated without the primary antibody were used as negative controls to exclude nonspecific binding of the secondary antibody. Pictures were taken at 20 times magnification using a Leica DM5000B microscope equipped with a Leica DFC500 camera and Image Pro Plus software (vs 5.02; Media Cybernetics). Number of podocytes per field were counted manually and the percentage of CD31 positivity per glomerulus was analysed using imageJ software (U.S. National Institutes of Health, MD, USA) in 25 glomeruli per mouse.

RNA isolation and RT qPCR

For gene expression analysis, total kidney mRNA was isolated using TriReagent isolation reagent (#11667165001; Roche Diagnostics, Almere, Netherlands) according to the manufacturer's recommendations. All mRNA samples were quantified by spectrophotometry and stored at -80°C until further analysis. 1 μ g of mRNA was treated with DNase using the RQ1 DNase kit (M6101, Promega, WI, USA) and subsequently converted to cDNA using M-MLV reverse transcriptase (M1705, Promega, WI, USA) and random hexamer primers (#S0142,

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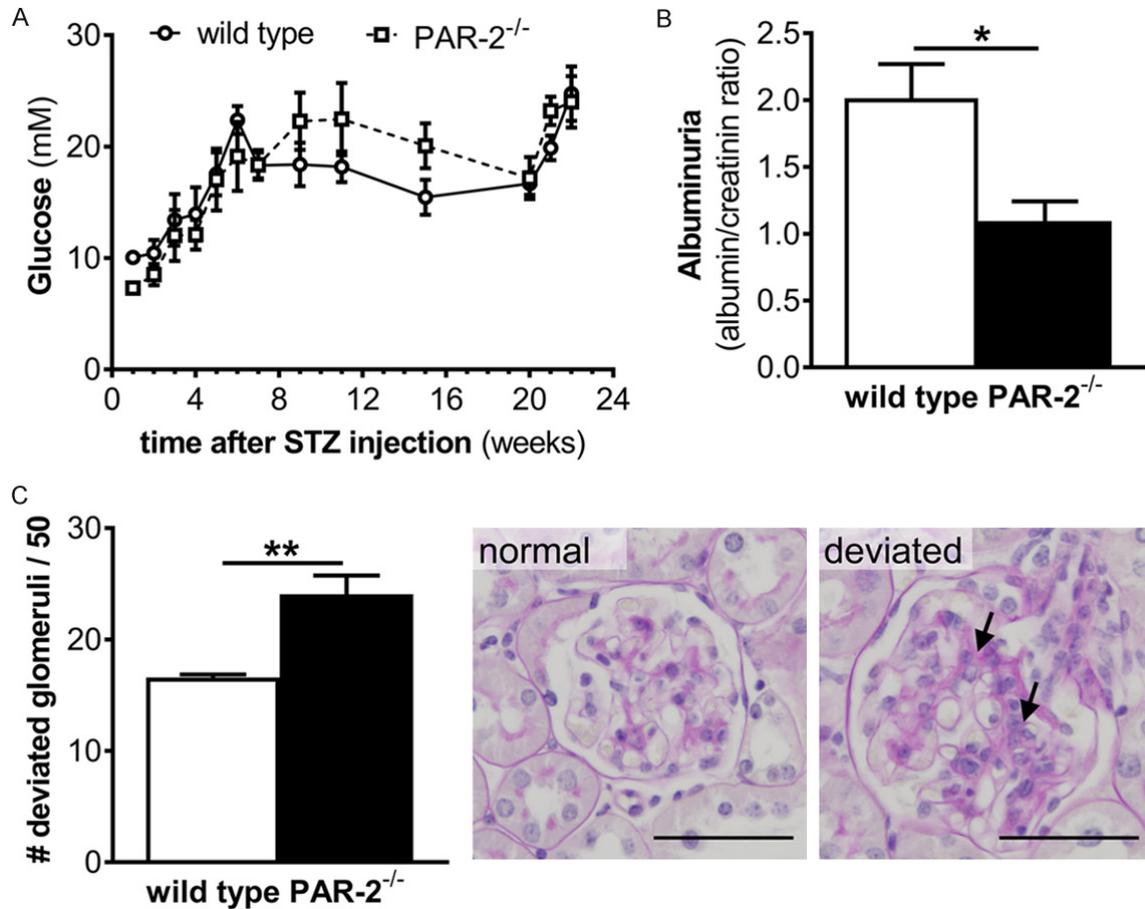


