Protease-activated receptors in diabetic nephropathy and renal fibrosis

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Chapter 8

Summary & general discussion

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Summary

In the Netherlands, 1.700.000 people suffer from chronic kidney disease (CKD), a condition in which fibrotic tissue builds up in the kidneys, ultimately destroying kidney function. Diabetic nephropathy is the biggest sole cause of chronic kidney disease. In order to find potential targets for therapeutic interventions, we need to better understand the development of diabetic nephropathy and renal fibrosis.

In this thesis, chapter 1 gives an introduction to the kidneys, renal fibrosis development, diabetic nephropathy, the coagulation system during diabetes, and protease-activated receptors (PARs). As PARs are described to play a role in fibrosis development in liver and lungs, we hypothesized that PARs may also play a role in the development of renal fibrosis associated with diabetic nephropathy. In chapters 2 and 3, we explored the role of PAR-1 and PAR-2 (i.e. two of the four PAR family members with known expression in the kidney) in diabetic nephropathy development. By comparing diabetic PAR-1 deficient mice to diabetic wild type mice, we show that PAR-1 deficient mice develop less renal damage compared to wild type mice (chapter 2). Histopathological analysis revealed that reduced renal damage in diabetic PAR-1 deficient mice was accompanied by diminished mesangial expansion. To determine whether PAR-1 stimulation indeed leads to mesangial expansion, a series of in vitro experiments was performed showing that PAR-1 signalling in mesangial cells led to increased proliferation and expression of extracellular matrix proteins. Overall, we thus concluded that PAR-1 plays an important role in the development of diabetic nephropathy by inducing mesangial expansion. Similar experiments comparing nephropathy in diabetic wild type and PAR-2 deficient mice are described in chapter 3. In contrast to PAR-1 deficient mice, PAR-2 deficient mice developed more mesangial expansion. Indeed, 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. In chapter 4, we continue with the finding that PAR-1 deficient mice are protected against diabetic nephropathy and investigate whether pharmacological inhibition of PAR-1 using vorapaxar would limit diabetic nephropathy. Indeed, diabetic mice on vorapaxar treatment did not show...
any signs of kidney damage despite having similar levels of hyperglycemia. We thus concluded that vorapaxar treatment prevents the development of diabetic nephropathy in streptozotocin-induced diabetic mice, and pinpoint PAR-1 as a novel therapeutic target to pursue in the setting of diabetic nephropathy.

The promising results of chapter 4 are based on a model for experimental type 1 diabetes, while the majority of diabetic patients suffer from type 2 diabetes. Next, we therefore tested the effect of vorapaxar treatment in an state-of-the-art BTBR\textsuperscript{ob/ob} mouse model for type 2 diabetic nephropathy (chapter 5). We show that vorapaxar treated BTBR\textsuperscript{ob/ob} mice develop less hyperglycaemia compared to matched controls. Moreover, vorapaxar limits mesangial expansion, suggesting that PAR-1 drives mesangial expansion in both type 1 and type 2 diabetes. However, despite limited hyperglycaemia and mesangial expansion, vorapaxar did not improve albuminuria or podocyte loss. From these results we conclude that, as opposed to its potential therapeutic role in type 1 diabetes-induced nephropathy, vorapaxar does not seem an attractive intervention to pursue in the setting of type 2 diabetes and stresses the importance to treat both entities differently in order to pursue best clinical result for the patient.

Although thrombin is the prototypical PAR-1 agonist, anticoagulant treatment does not limit diabetic nephropathy in experimental animal models suggesting that thrombin is not the endogenous PAR-1 agonist driving diabetic nephropathy. To identify the endogenous PAR-1 ligand that potentiates diabetic nephropathy, we consequently analysed the expression of all proteases in glomeruli isolated from human kidneys with and without diabetic nephropathy (chapter 6). The recently identified PAR-1 agonist plasminogen, together with its physiological activator tissue plasminogen activator, were among the highest expressed proteases. However, stimulation of mesangial cells with plasmin resulted in a decrease rather than an increase of fibronectin deposition. We therefore concluded that plasmin may not be the endogenous PAR-1 agonist that potentiates diabetic nephropathy, and the responsible ligand remains elusive.
Finally, in **Chapter 7** we assessed the role of PAR-1 in tubulo-interstitial fibrosis. We subjected PAR-1 deficient mice to unilateral ureter obstruction, which is a model for obstructive nephropathy and leads to a rapid development of tubulo-interstitial fibrosis. Most importantly, PAR-1 deficient mice developed less fibrosis compared to wild type mice. Further experiments revealed that this is likely due to PAR-1-dependent pro-fibrotic cytokine production rather that mesenchymal transition of tubular epithelial cells.
General discussion

