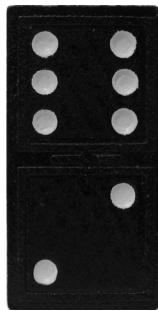


CHAPTER 8



Summary, General discussion and Future directions

Summary

The incidence and prevalence of end-stage renal disease have steadily increased within the recent decennia despite the development of powerful pharmacological tools such as ACE inhibitors and AT1 receptor blockers. Hence, it is required to identify additional targets for intervention in renal disease. Current therapeutic strategies aiming to limit progression of renal disease mainly act through drug-target interactions at *extracellular* (or receptor) level (e.g. ACE inhibitors, AT1 receptor antagonists). This thesis addresses the question whether *intracellular* signaling molecules, and especially protein kinases, could be suitable targets for intervention in renal disease.

Part I of this thesis addresses the various functions of protein kinases in the kidney, both under normal and under disease conditions, and strategies to investigate their role. Protein kinases connect signals outside the cell (e.g. receptor ligation) to actions within the cell (e.g. gene transcription). By these means, protein kinases are physiological messengers involved in signaling pathways regulating virtually all physiological mechanisms. One of the most extensively studied groups of protein kinases are the mitogen-activated protein (MAP) kinases, as reviewed in **chapter 2**. The main members of the MAP kinase superfamily are p38, extracellular signal-activated kinase (ERK) and c-Jun N-terminal kinase (JNK). MAP kinases are present in all renal cells types. Stimuli that can induce MAP kinase activity *in vitro* include growth factors like EGF and PDGF, cytokines like TNF- α and IL-1 β , hormones like angiotensin II, insulin, bradykinin, and various types of stress including osmotic, mechanic and oxidative stress (1). Although most of these factors can induce p38, ERK and JNK, relative specificity towards one MAP kinase pathway is common. Upon activation, MAP kinases can either interact with proteins in the cytoplasm or transduce signals to the nucleus (generally through activation of transcription factors). In normal cells MAP kinases play a role in regulation of physiological functions. Under disease conditions, MAP kinase activation is often strongly increased, suggesting that modulation of this activation could have therapeutic potential. Selective blockade of MAP kinase family members has become possible with the availability of highly specific inhibitors. Several recent studies have demonstrated that MAP kinase inhibition reduces renal damage in various models including experimental

glomerulonephritis and renal fibrosis, suggesting that MAP kinases actively modulate renal disease.

However, protein kinases display many interactions, forming complex signaling cascades. Therefore, it might be more fruitful to consider multiple protein kinases simultaneously, rather than single kinases. For instance, inhibition of one kinase can be compensated by parallel pathways, or can adversely affect other pathways. One example *in vivo* has been presented in the remnant kidney model, where administration of a p38 inhibitor did not improve but worsened renal function, proteinuria, glomerulosclerosis and tubulointerstitial injury (2). The ERK pathway was highly activated in kidneys of rats treated with the p38 inhibitor, thus, it was concluded that p38 inhibition induced ERK activation in this model. Although alternative mechanisms were not excluded in that study, cross-talk between kinase pathways has indeed been widely established *in vitro*. Hence, it would be crucial to study not just the activity of one particular kinase, but rather a more extensive kinase profile.

Profiling techniques, allowing to address patterns rather than single factors have emerged over the last years for several biological levels (i.e. genomics, proteomics). In line with these developments, an array technique has been developed allowing simultaneous analysis of a large number of kinases (**chapter 3**). The array, containing 1100 peptide sequences specific to known kinase substrates spotted on a chip, can be exposed to renal lysates, measuring the enzymatic activity per particular kinase. In this manner, the chip can profile activities of signaling pathways, as had already been demonstrated in isolated inflammatory cells and adipocytes (3T3-L1) (3;4). We have been the first to use this kinase array on renal tissue, to profile a considerable part of the renal "kinome" (i.e. all mammalian kinases). We profiled the kinase activities in lysates from homozygous Ren2 rats, characterized by a strongly activated renin-angiotensin-aldosterone system, hypertension and AngII-mediated renal damage. We found that p38 MAP kinase and PDGFR β were increased in untreated Ren2 compared to control rats, which was reversed by ACEi. These findings were both confirmed by conventional techniques and supported by the literature since p38 MAP kinase inhibition and PDGFR β blockade both provide renoprotection in the homozygous Ren2 model (5;6).

Although identification of increased kinase activation states may suggest their involvement in renal disease, specific intervention is required to establish a functional role. **Part II** addressed

the effects of intervention with specific MAP kinase inhibitors in renal disease. Selective inhibition of each of the MAP kinase family members (p38, ERK, JNK) has been studied in experimental renal disease and *in vitro* in renal cells.

