Cell Type Specific Mechanisms of Mineralocorticoid Receptor Mediated Renal Injury

Thesis submitted to the Faculty of Medicine,
Monash University in fulfilment of the requirements for the degree of Doctor of Philosophy (PhD)

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Submitted: October 2013

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Abstract

The mineralocorticoid receptor (MR) has been found to exhibit both physiological roles in epithelial cells, and pathological roles in non epithelial kidney cells such as macrophages, podocytes and fibroblasts. Recent evidence has shown that activation of MR in these cells promotes inflammation, apoptosis and fibrosis – key pathological processes that contribute to declining renal function, albuminuria and progression of kidney disease. Antagonists of MR have been available for clinical use for decades; however their utility in renal patients has been impeded by hyperkalaemia due to tubular MR blockade. Therefore, an understanding of what cells mediate pathological effects of MR during kidney disease may allow targeted MR blockade, thus avoiding complications of salt disturbance.

In order to assess the cell specific effects of MR signalling in kidney disease, we examined renal cells that express MR, such as renal fibroblasts, macrophages and podocytes. Initially, the role of MR activation on renal fibroblast proliferation was examined *in vitro* (Chapter 2). Rat renal fibroblasts, as well as primary mouse renal fibroblasts harvested from a kidney undergoing fibrosis, were exposed to aldosterone in serum free conditions. Increased proliferation and cell counts were observed in both cell types in a dose-dependent manner. Mechanistic experiments revealed MR activation leading to transactivation of growth factor receptors and subsequent activation of mitogen-activated protein kinases, to increase fibroblast proliferation. These results suggest that

aldosterone-induced fibroblast proliferation may be another mechanism by which MR contributes to renal fibrosis.

Secondly, we investigated the role of macrophages MR signalling in models of acute glomerulonephritis and diabetic nephropathy. In the glomerulonephritis model (Chapter 3), mice with selective MR deletions in macrophages had reduced overall renal injury as determined by kidney inflammatory infiltrates, glomerular and interstitial fibrosis and preservation of renal function. The degree of renal protection from targeted macrophage MR inhibition was similar to, and often superior to that of systemic MR antagonism. Thus, our findings demonstrate an important role of macrophage MR in the pathogenesis of acute glomerulonephritis, and may be a potential target for targeted MR blockade.

Mice were then subjected to streptozotocin induced type 1 diabetes in a model of diabetic nephropathy (Chapter 4). Selective macrophage MR deletion markedly attenuated evidence of glomerular hyperfiltration, with reductions in glomerulomegaly and hypercellularity during diabetic nephropathy. Furthermore, glomerular macrophage infiltration and tubulointerstitial apoptosis were also attenuated in the knock-out mice. Despite these changes, renal function and albuminuria were not affected by macrophage MR deletion, suggesting a limited role for myeloid MR during early diabetic nephropathy.

Lastly, we examined the relative importance of podocyte MR signalling in a model of glomerulonephritis (Chapter 3). Mice with selective MR deletions in podocytes developed severe renal injury comparable to wild-type mice, with no protection seen in albuminuria, renal dysfunction or renal fibrosis. These results suggest that podocyte MR signalling plays a limited role in renal injury during acute glomerulonephritis; contrasting to that of myeloid MR signalling.

In conclusion, this series of experiments have increased our knowledge of the pathological role of MR activation in non-epithelial renal cells. Our results are unique, in that for the first time, we were able to delineate the effects of MR activation in selective cells, during kidney disease. An understanding of cell specific MR signalling may lead to cell selective MR inhibition, or its downstream signalling pathways, and avoid unwanted adverse effects such as hyperkalaemia. Not only will this improve treatment of patients with kidney disease, it may also have implications for the treatment of cardiovascular and cerebrovascular diseases.

