CHAPTER 1

General introduction and aims of the thesis
Chapter 1
Chronic kidney disease

Chronic kidney disease (CKD), characterized by progressive renal function loss, is a major cause of morbidity and mortality worldwide. Many patients who suffer from CKD will ultimately develop end-stage renal disease (ESRD) with the deplorable need for dialysis or transplantation. The incidence and prevalence of ESRD increased at an alarming rate during the last decades due to ageing of the population, reduced cardiovascular mortality of subjects with CKD, and the increasing incidence of type 2 diabetes and obesity. Patients with ESRD have an extremely elevated risk for premature death of cardiovascular disease and endure an impaired quality of life when treated with dialysis or transplantation. Altogether, the consequences for patients and society are tremendous. Improvement of the current intervention strategies in CKD is therefore highly important to prevent progression towards ESRD in renal patients. To this purpose, better insights in the pathophysiology of CKD and the effects of current renoprotective intervention strategies are warranted.

The renin-angiotensin aldosterone system (RAAS) plays an important role in the initiation and progression of CKD predominantly via angiotensin (Ang) II (textbox 1). Ang II generates intrarenal hemodynamic and inflammatory changes that promote proteinuria, growth of glomerular and tubular cells, inhibit NO synthesis, stimulate extracellular matrix synthesis and induce chemokines, reactive oxygen species and apoptosis. Pharmacological blockade of the RAAS, as outlined below, is therefore a main tool in renoprotective intervention.

The main clinical risk factors for progressive renal function loss in both diabetic and non-diabetic renal diseases are blood pressure, and in particular, proteinuria. Proteinuria was formerly assumed to be a mere reflection of the severity of renal damage. However, currently, it is well-established that proteinuria triggers a sequence of events promoting further renal damage, thereby contributing to a vicious circle of ongoing renal function loss (textbox 2). Therefore, renoprotective therapies aim at optimal reduction of proteinuria.

RAAS blockade: benefits and limitations

Pharmacological blockade of the RAAS, by Angiotensin-converting enzyme (ACE) inhibitors or Angiotensin receptor type 1 blockers (ARB), reduces blood pressure, and moreover is the most powerful antiproteinuric treatment currently available. In the 1980’s the idea behind the mechanism of action of ACE inhibition was fairly straightforward. ACE inhibitors block the conversion of Ang I to Ang II, thereby decreasing blood pressure. In addition to its antihypertensive properties, ACE inhibitors also decrease intraglomerular pressure, improve glomerular barrier size selectivity and consequently reduce proteinuria. Indeed, a tremendous body of research, both clinical and experimental, unequivocally showed that ACE
inhibitors slow progression of renal function loss more effectively than other non-RAAS blocking antihypertensive therapies. Several measures can be taken to improve the effect of RAAS blockade and optimize renoprotective therapy, such as increasing the dosage of ACE inhibitors and combining ACE inhibitors with ARBs. Furthermore, it is known that correction of volume overload or induction of mild volume depletion by diuretics and/or dietary sodium restriction enhances the therapeutic efficacy of ACE inhibitors on the intermediate parameters proteinuria and blood pressure.

Although RAAS blockade has been shown to improve renal outcome in most studies, prognosis in CKD is still poor, as generally RAAS blockade only delays, but not prevents ESRD. In the RENAAL study for instance, postponement of renal endpoints by ARB was approximately 18 months. Thus, in spite of a clear-cut response to RAAS blockade, reflected by reduction of blood pressure and proteinuria, the protection against ongoing renal damage is incomplete in many cases.

This raises the question why renoprotection by RAAS blockade so often fails in the end. Animal studies from our lab and others suggest that RAAS blockade slows down progression of glomerular injury, but might be insufficiently effective in slowing down interstitial fibrosis, even when it exerts a powerful antiproteinuric effect. The predictive effect of morphological characteristics of renal damage for long term outcome has been less extensively documented than the predictive effects of proteinuria, at least in human. Yet, the available data is highly consistent, showing that progression of interstitial fibrosis over time is directly correlated with renal function loss and predicts renal outcome.