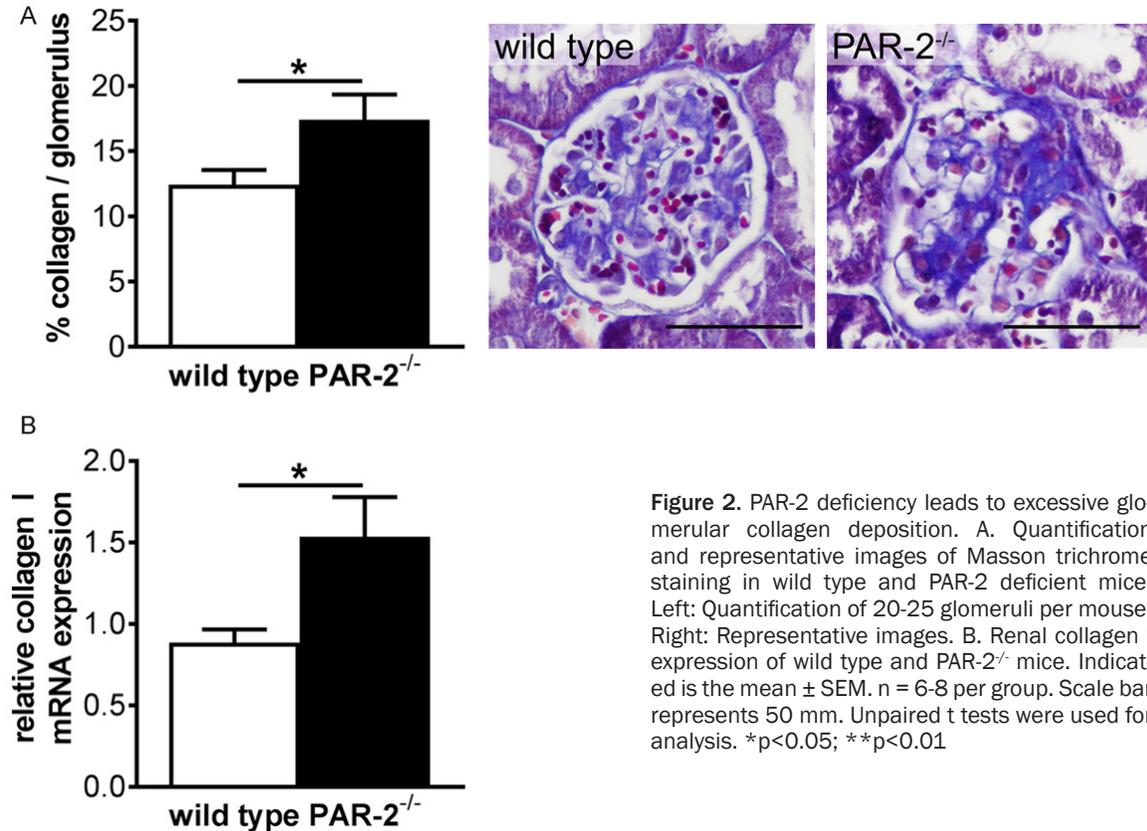
Figure 1. PAR-2 deficiency limits albuminuria in diabetic mice. A. Blood glucose levels of diabetic wild type and PAR-2 deficient (PAR-2^{-/-}) mice. B. Urinary albumin/creatinine ratio of diabetic wild type and PAR-2^{-/-} mice. C. Quantification and representative images of deviated glomeruli in wild type and PAR-2^{-/-} mice. Indicated is the mean ± SEM. n = 6-8 per group. Scale bar represents 50 mm. Unpaired t tests were used for analysis. *p<0.05; **p<0.01.