General Declaration

Monash University

Declaration for thesis based or partially based on conjointly published or unpublished work

In accordance with Monash University Doctorate Regulation 17.2 Doctor of Philosophy and Research Master's regulations the following declarations are made:

I hereby declare that this thesis contains no material which has been accepted for the award of any other degree or diploma at any university or equivalent institution and that, to the best of my knowledge and belief, this thesis contains no material previously published or written by another person, except where due reference is made in the text of the thesis.

This thesis includes 1 original papers published in peer reviewed journals and 2 unpublished publications. The core theme of the thesis is cell type specific mechanisms of mineralocorticoid receptor mediated kidney injury. The ideas, development and writing up of all the papers in the thesis were the principal responsibility of myself, the candidate, working within the Department of Medicine, under the supervision of Dr. Greg H. Tesch.

In the case of chapter 2-4, my contribution to the work involved the following:

Thesis chapter	Publication title	Publication status*	Nature and extent of candidate's contribution
2	Aldosterone induces kidney fibroblast proliferation via activation of growth factor receptors and PI3K/MAPK signalling	Published	Performed cell culture and proliferation assays. Prepared figures, statistical analyses and writing of the manuscript. Contribution 70%
3	Mineralocorticoid Receptor Activation in Macrophages Contributes to Progressive Kidney Disease	Returned for revision	Maintained animals. Performed majority of histological and molecular biology analyses. Prepared manuscript. Contribution 65%
4	Role of macrophage mineralocorticoid receptor in early mouse streptozotocin induced-diabetic nephropathy	Manuscript in draft	Maintained animals. Performed majority of histological and molecular biology analyses. Prepared manuscript. Contribution 70%

I have not renumbered sections of submitted or published papers in order to generate a consistent presentation within the thesis.

Signed: ……

Date: ……

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Conference Presentations

2011

Australian and New Zealand Society of Nephrology 47th Annual Scientific Meeting, Adelaide, South Australia (September 2011).

***Huang L**, Nikolic-Paterson DJ, Ma FY, Tesch GH*. Aldosterone Promotes Proliferation of Cultured Renal Fibroblasts via Activation of PDGF receptor and PI3 Kinase.

Oral presentation.

2012

Australian and New Zealand Society of Nephrology 48th Annual Scientific Meeting, Auckland, New Zealand (August 2012).

***Huang L**, Nikolic-Paterson DJ, Han YJ, Ozols E, Young M, Tesch GH*. Mineralocorticoid Receptor Activation in Macrophages Contributes to Renal Injury in Progressive Kidney Disease.

Oral presentation (**Winner** of the Basic Science Young Investigator Award)

Publications

Huang LL, Nikolic-Paterson DJ, Ma FY, Tesch GH. Aldosterone Induces Kidney Fibroblast Proliferation via Activation of Growth Factor Receptors and PI3K/MAPK Signalling. *Nephron Exp Nephrol*. 2012;120: e114-e121

Huang LL, Nikolic-Paterson DJ, Han YJ, Ozols E, Young M, Tesch GH. Mineralocorticoid Receptor Activation in Macrophages Contributes to Renal Injury in Progressive Kidney Disease. *J Am Soc Nephrol*. (second revisions)

Acknowledgements

Dr. Greg Tesch (principle supervisor) for his dedication, thoughtful advice, attention to detail and strategies for keeping this thesis on time. His supervision and guidance in experimental planning and execution has provided me with insightful knowledge that I will be able to use for future medical research. **Associate Professor David Nikolic-Paterson** for his considered approach in cultivating independent thinking, hypothesis generation and humour.

Professor Peter G Kerr for his advice, constant support, and instilling the importance of medical research, which has created many opportunities for me as a Nephrologist.

Dr. Yingjie Han and **Dr. Frank Ma** for their assistance and teachings in animal experimentation and histological techniques. **Ms Elyce Ozols** for her assistance in the breeding and maintenance of mice. **Ms Naomi Woodman** for her technical assistance in matters of cell culture and molecular chemistry. **Ms Tara Robertson** and **Ms Lois Jones** for their advice, administrative and laboratory assistance.