In human, it is usually not feasible to monitor renal structural damage during therapy. Consequently, the intermediate parameters proteinuria and blood pressure are the only clinically available criteria to monitor therapy response. Whereas tubulointerstitial injury and glomerular damage often go together, this may not be invariably so. In experimental models such as adriamycin nephrosis and experimental transplantation the responses of blood pressure and proteinuria to RAAS blockade, while nicely reflecting glomerular protection, were dissociated from the tubulointerstitial findings. This suggests that during RAAS blockade, progressive interstitial injury could go largely undetected from clinical parameters. The mechanism underlying the discrepancy between the clinical and glomerular parameters on the one hand, and the interstitial fibrosis on the other is not understood. If these findings are applicable to human, it could well be an explanation for limited long term efficacy of RAAS blockade to prevent ESRD. It is therefore extremely important to unravel the pathophysiological mechanisms underlying this phenomenon.
Novel insights in the RAAS

In the last decade it became clear that the RAAS is far more complicated than previously thought and that there is more to ACE inhibitors than simply inhibiting Ang II formation. Recently, novel players in the RAAS have been recognised, including new metabolising enzymes (e.g. ACE2) and smaller angiotensin peptides [e.g. Ang(1-7)] that are biologically relevant and can modulate and antagonize the effect of Ang II (figure 1). Effects on these components might thus be relevant to the efficacy of RAAS blockade.
**A second arm in the RAAS**

Ang(1-7) has vasodilator and anti-proliferative properties and can be formed from Ang I via subsequent cleavage by ACE and ACE2, or directly from Ang I by endopeptidases (figure 1). Ang(1-7) has been proposed to contribute to the beneficial effects of RAAS blockade, as both systemic and tissue Ang(1-7) are considerably elevated during treatment with ACEi or ARB20-32.

An important novel enzyme involved in the generation of Ang(1-7) is ACE233,34. ACE2, a homologue of ACE, functions as a carboxymonopeptidase cleaving Ang I into Ang (1-9), which can subsequently be converted to Ang(1-7) by ACE33,34 (figure 1). ACE2 can also directly break down Ang II to generate Ang(1-7)33,34. ACE2 acts in a counter-regulatory manner to ACE by shifting the balance between Ang II and Ang(1-7). Initially, it was hypothesized that a disrupted balance between ACE2 and ACE would lead to hypertension and end organ damage35. Various studies indeed provided evidence that ACE2 is implicated in cardiovascular and renal (patho)physiology, diabetes, pregnancy and lung disease36-39. However, its precise role in organ pathology and therapy response is still under investigation. Unexpectedly, ACE2 also serves as the cellular receptor for the severe acute respiratory syndrome (SARS) virus40.

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**Figure 1.** Schematic outline of the renin-angiotensin aldosterone system (RAAS)
Altogether, the RAAS is no longer considered a single-arm cascade, but rather a two-arm cascade, with a delicate balance between both arms. Ang(1-7) and ACE2 are implicated in what is considered the beneficial arm, namely the ACE2-Ang(1-7)-mas receptor axis, that opposes the effects of the detrimental arm of the RAAS i.e. the ACE-Ang II-AT₁ receptor axis. The balance between these two opposing arms will ultimately determine its net effects on (patho)physiological processes. Thus, it might be fruitful to consider the effects of RAAS blockade from the perspective of the balance in this two-arm system. It has been suggested that modulation of the balance between intrarenal ACE and ACE2 with consequent low levels of Ang II and high levels of Ang(1-7) contributes to the renoprotective mechanisms of RAAS blockade. If so, it is not only reduction of Ang II that matters, but also the effects of the RAAS blockade on the opposing arm.

**Textbox 2. Proteinuria and tubulointerstitial fibrosis**

Chronic kidney disease can result from a wide variety of causes, however, an increased intraglomerular pressure is a key event in the progression of renal disease. According to Brenner et al, after an initial renal pathological event, the loss of nephrons results in a compensatory response of the remaining nephrons, which consists of an increase in intraglomerular pressure that facilitates hyperfiltration. However, an increased intraglomerular pressure also results in abnormal loss of proteins in the urine (proteinuria), glomerulosclerosis and, on the long-term, gradual decline of glomerular filtration rate (GFR).

Proteinuria is thus considered as a marker of glomerular damage, but also plays a direct role in the progression of renal disease by causing tubulointerstitial injury as it passes down the tubular lumens. In reaction to plasma proteins, tubular epithelial cells become activated and release a variety of chemokines (monocyte chemoattractant protein-1 and osteopontin), which attract inflammatory cells to the renal interstitium and lead to their activation. Macrophages contribute to interstitial injury by releasing growth factors, reactive oxidant species and cytokines. This inflammatory reaction induces apoptosis and flattening of tubular epithelial cells (atrophy). Moreover, through release of cytokines local peritubular and bone-marrow derived fibroblasts are stimulated to proliferate. During this process they express contractile proteins resulting in a conversion to a myofibroblast phenotype. Some tubular epithelial cells transdifferentiate and migrate to the interstitium to transform into myofibroblasts, a process called epithelial mesenchymal transformation (EMT). Further in the process of activation these interstitial myofibroblasts produce collagen and other extracellular matrix proteins, which ultimately results in tubulointerstitial fibrosis and loss of peritubular capillaries. Several studies showed that the severity of tubulointerstitial disease correlates with renal function loss and predicts the long-term renal outcome.

