Protein kinase signaling

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CHAPTER 1

General introduction & Aims of the thesis

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Hypertensive renal damage: pathophysiology and prevention
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The incidence and prevalence of end-stage renal disease (ESRD) are increasing worldwide, forcing growing numbers of people to start dialysis or undergo kidney transplantation (1). In the United States, the incidence of ESRD increased dramatically within the last decades, although the curve seems to have become less steep within the last years (Figure 1, gray bars). Data from the Netherlands indicate a parallel trend, albeit fortunately at a substantially lower level (www.renine.nl). The prevalence of ESRD increases steadily, mainly due to the aging population and the drastically reduced cardiovascular mortality (Figure 1, black bars), also in renal patients. Based on demographics, i.e. the ageing population and the emerging epidemic of type 2 diabetes mellitus, further increases in ESRD are anticipated, requiring substantial financial and human resources to care for these patients in the future (2). Together, these alarming data underline the need to develop novel powerful interventions in order to combat renal disease.

Antihypertensive treatment has been the cornerstone of renoprotective intervention over the last decades. In addition, it was increasingly recognized that reduction of proteinuria is a treatment target in itself, and that blood pressure reduction could result in lowering of proteinuria, slowing progression of renal damage. However, although improved blood pressure control importantly contributed to a strong reduction of cardiovascular mortality, it has not prevented increasing numbers of patients to develop ESRD (Figure 1).

Figure 1. Trends of ESRD incidence and cardiovascular mortality in the US
Age-adjusted incidence of ESRD (gray bars) and age-adjusted cardiovascular mortality (black) over the last decades in the United States. ESRD data were adopted from the United States Renal Database System (www.usrds.org). Cardiovascular mortality data are from the Global Cardiovascular Infobase (www.cvdinfobase.ca); originally derived from the World Health Organization (www.who.int).
Furthermore, blockade of the renin-angiotensin-aldosterone system (RAAS) has been identified as a powerful tool to reduce proteinuria and preserve renal function in both non-diabetic (3) and diabetic patients (4-6). Currently, angiotensin converting enzyme (ACE) inhibitors and AT1 receptor blockers are widely used in subjects with renal disease to delay or prevent development of end-stage renal disease. In animal models, RAAS blockade can even provide regression of renal damage (7).

Yet, in spite of the availability of powerful tools such as RAAS blockade progression of renal function loss towards ESRD can apparently not be prevented in many patients. Improved RAAS blockade through dosage increase (8), combined ACE inhibition and AT1 receptor blockade (9), or addition of a low-salt diet (10) are advocated to provide optimal renoprotection, based on the response of the intermediate parameters blood pressure and proteinuria, and hopefully, better implementation of these measures will result in improved renoprotective efficacy. However, individual titration for maximal RAAS blockade is associated with frequent side-effects and poor tolerability (11). Moreover, although low sodium diet potentiates the reduction of blood pressure proteinuria by RAAS-blockade, in experimental studies this combination is associated with adverse renal effects in healthy and proteinuric rats (12). Thus, long term studies in human are required to substantiate improved renal outcome. Third, pre-treatment renal damage, which is often present in renal patients at presentation, blunts the efficacy of RAAS blockade, even when it is still mild (13). Taken together, it is unsure whether the currently available intervention strategies will be sufficient to eliminate progressive renal function loss. Novel, alternative and/or additional modes of intervention are therefore needed, directly targeting the pathophysiological pathways involved in progressive renal structural damage. Better insights into the molecular mechanisms of progressive renal damage can be a powerful tool to identify novel targets for intervention.

In progressive renal disease, angiotensin II (AngII) plays a central role, not only through its effect on vascular tone, but also as a growth factor and a pro-inflammatory mediator. In response to tissue damage, renal levels of AngII increase and may up-regulate the expression of other factors such as transforming growth factor-β1 (TGF-β1), tumor necrosis factor-α (TNF-α), osteopontin, and vascular cell adhesion molecule-1 (VCAM-1), in order to
repair tissue damage (14). If increased renal levels of AngII are sustained, these repair mechanisms can be deranged and result in renal inflammation and fibrosis. The currently available interventions in renal disease (e.g. ACE inhibitors, AT1 receptor antagonists) predominantly act through drug-target interactions at extracellular (or receptor) level. This thesis investigates whether *intracellular* signaling molecules are suitable targets in renal disease. At the cellular level, the expression of chemokines, cytokines, adhesion molecules and matrix proteins in response to, for example, binding of AngII to the AT1 receptor, is regulated by complex networks of signal transduction molecules (Figure 2). As such, intracellular signal transduction molecules are messengers that concertedly regulate cellular actions such as proliferation, chemoattraction or matrix production, in response to extracellular stimuli.