My fellow PhD students **Dr. Jessica Ryan**, **Dr. Matthew Damasiewicz** and **Dr. Sharmila Ramessur** for their comradery, encouragements, and support throughout the PhD.

My daughter **Caitlin**, who provided me with the inspiration and courage to take bold steps forward, even in times of uncertainty.

I would like to thank my dear wife **Tracy** for her constant love, unfaltering support and encouragements that provided me with the determination to complete this thesis.

Lastly, I would like to acknowledge the generous funding from the Jacquot Research Entry Scholarship and from the NHMRC Medical post-graduate Scholarship.

Abbreviations

11β-HSD	11-beta hydroxysteroid dehydrogenase
ACEi	Angiotensin converting enzyme inhibitor
ACR	Albumin to creatinine ratio
Akt	Protein kinase B
AngII	Angiotensin II
ARB	Angiotensin receptor blocker
BASP1	Brain acid-soluble protein-1
BSA	Bovine serum albumin
CKD	Chronic kidney disease
CTGF	Connective tissue growth factor
CVD	Cardiovascular disease
DAB	3,3-diaminobenzidine
DMEM	Dulbecco’s modified Eagle’s medium
DN	Diabetic nephropathy
DNA	Deoxyribonucleic acid
ECM	Extacellular matrix
EGF	Epidermal growth factor
EGFR	Receptor for epidermal growth factor
EGTA	Ethylene glycol tetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
EMT	Epithelial to mesenchymal transition
EPHESUS	Eplerenone Post-Acute Myocardial Infarction Heart Failure Efficacy and Survival Study
EPL	Eplerenone
ERK1/2	Extracellular signal-regulated kinase

ESRF	End stage renal failure
FCS	Fetal calf serum
FSGS	Focal and segmental glomerulosclerosis
FSP-1	Fibroblast specific protein-1
GBM	Glomerular basement membrane
GCS	Glomerular cross section
GFR	Glomerular filtration rate
GN	Glomerulonephritis
HbA_{1C}	Glycated haemoglobin
HSP	Heat shock protein
ICAM-1	Intercellular adhesion molecule-1
IFN-γ	Interferon-gamma
Ig	Immunoglobulin
IRI	Ischaemic reperfusion injury
JNK	c-Jun N-terminal kinase
KO	Knock-out
LBD	Ligand binding domain
LysM	Lysozyme M
MAPK	Mitogen-activated protein kinase
MCP-1	Monocyte chemoattractant protein-1
MKK3	Mitogen activated protein kinase kinase 3
MR	Mineralocorticoid receptor
mRNA	Messenger ribonucleic acid
MyMRKO	MR gene deletion in myeloid cells
МΦ	Macrophage
NC	Negative control

NFκB	Nuclear factor-kappa B
NRK-49F	Normal rat kidney fibroblasts
PAI-1	Plasminogen activator inhibitor-1
PAP	Peroxidise-conjugated anti-peroxidase
PAS	Periodic acid Schiff's reagent
PBS	Phosphate buffered saline
PCNA	Proliferating cell nuclear antigen
PCR	Polymerase chain reaction
PDGF	Platelet derived growth factor
PDGFR	Receptor for platelet derived growth factor
PI3K	Phosphoinositide 3-kinase
PLP	Paraformaldehyde-lysine-periodate
PodMRKO	MR gene deletion in podocytes
PTC	Proximal tubular cell
RALES	Randomised Aldactone Evaluation Study
RANTES	Regulated upon activation: normal T cell expressed/secreted
RNA	Ribonucleic acid
ROS	Reactive oxygen species
SEM	Standard error of the mean
Sgk1	Serum and glucocorticoid-regulated kinase 1
SMA	Smooth muscle actin
STZ	Streptozotocin
TGF-β	Transforming growth factor-beta
TNF-α	Tumour necrosis factor-alpha
TRAIL	Tumour necrosis factor related apoptosis inducing ligand
UUO	Unilateral ureteral obstruction

VEGF	Vascular endothelial growth factor
VSMC	Vascular smooth muscle cell
WT	Wild type
WT-1	Wilms’ tumour-1