In **chapters 4 and 5**, we investigated whether treatment of homozygous Ren2 rats with either a p38 or an ERK inhibitor would reduce glomerular and tubulointerstitial damage. The homozygous Ren2 model was chosen since p38 and ERK are operative downstream of the AT1 receptor and the RAAS is highly activated in these animals. We demonstrated reduced markers of subtle glomerular injury (e.g. desmin expression and mesangial matrix expansion) by p38 or ERK inhibition, compared with untreated Ren2. Moreover, treatment with a p38 inhibitor reduced the tubulointerstitial expression of α -smooth muscle actin, osteopontin and Kim-1, as well as interstitial fibrosis; thus, p38 inhibition reduced tubulointerstitial injury in the Ren2 model. These effects of MAP kinase inhibition were independent of blood pressure. Together, they suggest that MAP kinase inhibition has renoprotective potential in angiotensin II-induced renal damage.

The role of the JNK pathway in renal disease is discussed in **chapters 6 and 7**. Although the role of ERK and p38 activation has been studied in various renal models, much less is known about JNK. In **chapter 6** we describe that in renal biopsies from patients with various renal disorders, JNK is activated in both glomerular and tubular cells. The extent of tubular JNK activation correlated with interstitial macrophage accumulation and other parameters of tubulointerstitial damage. To investigate whether these associations reflect pathogenetic mechanisms, we further addressed the role of JNK in experimental renal disease. In the rat unilateral ischemia/reperfusion (I/R) model, we studied the time course of JNK and c-Jun (a transcription factor and major JNK substrate) activation. We found that already at 30 minutes after I/R, JNK and c-Jun were strongly activated in tubular epithelial cells. The extent of tubular JNK and c-Jun activation over time correlated with interstitial macrophage accumulation. Moreover, in the I/R model, the JNK inhibitor SP600125 strongly reduced renal c-Jun activation, MCP-1 gene expression and interstitial macrophage accumulation, both at 4 and 15 days after I/R. In cultured renal tubular epithelial cells, inhibition of JNK with the specific inhibitor SP600125 strongly reduced MCP-1 gene expression. Together, these data suggest that JNK is involved in MCP-1-mediated interstitial macrophage accumulation in response to tubular injury (Figure 1).

The role of activation of the transcription factor c-Jun, a major signaling target of JNK, in human renal disease is addressed in **chapter 7**. In renal biopsies of subjects with various renal disorders, we demonstrated strong induction of c-Jun activation. To identify a potential mechanism through which c-Jun may be involved in human renal disease, we studied upstream inhibition of c-Jun using the JNK inhibitor SP600125 in cultured human tubular epithelial cells. Inhibition of c-Jun activation reduced expression of MCP-1 and procollagen-1 α 1 in human tubular epithelial cells, which suggests involvement in inflammatory and profibrotic routes in these cells. The fact that *in vivo* c-Jun was also activated in minimal change disease suggests that c-Jun may be activated by proteinuria, and that activation of c-Jun may be an early event in pathophysiology, regulating the expression of genes involved in renal damage.