Fisher Scientific, Landsmeer, 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 to TBP expression. The following primer sequences were used: mPAR-1 forward: 5'-GT-TGATCGTTTCCACGGTCT-3'; mPAR-1 reverse: 5'-ACGCAGAGGAGGTAAGCAA-3'; mTBP forward: 5'-GGAGAATCATGGACCAGAACA-3' mTBP reverse: 5'-GATGGGAATCCAGGAGTCA-3'; GAPDH forward: 5'-CTCATGACCACAGTCCATGC-3' GAPDH reverse: 5'-CACATTGGGGGTAGGAACAC-3'; SERPINE1 forward: 5'-GGGCTCGAGTATGACGTTGT-3' SERPINE1 reverse: 5'-GGTCATGTTGCCCTTCCAT-3'.

Multiplex ligation dependent probe amplification (MLPA)

Coagulation, inflammation, and apoptosis related gene expression levels were determined in isolated RNA of the mouse kidneys. 50 ng/ul RNA were analyzed as described previously [6]. Several genes involved in apoptosis, inflammation and coagulation were analysed, using Beta-2-Microglobulin (B2M) and TATA-Box Binding Protein (TBP) as reference genes: apoptosis, BCL2 Like 2 (BCL2), BCL2L2, BCL2 Like 1 (BCL2L1), BOK (BCL2L9), BCL2 Like 11 (BIM), BCL2 Like 13 (BCL2L13), Bcl2 Modifying Factor (BMF), Serpin Family B Member 9 (SERPINB9), CASP8 And FADD Like Apoptosis Regulator (CFLAR), BCL2 Family Apoptosis Regulator (MCL1), BCL2 Associated X (BAX), BCL2 Antagonist/Killer 1 (BAK1), BH3 Interacting Domain Death Agonist (BID), Associated Agonist

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Of Cell Death (BAD), Bcl-2-interacting Killer (BIKLK), Modulator Of Apoptosis 1 (MOAP1), Apoptotic Peptidase Activating Factor 1 (APAF1), Apoptosis Inducing Factor Mitochondria Associated 1 (PDCD8), Phorbol-12-Myristate-13-Acetate-Induced Protein 1 (PMAIP1), Baculoviral IAP Repeat Containing 3 (BIRC3), Baculoviral IAP Repeat Containing 6 (BIRC6), HTRA2 (PRSS25), Diablo IAP-Binding Mitochondrial Protein (DIABLO), P21 Activated Kinase 2 (PAK2); inflammation, MIP-1-Alpha (CCL3), Interleukin 1 Beta (IL1B), Toll Like Receptor 2 (TLR2), Toll Like Receptor 4 (TLR4), Toll Like Receptor 9 (TLR9), CD14, Lymphocyte Antigen 96 (LY96), Interleukin 1 Receptor Associated Kinase 1 (IRAK1), Interleukin 1 Receptor Associated Kinase 3 (IRAK3), Nuclear Factor Kappa B (NFkB1), Nitric Oxide Synthase 3 (NOS3), Intercellular Adhesion Molecule 1 (ICAM1), E-Selectin (SELE), Vascular Cell Adhesion Molecule 1 (VCAM1), Hypoxia Inducible Factor 1 Alpha (HIF1A); coagulation, Tissue Factor Pathway Inhibitor (TFPI), Tissue Factor (F3), Protein C Receptor (PROCR), Plasminogen Activator Inhibitor 1 (SERPINE1), Tissue Type Pla-

smi-nogen Activator (PLAT), and Urokinase Plasminogen Activator Receptor (PLAUR).

Statistical analysis

All values are expressed as mean ± SEM. All groups were tested for normality and for outliers using the D'Agostino-Pearson omnibus normality test and Grubbs outlier test. Detected outliers were excluded from analysis. Differences between two groups were analysed using a t-test if data were normally distributed, or a Mann-Whitney U-test for non-parametric data. All analyses were performed using GraphPad Prism version 5.01.

Results

Four weeks after streptozotocin injections, diabetes was established in all mice as evident from blood glucose levels of >15 mmol/L. Importantly, PAR-2 deficiency did not affect the development of diabetes as glucose levels were similar in wild type and the PAR-2 deficient mice over the time frame of the experiment (**Figure 1A**). Despite a similar degree of