1 Literature review

1.1 Kidney disease burden

Kidney disease represents an increasing socio-economic burden on the community. Patients affected by chronic kidney disease have an increased mortality and morbidity. Restricted fluid intake, strict dietary modifications to avoid potassium, salt and phosphate dictate patients' lifestyles. Despite being able to prolong the life span of patients with end stage kidney disease through renal replacement therapies such as dialysis and renal transplantation, these treatments still have considerable limitations. In Australia and New Zealand, haemodialysis patients have a five year survival of 45% (1), while older patients experience even worse survival. The leading cause of death for dialysis patients are attributable to cardiovascular disease such as ischaemic heart disease, stroke and peripheral vascular disease. Although renal transplantation improves life expectancy, issues such as graft rejection, opportunistic infections and malignancies are its limiting factors. These factors suggest that the prevention of end stage renal failure (ESRF) is paramount in managing this illness.

1.2 Aetiology of progressive kidney disease

The commonest causes of progressive kidney diseases leading to ESRF in the industrialised world are diabetic nephropathy (DN) and glomerulonephritis (GN). With the explosive increase in non-insulin dependent diabetes mellitus to epidemic proportions, there has been a corresponding increase of patients

diagnosed with DN. In fact, it has overtaken GN as the leading cause of ESRF in the last decade (Table 1.1). Other common aetiologies include hypertensive nephrosclerosis, reflux nephropathy and autosomal dominant polycystic kidney disease.

Table 1.1 Causes of ESRF over a decade in Australia

Disease	Number (% patients)	
	2000	*2010*
Diabetic nephropathy	381 (22%)	798 (35%)
Glomerulonephritis	518 (30%)	489 (22%)
Hypertension	239 (14%)	308 (14%)
Polycystic kidney disease	109 (6%)	160 (7%)
Reflux nephropathy	89 (5%)	59 (3%)
Analgesic nephropathy	79 (5%)	37 (2%)
Others	190 (11%)	272 (12%)
Uncertain	118 (7%)	134 (6%)
Total	**1723**	**2257**

Adapted from ANZDATA Annual Report 2011

1.2.1 Diabetic Nephropathy

Diabetes mellitus from either insulin resistance, or the failure of pancreatic β-cells responsible for producing endogenous insulin, leads to hyperglycaemia. Despite extensive research, the exact pathological mechanism of DN remains unknown. The progression from having hyperglycaemia during diabetes, leading to DN involves multiple processes and is still being investigated. These include the biological effects of advanced glycation end-products (2), oxidative stress (3-4), genetic predisposition (3, 5) including angiotensin converting

enzyme I/D polymorphism (6), and environmental stressors (3). The cardinal clinical features of DN are: albuminuria, which can range from minute quantities, i.e. microalbuminuria, to overt proteinuria; and the accelerated loss of renal function over time. Patients with type 1 diabetes who also have overt proteinuria can have reductions in the glomerular filtration rate (GFR) by as much as 20 ml/min/year and have a 10-year ESRF incidence of approximately 50 percent. Type 2 diabetics with overt proteinuria have a 20% chance of reaching ESRF; however due to lead time bias, it is difficult to determine the temporal relationship. Diabetic nephropathy also has distinct histological changes that have recently been formally classified (7). These include glomerular alterations such as: glomerular basement membrane thickening; mesangial expansion; formation of nodular sclerosis or Kimmelstiel-Wilson lesions and glomerulosclerosis (Table 1.2); as well as tubulointerstitial changes of tubular atrophy, interstitial fibrosis and arteriosclerosis. Patients with DN also have a strong predisposition to vascular disease in other end organs, causing retinopathy, motor and sensory polyneuropathy, ischaemic heart disease and stroke (8-9).