According to the world health organisation, 422 million people were diagnosed with diabetes in 2014. The health implications of this endemic are expected to be large as diabetic patients frequently develop complications like (among others) diabetic nephropathy leading to renal fibrosis and end-stage renal disease (ESRD)\(^3\). Diabetic nephropathy actually emerged as the major causative pathology in patients entering end-stage renal disease worldwide and it is responsible for almost half of all ESRD cases. Although long-recognized as a devastating complication of diabetes, current treatment options to combat diabetes-induced nephropathy are limited and alternative clinical strategies are eagerly awaited for. As protease-activated receptors recently emerged as key players in fibrosis, the aim of this thesis was to investigate whether protease activated receptors would be good targets for novel interventions that prevent the decay of kidney function in diabetic patients.

Based on the results described in this thesis, we could conclude that PAR-1 inhibition may be a potential target to prevent diabetic nephropathy development in type 1 diabetic patients. Indeed, we show that PAR-1 contributes to diabetic nephropathy development by inducing mesangial expansion and that PAR-1 deficiency prevents the development of diabetic nephropathy in streptozotocin-induced type 1 diabetic mice (chapter 2). Importantly, we also show that pharmacological inhibition of PAR-1 using vorapaxar reduces nephropathy in the streptozotocin-induced type 1 diabetes mouse model (chapter 3). As vorapaxar (under its trade name Zontivity) obtained FDA approval as anticoagulant treatment for patients with secondary ischemic events in 2014, the extrapolation to diabetic patients at risk for diabetic nephropathy may even be feasible on short notice. Interestingly, diabetic patients are at increased risk for cardiovascular events and treatment with vorapaxar in the TRA-2\(^0\)P-TIMI trial appeared especially beneficial in patients with diabetes\(^4\). This is particularly relevant, as it may thus be possible to reduce both the risk for cardiovascular events and diabetic nephropathy development with the addition of vorapaxar to the standard care. Importantly however, vorapaxar treatment increases the risk of bleedings, as thrombin-mediated PAR-1 signalling on platelets
stimulates coagulation. In fact, the TRA*CER trial, in which high risk acute coronary syndrome patients were treated with vorapaxar on top of standard anticoagulant treatment, has been terminated because of a high risk of bleeding and doubling in risk of intracranial haemorrhage\textsuperscript{5}. Therefore vorapaxar should not be prescribed to patients with a history of transient ischemic attack (TIA) or stroke. In the TRA 2\textsuperscript{o}P-TIMI trial vorapaxar treatment resulted in a positive benefit-to-risk ratio for prevention of secondary cardiovascular events. Whether long term treatment with vorapaxar in diabetic patients can outweigh the risk of bleedings, remains to be determined. Finally, this risk should be considered for every individual and patients on vorapaxar treatment should be carefully monitored.