*Figure 2. Diagram illustrating intracellular protein kinase signaling*  
Protein kinases, e.g. MAP kinases, can be activated by a stimulus (e.g. receptor binding). Upon activation, protein kinases can be transported to the nucleus or interact locally with cytoplasmic proteins. In the nucleus, activated kinases phosphorylate (i.e. activate) various transcription factors, resulting in modulation of gene expression (associated with proliferation, apoptosis, inflammation etc). The arrows indicate the various levels of intervention in renal disease: ACE inhibitors reduce formation of angiotensin II, AT1 receptor blockers reduce binding of angiotensin II to the AT1 receptor, and protein kinase inhibitors reduce intracellular signaling downstream of the AT1 receptor.
Protein kinase signaling in the kidney

This thesis will focus on the role of protein kinases, an extensive and diverse group of signal transduction molecules, in renal disease. Protein kinases are crucial to many physiological functions of the normal cell, however increased activity of various protein kinases has been associated with pathological mechanisms such as inflammation and fibrosis (15). Through activation of transcription factors, protein kinases regulate the expression of a large number of genes (Figure 2), which can have either beneficial or detrimental effects. We hypothesized that modulation of the activity of specific kinases would reduce expression of pathophysiologically relevant genes, and hence, renal damage.

Protein kinases are intracellular signal transduction enzymes that can attach a phosphate group to target proteins, using kinetic energy (therefore termed “kinases”). Their main function is to connect cell-surface receptor signals to intracellular effects such as modulation of gene expression (Figure 2) (16). Protein kinases can be activated by a wide range of events such as receptor ligation (e.g. AngII – AT1 receptor), hormones, cytokines, or various types of stress. Interestingly, protein kinases display specificity towards their substrates, although strictness of specificity may vary per kinase. Substrate phosphorylation may result in altered enzymatic activity of the target protein, affecting its interactions with other proteins, cellular location, or degradability by proteases. Target proteins include other protein kinases, phospholipases, transcription factors, and cytoskeletal proteins (17).

One of the best-defined groups of protein kinases is the mitogen-activated protein (MAP) kinase superfamily, consisting of p38 MAP kinase, extracellular signal-regulated kinase (ERK) and c-Jun N-terminal kinase (JNK) (Figure 3). MAP kinases are expressed in normal cells since they are involved in various physiological cellular processes such as proliferation or cell growth. Moreover, since MAP kinases are also operative downstream of the AT1 receptor, they may play a role in the renal effects of AngII. AngII, but also growth factors such as TGF-\(\beta\) are able to activate p38, ERK and JNK in various renal and non-renal cell types, modulating expression of genes involved in inflammation and proliferation through activation of transcription factors (e.g. c-Jun) (Figure 3). Vice versa, increased expression of inflammatory
Figure 3. Overview of mitogen-activated protein (MAP) kinase signaling cascades
Many different types of receptors are able to induce MAP kinase activation through activation (i.e. phosphorylation) of MAP kinase kinase kinases, which activate MAP kinase kinases, activating in turn MAP kinases. Downstream of MAP kinases, the actual regulation of gene expression mainly occurs through the activation of transcription factors by MAP kinases.

and fibrotic factors can (further) induce MAP kinase activation (15). Through such autocrine or paracrine positive feedback loops, protein kinase signalling may contribute to the development and/or maintenance of renal injury. As such, regardless of the initial stimulus (e.g. proteinuria, hypertension) MAP kinases play a role in general mechanisms (fibrosis, inflammation) contributing to renal injury. Thus, MAP kinase inhibition could be beneficial in a wide range of renal diseases. Indeed, their activity is strongly increased in areas of renal
damage, and inhibition of MAP kinase activation is renoprotective in models of inflammatory and fibrotic renal disease (15).

In human renal disease, MAP kinases p38 and ERK are strongly activated compared with normal renal tissue, and their activities correlated to parameters of renal injury (18;19). However, it is unknown whether activation of the MAP kinase JNK or its downstream transcription factor c-Jun is also associated with human renal disease.

Thus, intracellular protein kinase signaling may be a target to reduce inflammatory and fibrotic renal damage. Whereas recent studies indicate that inhibition of p38 MAP kinase or ERK can indeed ameliorate inflammatory and/or fibrotic renal injury (20;21), their role in AngII-mediated renal damage is unclear. Furthermore, little is known about the role of the JNK pathway in renal disease.