Thus, proteinuria is cardinal in the progression of glomerular and interstitial damage and therefore renoprotective therapies primarily focus on the reduction of urinary protein excretion. The RAAS is an important mediator of proteinuria and the progression of renal damage and is considered an important target for renoprotective intervention.
Bypassing ACE, Ang II and the AT\textsubscript{1} receptor

The limited effectiveness of RAAS blockade may be explained by several novel factors in the RAAS that provide potential escape mechanisms. A substantial proportion of Ang II is generated from Ang I by enzymes other than ACE, such as chymase and endopeptidases, which are not susceptible to ACE inhibition\textsuperscript{41}. This explains the rationale for using ARBs, because ARBs can inhibit binding of Ang II to \textit{AT}_{1} receptors\textsuperscript{42}. Nevertheless, in contrast to ARBs, ACE inhibitors have several additional beneficial effects; they increase plasma bradykinin and other substances catabolized by ACE, such as N-acetyl-Ser-Asp-Lys-Pro (AcSDKP), which has strong antifibrotic properties\textsuperscript{43,44}. Current renoprotective strategies aim at preventing the fibrotic actions of Ang II. However, Ang II is not the only effector hormone of the RAAS. The mineralocorticoid hormone aldosterone, which is stimulated by Ang II, is a potent inducer of endothelial dysfunction and stimulates inflammation, proteinuria and fibrosis\textsuperscript{45}. ACE inhibitors induce an acute decrease in plasma aldosterone. This effect, however, is usually transient and followed by a progressive rise in aldosterone levels despite continued therapy. During long term ACE inhibition, plasma aldosterone can ultimately reach normal or even elevated concentrations, especially during low-sodium intake\textsuperscript{46}. The presence of some potassium retention may contribute to this “aldosterone escape”. Considering the evidence for pro-fibrotic effects of aldosterone, this aldosterone escape may well contribute to ongoing renal function loss during RAAS-blockade. Aldosterone antagonist therapy may therefore provide additional renoprotection during ACE inhibition or ARB.

\textbf{Figure 2.} Renin and prorenin binding to the (pro)renin-receptor (PRR), with subsequent activation of intracellular MAP-kinases ERK 1/2, which initiates transcription of PAI-1 and TGF-\(\beta\) independent of Angiotensin (Ang) II and the \textit{AT}_{1} receptor. Furthermore, receptor bound prorenin is non-proteolytically activated and can convert angiotensinogen to Ang I.
The discovery of a novel (pro)renin-receptor provides another possible escape mechanism during ACE inhibition and ARB. (Pro)renin levels are elevated during ACE inhibition and ARB due to interruption of the negative feedback between Ang II and renin. Binding of renin to this (pro)renin-receptor induces a 4-fold increase of the catalytic efficiency of angiotensinogen conversion to Ang I (figure 2). Moreover, receptor-bound prorenin is non-proteolytically activated and becomes fully enzymatically active (figure 2). This indicates that the (pro)renin-receptor may be involved in Ang II formation on the cell surface, allowing Ang II to bind immediately to AT₁ receptors. In addition, receptor bound (pro)renin activates profibrotic pathways via second messenger pathways independent of angiotensin II generation or action (figure 2). Experimental data derived from transgenic rats confirmed the link between overexpression of the (pro)renin-receptor and cardiovascular and renal pathology, possibly involving direct activation of the receptor by (pro)renin. Thus, a physiological role for (pro)renin might be established, which is important in view of the high (pro)renin levels during ACE inhibition. It is still unknown how the (pro)renin-receptor is regulated and whether high substrate conditions influence its expression. Although the profibrotic actions of the (pro)renin-receptor have been established in vitro, animal and human data are still lacking. Taking together all these mechanisms, one can see that there are several new insights in the actions of RAAS blockade that might explain its limited effectiveness.

**Aim and scope of the thesis**

The effects of ACE inhibitors have been studied extensively and its renoprotective effects are widely accepted. However, as noted above, renal function loss is progressive in many patients despite this powerful antiproteinuric treatment, suggesting ongoing renal damage due to disease progression, escape mechanisms during ACE inhibition or other unknown effects. The overall aim of this thesis is to study possible underlying pathophysiological mechanisms of limited renoprotection by ACE inhibitors during disease. The recent discovery of novel components within the RAAS (ACE2 and (pro)renin-receptor) might allow for new insights into the mechanism of action of ACE inhibitors and resistance against their renoprotective effects.