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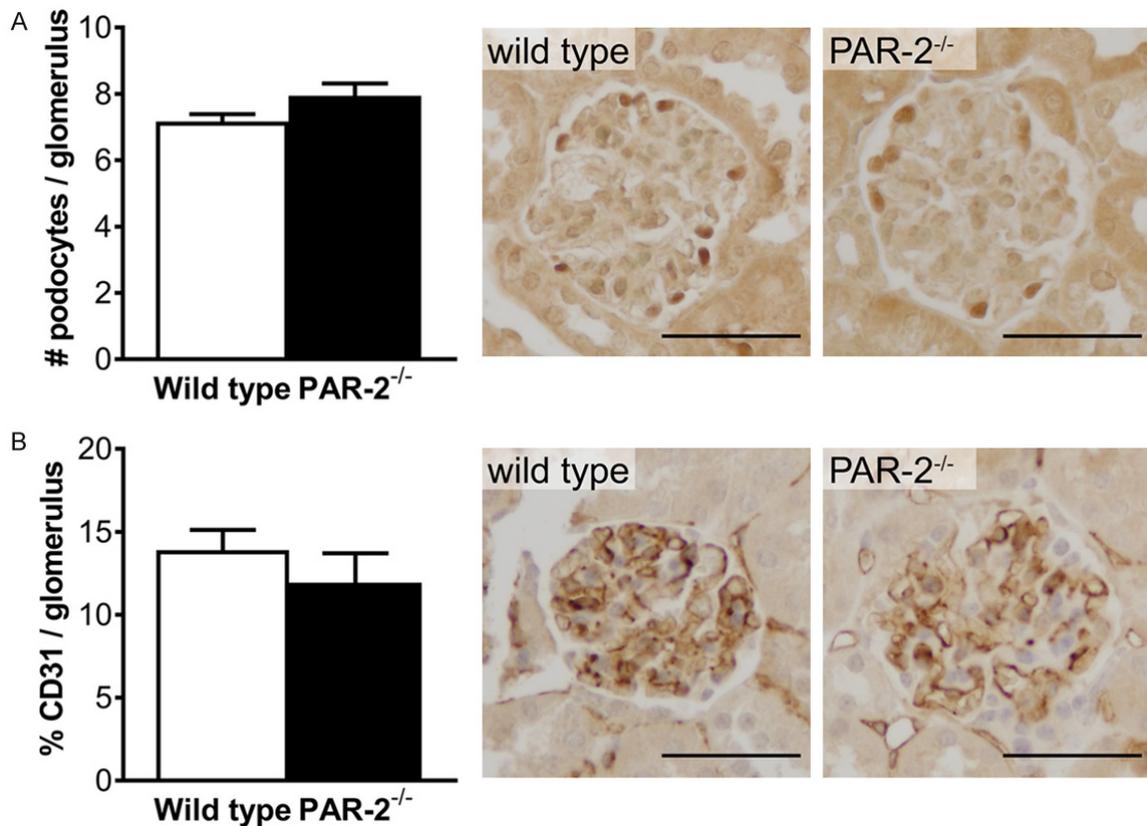


Figure 3. The effect of PAR-2 deficiency on podocyte density and glomerular vasculature. (A) Number of podocytes and (B) percentage of CD31 positivity per glomerulus of diabetic wild type and PAR-2 deficient (PAR-2^{-/-}) mice. Left: Quantification of 20-25 glomeruli per mouse; Right: Representative images. Indicated is the mean \pm SEM. n = 6-8 per group. Scale bar represents 50 μ m. Unpaired t tests were used for analysis. *p<0.05.

hyperglycemia, PAR-2 deficient mice showed significantly reduced albumin leakage into the urine compared to the wild type mice six months after the induction of diabetes (**Figure 1B**). Interestingly however, mesangial expansion, as depicted by the number of injured glomeruli, was increased in diabetic PAR-2 deficient mice compared to the diabetic wild type mice (**Figure 1C**). Accordingly, an increase in collagen deposition was observed in Masson Trichrome stained glomeruli of PAR-2 deficient mice, as well as an increase in collagen I mRNA expression in whole kidney homogenates (**Figure 2A, 2B**).