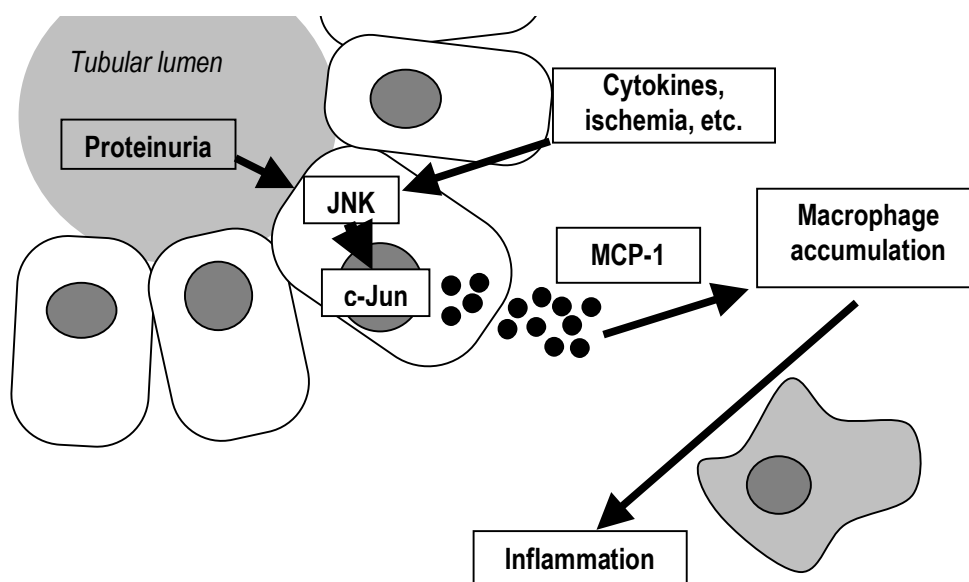


Figure 1. Proposed role for JNK activation in renal injury

Schematic representation of a proposed role for JNK activation in renal inflammation. In response to injury, e.g. as a result of proteinuria (or ischemia/reperfusion), JNK is activated in tubular epithelial cells. In turn, JNK activates the transcription factor c-Jun, which increases expression of the MCP-1 gene. Increased amounts of MCP-1 are released, resulting in the attraction of macrophages towards the interstitium, importantly contributing to renal inflammation.

General discussion and future perspectives

The studies described in this thesis provide support for a role of MAP kinases in renal damage – and suggest that intervention may have therapeutic potential. Renoprotective effects of p38 and ERK inhibition, which we demonstrated in the Ren2 model, have similarly been shown in a number of other models (7-10). In addition, we are the first to demonstrate that systemic JNK inhibition is also beneficial in the renal ischemia/reperfusion model through reducing renal inflammation. Our studies provide a conceptual sequence of events, in which JNK activation in tubular epithelial cells in response to ischemia/reperfusion results in recruitment of macrophages from the circulation through the chemoattractant MCP-1 (monocyte chemoattractant protein-1; Figure 1). The renal inflammatory response upon ischemia/reperfusion could be strongly reduced by renal blockade of JNK activity through systemic administration of a JNK inhibitor.

The current studies thus indicate that MAP kinases could be considered targets for intervention in renal disease. However, many questions remain unanswered, even when the perspective is limited to experimental renal disease. For example, the long-term effects (e.g. in chronic renal disease) of MAP kinase inhibition are still largely unknown. Furthermore, it would be important to study whether blockade of the JNK pathway also reduces glomerular injury, and if proteinuria can be ameliorated by JNK inhibition. Reduction of proteinuria and podocyte injury by a selective p38 inhibitor in experimental nephritic syndrome has already been demonstrated (7).

It is tempting to speculate on future application of MAP kinase inhibitors in clinical renal disease. Based on promising results from animal studies, renoprotective effects may also be expected in patients. However, the general role of MAP kinases in the normal cell physiology implicates that side-effects may be an important drawback for the clinical use of this type of inhibitors. Nevertheless, (non-renal) phase I and II trials with p38 inhibitors are currently ongoing, for example in patients with rheumatoid arthritis (ClinicalTrials.gov identifier NCT00303563). Moreover, in the field of oncology, powerful protein kinase inhibitors have already been used in numerous clinical trials with promising results and, surprisingly, relatively mild side-effects (11-13).

An interesting development to reduce possible side-effects is the concept of renal drug targeting. As demonstrated by studies of Prakash et al, a compound can be coupled to a carrier molecule that selectively delivers the drug to the kidney. In this manner, a p38 inhibitor has been successfully targeted to the kidney (14), moreover, renal targeting of a low single dose of a TGF- β kinase inhibitor reduced interstitial macrophage accumulation and fibrosis in the unilateral ureteral obstruction (UUO) model (15). These studies are promising, not only regarding the targeting of kinase inhibitors, as the availability of any powerful drug that could be targeted towards damaged organs without systemic side-effects would have major impact on clinical practice. Future studies will have to further evaluate the efficacy and safety of drug targeting.

Another relevant question is whether drugs that specifically inhibit kinase activation should be used instead, or on top of RAAS blockade, currently a major therapeutic strategy in renal disease. Actually, to our knowledge, no studies on the additional effect of protein kinase inhibitors on top of RAAS blockade have been published; since relevant experimental models displaying reduced efficacy of RAAS blockade are available (16), such studies may well be performed in the near future. Furthermore, it may be interesting to study combinations of protein kinase inhibitors, given the fact that inhibition of a single kinase may induce parallel signaling pathways. Arraying techniques may well be of use in the selection of appropriate protein kinases as targets for intervention under specific conditions.

In conclusion, although MAP kinase inhibition in human renal disease is currently still a bridge too far, the studies presented in this thesis indicate that p38, ERK and JNK play a central role in pathophysiological processes in the kidney. Future studies providing more insight in their functions in renal physiology and disease, especially concerning the JNK pathway, are required in order to further explore their potential roles as targets for intervention in renal disease.

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