A potential way to circumvent the bleeding risk of vorapaxar treatment would be to find and specifically inhibit the endogenous PAR-1 ligand that potentiates diabetic nephropathy development. Thrombin is the prototypical PAR-1 agonist, but other proteases such as APC, Factor X, plasmin, MMP-1, and MMP-13 can activate the receptor as well\textsuperscript{6-10}. These proteases cleave and activate PAR-1 all in a slightly different manner, resulting in divergent signalling (a process called biased agonism)\textsuperscript{6}. Although thrombin levels are increased during diabetes, thrombin inhibition did not reduce diabetic nephropathy development in experimental animal models\textsuperscript{11,12}. It is thus likely that another protease triggers diabetic nephropathy development. Inhibiting the endogenous ligand that potentiates diabetic nephropathy development rather than PAR-1 itself will leave the PAR-1-thrombin mediated signalling required for proper coagulation intact. In this thesis, we performed an unbiased screening of all proteases that are expressed in the glomeruli of patients with diabetic nephropathy. Although we identified plasmin(ogen) as a potential PAR-1 agonist in the setting of diabetic nephropathy, \textit{in vitro} testing refuted plasmin as the harmful ligand driving mesangial expansion. Of the other proteases that were highly expressed during diabetic nephropathy, none have yet been described as a PAR-1 agonist and/or to play a role in diabetic nephropathy development. Importantly, the endogenous PAR-1 agonist driving diabetic nephropathy may not be synthesized locally in the glomeruli and consequently would not be picked up in our screen. Indeed, due to early microvascular injury, circulating proteases produced at distinct sites may reach and activate PAR-1 on mesangial
cells. Our approach, analysing mRNA expression levels in glomeruli, was unfortunately not qualified to identify such proteases, and the endogenous PAR-1 ligand in the setting of diabetic nephropathy therefore remains elusive.

Although we found that pharmacological inhibition of PAR-1 using vorapaxar resulted in improved renal function in the streptozotocin-induced diabetes mouse model for type 1 diabetes (chapter 4), vorapaxar treatment was not sufficient to improve renal function in BTBR\textsuperscript{ob/ob} mice with type 2 diabetes (chapter 5). One explanation for this discrepancy may be the multifactorial nature of type 2 diabetes compared to type 1 diabetes. In contrast to type 1 diabetes, type 2 diabetes is often associated with obesity, high blood pressure, and/or inflammation; all of which are risk factors for developing chronic kidney disease\textsuperscript{13}. Likewise, these factors all contribute to nephropathy development via different mechanisms and signalling pathways. As the BTBR\textsuperscript{ob/ob} mice indeed developed obesity and increased inflammation, these factors possibly obscured the beneficial effect of PAR-1 inhibition. Irrespective of the actual reason why vorapaxar treatment did not improve renal function in type 2 diabetic mice, our results highlight that diabetic nephropathy in type 1 and type 2 diabetes should be considered distinct diseases and need to be treated accordingly.

Based on the notion that PAR-2 contributes to diabetic nephropathy development in db/db mice and eNOS\textsuperscript{+/−} Akita mice\textsuperscript{14,15}, we hypothesized that PAR-2 deficient mice subjected to streptozotocin would be protected against diabetic nephropathy as well. Indeed, we observed a reduction in albuminuria in diabetic PAR-2 deficient mice compared to diabetic wild type mice (chapter 3). However, mesangial expansion was increased rather than reduced in these mice. This is in contrast to a decrease in mesangial expansion observed in PAR-2 deficient eNOS\textsuperscript{+/−} Akita mice and in db/db mice upon treatment with a PAR-2 inhibitor\textsuperscript{14,15}. Although based on the latter studies it was suggested that PAR-2 inhibition could be a novel strategy to prevent diabetic nephropathy, our data indicate that PAR-2 is probably not the eagerly awaited target for diabetic nephropathy treatment. More importantly, 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 preclinical models.

Chronic kidney disease (CKD) progresses via renal fibrosis towards end stage renal disease, independent of the underlying cause\textsuperscript{16}. To explore the role of PAR-1 in CKD broader that diabetic nephropathy, we subjected PAR-1 deficient mice to unilateral ureter obstruction – a model for obstructive nephropathy and leads to a rapid development of tubulo-interstitial fibrosis (chapter 7). We show that PAR-1 not only prevents glomerulosclerosis in the setting of diabetic nephropathy, but also diminishes tubulo-interstitial fibrosis in the setting of obstructive nephropathy. This suggests that PAR-1 inhibition may be beneficial in a broader range of chronic kidney diseases, although pharmacological inhibition of PAR-1 in obstructive nephropathy is necessary before extrapolating these finding towards the clinical setting.

Overall, PAR-1 seems to play an important role in the development of diabetic nephropathy and vorapaxar treatment might be a feasible treatment for type 1 diabetic patients at risk for developing diabetic nephropathy, although only if bleeding complications associated with vorapaxar treatment are not too severe. Finding the endogenous PAR-1 ligand that potentiates diabetic nephropathy may lead to a treatment strategy that limits diabetic nephropathy without the risk of severe bleedings.
References