As PAR-2 was recently shown to disrupt glomerular integrity by inducing podocyte loss and microvascular injury [4, 5], we next evaluated the vascular density and podocyte numbers in the glomeruli of wild type and PAR-2 deficient mice. As shown in **Figure 3A**, podocyte numbers were similar in diabetic wild type and

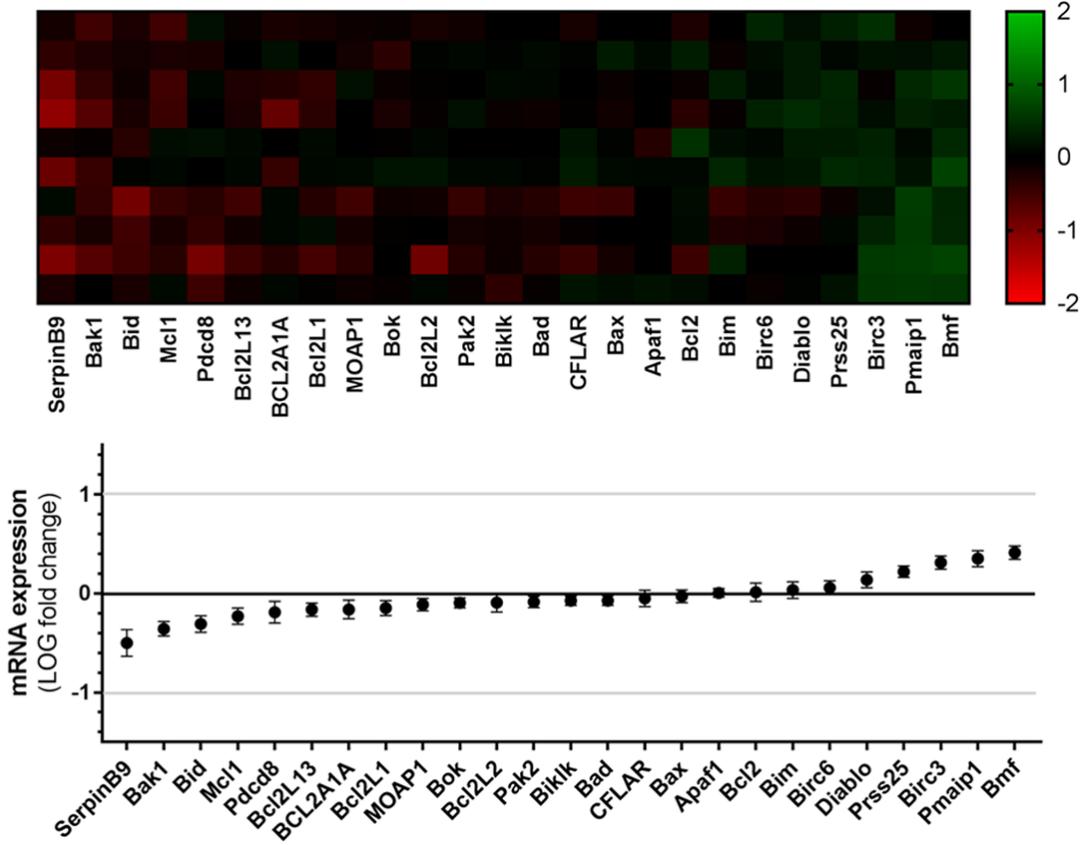
PAR-2 deficient mice. Likewise, glomerular microvascular density, as measured by CD31 positivity, was similar in diabetic wild type and PAR-2 deficient mice (**Figure 3B**).

To screen for other possible mechanisms that might explain our findings, MLPA analysis was performed using apoptosis, inflammation and coagulation gene panels [7]. Interestingly, of all genes analyzed, only SERPINE1 was differentially expressed by at least a factor 2 (**Figure 4**). To validate the observed increase in SERPINE1 expression, qPCR analysis was performed showing that SERPINE1 expression was increased 2.2 fold in diabetic PAR-2 deficient mice compared to diabetic wild type mice (**Figure 5A**).