Table 1.2 Glomerular classification of diabetic nephropathy

Class	Description	Criteria
I	GBM thickening	GBM >395 nm in females and >430 nm in males on electron microscopy
IIa	Mild mesangial expansion	Mild expansion in >25% of observed mesangium
IIb	Severe mesangial expansion	Expansion of mesangial area greater than mean capillary lumen area, and in >25% of observed mesangium
III	Nodular sclerosis	At least one observed Kimmelstiel-Wilson nodule
IV	Advanced glomerulosclerosis	Global sclerosis in >50% of glomeruli

Adapted from Tervaert et. al. 2010. (7)

1.2.2 Glomerulonephritis

Glomerulonephritis encompasses a group of kidney conditions that share the common pathological feature of glomerular inflammation. Although heterogeneous, GN can be classified on clinical or histological grounds. Nephritic presentations of GN are characterised by the presence of haematuria, mild to moderate proteinuria, acute loss of renal function and hypertension. Depending on the sub-type of nephritis, other clinical features such as pulmonary haemorrhage or nasal deformities can predominate – as in anti-glomerular basement membrane (GBM) GN or granulomatosis with polyangiitis. Nephrotic presentations of GN have features including moderate-severe proteinuria, oedema, hyperlipidaemia, hypoalbuminaemia and hypercoagulable state. Table 1.3 summarises the different types of GN.

Regardless of cause, GN generally cause accelerated loss of renal function. In the case of rapidly progressive GN, severe acute kidney injury occurs and can lead to profound loss of renal function, resulting in ESRF. Pathological classification of GN is complex and is determined by the appearances on light microscopy, immunohistochemical staining and in certain instances, electron microscopy. From this classification, GN can appear normal under light microscopy, but have changes on electron microscopy i.e. minimal change disease. Alternatively, descriptive names are given to GN under light microscopy i.e. mesangiocapillary GN would infer glomerular inflammation associated with both mesangial and small vessel changes.

Table 1.3 Clinical classification of glomerulonephritis

Nephrotic presentation
Membranous glomerulonephritis
Minimal change disease
Focal segmental glomerulosclerosis
Lupus nephritis (class V)
Renal amyloidosis
Nephritic presentation
IgA nephropathy
Post Streptococcal glomerulonephritis
Hereditary nephritis (Alport syndrome)
Mesangial proliferative glomerulonephritis
Membranoproliferative glomerulonephritis
Rapidly progressive glomerulonephritis
Anti-GBM glomerulonephritis
Immune complex i.e. lupus nephritis (class IV)
Pauci-immune i.e. ANCA associated vasculitis

1.3 Limitations of current therapies for kidney disease

The management of chronic kidney disease is multifaceted. It involves the identification of patients at highest risk of kidney disease progression, treating hypertension and maintaining optimal glycaemic control in diabetic patients. Even before the onset of nephropathy, screening tests detecting the presence of minute protein in the urine i.e. microalbuminuria, will identify those at risk of developing subsequent nephropathy (8, 10). Intensive glycaemic control using hypoglycaemic agents is an important part of primary prevention of DN in both insulin dependent (11) and non-insulin dependent (12-14) diabetes. The former study clearly demonstrated the prevention of other microvascular diabetic complications such as retinopathy and nephropathy with intensive glycaemic control.

The most potent management to reduce the progression to DN is blood pressure reduction. Up to 70% of diabetics with nephropathy have coexisting hypertension (15). Hypertension accelerates renal damage during diabetes (16). Trials have shown that reduction of blood pressure with any agents can reduce the progression of kidney disease (17). More specifically, the addition of specific anti-hypertensives such as angiotensin converting enzyme inhibitors (ACEi) or angiotensin receptor blockers (ARB) have been shown to be reno-protective. Multiple clinical trials have shown ACEi/ARB to reduce proteinuria, often independent of blood pressure lowering effects, in diabetic (18) and non-diabetic kidney diseases (19). Use of ACEi/ARB also slowed the progression of

renal impairment (9, 20-21), reduced mortality or prevented ESRF (21-22) and could even prevent the onset of microalbuminuria (13). These beneficial effects of angiotensin II inhibition were superior compared to other anti-hypertensives such as calcium channel antagonists or β-adrenergic receptor blockers (23). In fact, the use of dihydropyridine calcium channel antagonists have been associated with worsening of proteinuria and renal function (24).