The first part of this thesis aims to dissociate the favourable effects of ACE inhibitors from their possible detrimental effects on kidney structure and function. In chapter 2 we test the hypothesis that ACE inhibitors, be it or not in combination with dietary sodium restriction, can exert adverse renal effects, i.e. can induce renal interstitial fibrosis and vascular abnormalities, despite reduction of proteinuria. Our hypothesis is based on the fact that the intermediate parameters proteinuria and blood pressure do not always correspond with better preservation of
renal interstitial morphology in experimental models, such as adriamycin nephrosis\textsuperscript{26,27} and experimental transplantation\textsuperscript{28}. The precise mechanism for these discrepant experimental findings is not completely understood. To dissociate between renal abnormalities attributed to specific characteristics of the renal disease and to the ACE inhibitor regimen itself, we treated both nephrotic and healthy rats with the ACE inhibitor lisinopril in combination with a normal or a low sodium diet and investigated renal glomerular, interstitial and vascular structures. In this study it turned out that indeed, the therapeutic regimen of ACE inhibitor plus low sodium diet induces renal interstitial lesions.

Chapter 3 investigates the functional impact of these adverse renal effects of ACE inhibitors in healthy rats. From experimental studies it is known that subtle renal interstitial injury of various origins, such as Ang II, cyclosporine and hypoxia, can lead to development of salt-sensitive hypertension\textsuperscript{54}. The renal lesions induced by ACE inhibition plus low sodium intake (chapter 2) are characterized by tubulointerstitial inflammation and fibrosis and mimic those seen after Ang II infusion. We therefore speculate that rats treated with ACE inhibition and a low sodium diet will develop salt-sensitive hypertension to the same extent as in the Ang II model. To this purpose, healthy rats were treated with these particular regimens for three weeks, followed by 8 weeks of high salt feeding. Besides blood pressure, renal structural and functional parameters were monitored.

In the second part of this thesis we will investigate whether knowledge on ACE2 can provide novel insights into the mechanisms of renal damage, and of the therapeutic action of ACE inhibitors. The role of ACE2 during ACE inhibition is still unclear, but Ang\textsubscript{(1-7)} has been proposed to contribute to the beneficial effects of RAAS blockade, as both systemic and tissue Ang\textsubscript{(1-7)} are considerably elevated during treatment with ACE inhibition\textsuperscript{29-32}.

First, in chapter 4 an overview of available information on genetic, structural and functional properties of ACE2 is presented. Its role in a variety of (patho)physiological conditions will be discussed and possible therapeutic options will be reviewed. It is known that ACE2 mRNA is present in virtually all human organs, including the kidney. However, the ACE2 protein expression pattern is largely unknown. Moreover, no data on expression of ACE2 in human renal disease is available so far. ACE2 has been identified as the functional receptor for the SARS coronavirus. Since identifying the possible route of infection has major implications for understanding the pathogenesis and future treatment strategies for SARS, we investigated ACE2 protein expression in various human organs in chapter 5. Moreover we studied ACE2 expression in renal biopsies from 58 patients with diverse primary and secondary renal diseases in chapter 6.

The reports on regulation of renal ACE2 in experimental models differ from each other and from those in human renal diseases. Some authors speculate that ACE and ACE2 localization and expression is regulated in parallel or synergistically\textsuperscript{55-57}. However, the concept of parallel
regulation has been questioned by others. To clarify the regulation of renal ACE2 and ACE, we investigated renal ACE and ACE2 expression during conditions of endogenous (low sodium diet) and exogenous (ACE inhibition) changes in RAAS activity (chapter 7).

The studies described in the third part of this thesis focus on the role of the newly identified (pro)renin-receptor as possible mediator of renal damage in high renin conditions. It is unknown how the (pro)renin-receptor is regulated in vivo, especially in high renin conditions. Therefore, we examined the regulation of both renin and the (pro)renin-receptor in the clipped kidney of Goldblatt rats (chapter 8). Moreover, the effect of blood pressure reduction by vasopeptidase inhibition on renin and (pro)renin-receptor in the clipped kidney of Goldblatt rats was studied in this chapter. Vasopeptidase inhibition increases renin further and induces renal damage in the clipped kidney.

RAAS blockade interrupts the normal feedback suppression of renin secretion from the kidneys, which results in elevated plasma renin levels during ACE inhibition, especially during dietary sodium restriction. For a long time it has been thought that the reactive rise in circulating (pro)renin during RAAS blockade had no deleterious effects. However, these increased levels of (pro)renin may exert profibrotic effects via activation of the (pro)renin-receptor independent of Ang II, and thus limit the eventual renoprotective benefit of these drugs by their antihypertensive and antiproteinuric effects. In chapter 9 we studied the possible profibrotic actions of the (pro)renin-receptor in the kidney during ACEi and strongly elevated renin levels induced by low sodium intake in healthy rats.

Finally, the results from the above studies are summarized and their implications discussed in chapter 10. Furthermore, this chapter will provide recommendations for future studies.
References