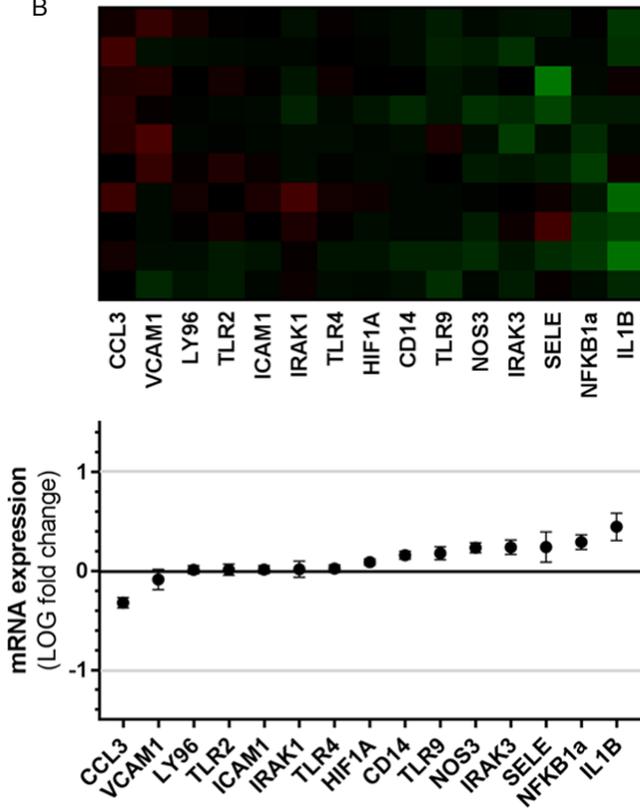
Finally, we determined renal PAR-1 expression levels as PAR-1 aggravates diabetic nephropathy by inducing mesangial expansion [8] and consequently a compensatory increase of PAR-1 in PAR-2 deficient mice would explain the

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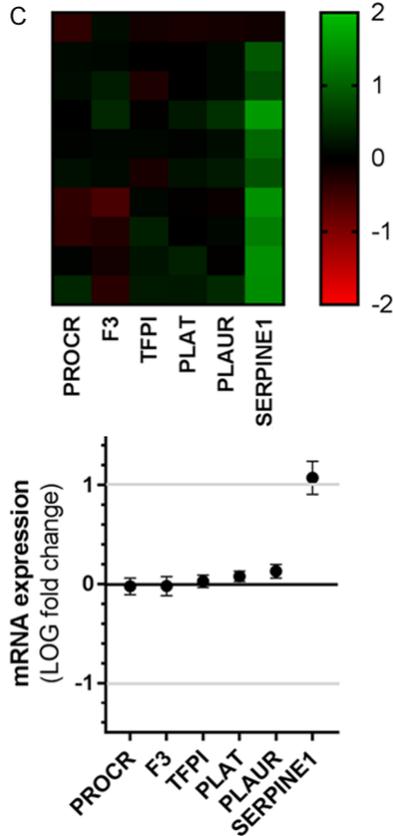
A



B



C



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Figure 4. Apoptosis, inflammation and coagulation related gene panel MLPA analysis. Expression profiling of apoptosis (A), inflammation (B) and coagulation (C) related gene panels in PAR-2 deficient diabetic mice. Upper panels show heat maps of differential expression between diabetic wild type and diabetic PAR-2 deficient mice. Lower panels show mean \pm SEM of LOG2 fold change. n = 6-8 per group.

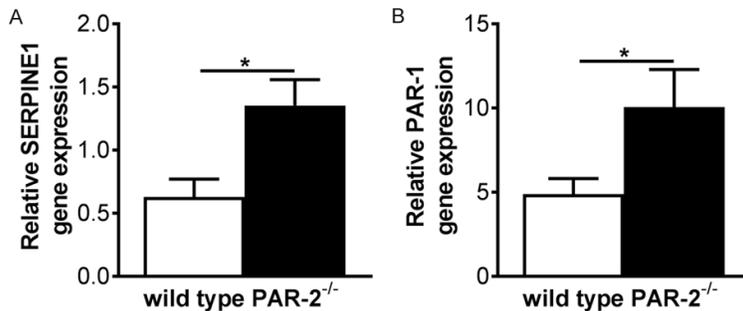


Figure 5. SERPINE1 and PAR-1 gene expression is upregulated in diabetic PAR-2^{-/-} mice. mRNA expression of SERPINE1 (A) and PAR-1 (B) in diabetic wild type and PAR-2 deficient (PAR-2^{-/-}) mice. Indicated is the mean \pm SEM. n = 6-8 per group. Unpaired t tests were used for analysis. *p < 0.05.

observed increase mesangial expansion. As shown in **Figure 5B**, PAR-1 mRNA levels were increased in PAR-2 deficient mice compared to wild type mice.