**Aim and scope of the thesis**

The overall aim of this thesis is to address the role of protein kinases in progressive renal damage. To this purpose, the first part of this thesis focuses on the identification of protein kinases that may be involved in renal damage (**Part I: Activation of protein kinase in renal disease**). **Chapter 2** presents an overview of the various functions of MAP kinases, their inducers and targets *in vitro* and *in vivo*, and the identified associations with renal damage in patients. Like most protein kinase pathways, MAP kinase pathways are very complex, since cross-talk is common. Thus, by studying the role of a single pathway in renal disease, the complexity of kinase signaling pathways is underestimated. Novel technologies to study multiple kinases activities simultaneously may provide more insight in the complex mechanisms of protein kinase signaling.

We have used such a novel technique, namely a kinase array which has been developed to simultaneously study kinase activities of over 1100 kinases (**chapter 3**). Thus far, this technology has only been used in cell culture studies in single cell type systems. We used this kinase array to study the activities of a large number of kinases (the “kinome”) in renal cortical tissue. We applied this novel technology to renal tissue from the homozygous Ren2
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model, a renin transgene rat model with Sprague Dawley background, characterized by angiotensin II-mediated renal damage. We compared the kinome profile of untreated Ren2 rats to both untreated Sprague Dawley control rats and Ren2 treated with an ACE inhibitor. In this manner, we tested whether our kinase array could detect protein kinases relevant to renal damage within our experimental model.

By arraying techniques, protein kinases activities can be associated with the degree of renal damage and thus candidate protein kinases can be selected. However, specific inhibition is required to establish whether a given protein kinase is actually involved in renal pathophysiology. The studies described in the second part of this thesis (Part II: Mitogen-activated protein kinase inhibition in renal disease) include interventions at several levels and in various in vivo and in vitro experimental settings relevant to renal damage. Since p38 MAP kinase was identified by the kinase array as potentially involved in AngII-mediated renal damage, and since MAP kinases are operative downstream of the AT1 receptor, we treated Ren2 rats, characterized by AngII-mediated glomerular and tubulointerstitial damage, with specific MAP kinase inhibitors.

First, we questioned whether p38 or ERK blockade would be able to reduce glomerular damage in the Ren2 model (chapter 4). To this extent, we quantified mesangial matrix expansion, but also subtle markers of mesangial (α-smooth muscle actin, SMA expression) or podocyte (desmin expression) injury in Ren2 (untreated and treated with p38 or ERK inhibitor) and control rats. Next, we studied effects of MAP kinase inhibition on tubulointerstitial damage in the same model (chapter 5). The effects of specific p38 inhibition on tubulointerstitial fibrosis and subtle markers interstitial SMA, osteopontin and Kim-1 (Kidney Injury Molecule-1), a recently identified marker of tubular injury, were investigated. We hypothesized that intervention with a p38 MAP kinase inhibitor would reduce tubular epithelial cell injury and tubulointerstitial fibrosis.

Whereas the role of p38 and ERK in renal damage has been subject of various studies, less is known about the third MAP kinase family member JNK (c-Jun N-terminal kinase) in renal disease. In non-renal cell types, JNK mediates expression of monocyte chemoattractant protein-1 (MCP-1), underlining its advocated role in inflammation. We addressed the question
whether JNK is involved in renal tubular MCP-1 expression and, subsequently, interstitial macrophage accumulation (chapter 6). This was studied in cultured tubular epithelial cells and in vivo. The renal expression of activated JNK and c-Jun, an important downstream transcription factor activated by JNK, was determined over time in the unilateral ischemia/reperfusion model, a model of tubular injury accompanied by interstitial macrophage accumulation. Furthermore, we intervened with a specific JNK inhibitor in the same model and studied its effect on renal MCP-1 expression and interstitial macrophage accumulation. Finally, we hypothesized that the numbers of tubular cells expressing activated JNK (pJNK) would be associated with renal macrophage accumulation and other parameters of renal damage in human renal disease.

In a separate study, we questioned whether renal activation of the transcription factor c-Jun, a major substrate of JNK would be associated with the severity of renal injury in patients (chapter 7). We studied the expression of activated c-Jun (pc-Jun) in biopsies from patients with various renal diseases and correlated this with parameters of renal disease including estimated glomerular filtration rate (eGFR), histopathological parameters such as focal glomerulosclerosis, and renal macrophage accumulation. In addition, we performed in vitro studies to investigate whether upstream inhibition of c-Jun activation would affect expression of inflammatory and fibrotic genes.

Results from the above studies will be summarized and its implications will be discussed in chapter 8. Furthermore, this chapter will provide directions for future studies on the role of protein kinase signaling in renal disease.

References