Discussion

Preclinical animal models recently showed that PAR-2 aggravates diabetic nephropathy based on which it has been suggested that PAR-2 inhibition would be a novel strategy to limit disease progression. Using an alternative model of type 1 diabetes, however, we here show that PAR-2 acts as a double edged sword by reducing albuminuria but increasing mesangial expansion.

A main finding of our study is the fact that PAR-2 deficiency limits albuminuria in a streptozotocin-induced diabetic nephropathy model. These data are in line with previous observations in PAR-2 deficient eNOS^{+/-} Akita mice which show reduced urinary albumin concentrations as compared to wild type eNOS^{+/-} Akita mice [4]. Based on these observations, and on the notion that pharmacological PAR-2 inhibition limits glomerular endothelial injury and prevents glomerular sclerosis in type 2 diabetic db/db mice, it is tempting to suggest that PAR-2 would be a therapeutic target to slow down the progression of diabetic nephropathy [4, 5].

Intriguingly however, reduced albuminuria was accompanied by increased mesangial expansion

in PAR-2 deficient mice subjected to the streptozotocin model which is in contrast to a small but significant decrease in mesangial expansion in PAR-2 deficient eNOS^{+/-} Akita mice [4]. The role of PAR-2 in diabetic nephropathy may therefore not be as straightforward as thought before. In order to better characterize the mechanisms underlying the observed phenotype of reduced albuminuria but increased glomerulosclerosis, we profiled kidney homogenates for differential

expression. Interestingly, of all genes analysed only SERPINE1, the gene coding for plasminogen activator inhibitor 1 (PAI-1), was significantly increased in PAR-2 deficient mice compared to wild type controls. This is particularly relevant as PAI-1 levels are increased in kidneys of diabetic nephropathy patients and these high PAI-1 levels are associated with excessive accumulation of extracellular matrix proteins [9]. Moreover, PAI-1 deficient mice subjected to STZ-induced diabetes develop less glomerulosclerosis and albuminuria compared to wild type mice [10]. Increased PAI-1 levels in PAR-2 deficient mice are probably not a direct effect of PAR-2 on SERPINE1 gene expression as previous studies show that PAR-2 stimulation/transactivation induces PAI-1 levels [11-13]. Although the cause of increased PAI-1 expression in diabetic PAR-2 deficient mice remains elusive, it is tempting to speculate it may be the underlying mechanism for the observed adverse effects on diabetic nephropathy in PAR-2 deficient mice.

In addition to PAR-2, its family member PAR-1 has also been implicated in diabetic nephropathy. Indeed, PAR-1 deficient mice develop less kidney damage after streptozotocin-induced diabetes, as evidenced by diminished albuminuria, plasma cystatin C levels, expansion of the mesangial area, and tubular atrophy [8]. This is particularly interesting as PAR-1 levels are differently regulated in PAR-2 deficient mice sub-

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jected to streptozotocin-induced diabetes or eNOS^{+/−} Akita-induced diabetes. Indeed, PAR-1 levels are decreased in PAR-2 deficient [4] whereas PAR-1 levels are increased in streptozotocin treated PAR-2 deficient mice. This differential regulation of PAR-1 may thus explain the observed difference in PAR-2-dependent mesangial expansion in the eNOS^{+/−} Akita model and our streptozotocin model. Alternatively, differential expression of PAR-2 agonists in the different preclinical diabetes models could be responsible for the observed discrepancy in mesangial expansion. PAR-2 is activated upon cleavage by different proteases like for instance factor X [4], cathepsin S [5], matriptase [14] and trypsin [15]. Importantly, the individual proteases induce divergent signaling pathways leading to differential functional consequences, a phenomenon known as biased agonism [15].

Irrespective the actual explanation for the opposite effect of PAR-2 deficiency on disease outcome in the different preclinical diabetes models, our data indicate that targeting PAR-2 in the setting of diabetic nephropathy should be pursued with care and is probably not the eagerly awaited novel treatment option for this common kidney disease. Moreover, our data underscore the fact that translation of preclinical data into clinical practise should be pursued only in case beneficial effects have been observed in multiple models.

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Disclosure of conflict of interest

None.

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