Although these treatments are now the standard of care, up to 40% of diabetic patients still have progression of their kidney disease (2, 5). This illustrates the incomplete understanding of disease mechanisms that contributes to diabetic nephropathy, thus relying on non-specific therapies. Similarly, the mainstay of GN treatment remains non-specific. Current therapies involve blood pressure control (25), blockade of the angiotensin II pathway (26) and immunosuppressive therapy using corticosteroids or toxic agents such as cyclophosphamide. Non-specific immunosuppression inadvertently exposes patients to an increased risk of obesity, diabetes mellitus, cancers, and opportunistic infections ranging from oral candidiasis to life threatening Pneumocystis jiroveci pneumonia.

Perhaps the most significant clinical limitation of using an ACEi or an ARB remains hyperkalaemia (27-28). As patients' kidney disease progresses, the ability of the remaining nephrons to excrete potassium becomes less efficient. Therefore, the addition of these potassium-sparring agents only worsens

hyperkalaemia, and could potentially cause life threatening cardiac arrhythmias (29). These limitations highlight the need for a better understanding of the pathogenesis behind DN and GN, as to provide specific, cell targeted treatments to truncate disease progression.

1.4 Major mechanisms of progressive kidney disease

There are scores of medical conditions that can induce chronic kidney disease through different modes of initiation. However, once the insult begins, they share similar downstream pathological processes. These processes are: inflammation leading to leukocytic infiltration of the kidney causing cell injury; cell death such as apoptosis of podocyte or tubular cells; and fibrosis, which includes glomerulosclerosis and tubulointerstitial fibrosis (30). These processes can be seen in the pathogenesis of both nephropathies stemming from DN and GN.

1.4.1 Role of inflammation in progressive kidney disease

Inflammation is a fundamental physiological response to tissue injury, infection and cancer. It involves the accumulation of inflammatory cells of both the innate and adaptive immune response to the site of tissue injury through complex systems of cell recruitment such as cytokines, chemokines and adhesion molecules. However, inflammation can also cause tissue injury itself, and if

uncontrolled or chronic, can contribute to renal impairment and eventual renal fibrosis (30).

1.4.1.1 Inflammation in diabetic nephropathy

Diabetic nephropathy is not considered a classical immune mediated disease, however there is increasing evidence to suggest an inflammatory component to its pathogenesis (3, 31-32). It has been shown in both human and animal models of DN that there is an early leukocytic glomerular infiltrate (33-36). The predominant cellular infiltrates consists of macrophages and to a lesser extent, lymphocytes (33, 37). Lymphocytes do not appear to be major contributors to the progression of DN; however, there is some evidence to suggest their involvement in the development of proteinuria (4, 38).

With progression of disease, glomerular macrophage count reduces, whilst tubulointerstitial macrophage infiltrate increases (32). Evidence of the pathologic role of accumulating macrophage derives from numerous animal experiments. Diabetic mice that are deficient in the monocyte chemotactic protein-1 (MCP-1) gene have a reduced kidney macrophage infiltrate which is associated with preserved plasma creatinine, reduced albuminuria and attenuated histological damage in glomeruli and tubules (35). Chow *et al.* also noted that MCP-1 deficiency reduced the proportion of activated macrophages, with reduced iNOS or sialoadhesin. Similarly, intercellular adhesion molecule type 1 (ICAM-1) deficiency in *db/db* mice – a model of type 2 diabetes – showed

a reduction in leukocytic infiltrate, preservation of renal function and markedly reduced parenchymal damage and fibrosis (36).

Another method used to inhibit the recruitment and function of macrophages is blockade of c-fms – the receptor for colony stimulating factor-1. C-fms signalling is crucial for monocyte/macrophage activation, proliferation and survival. Lim *et al.* demonstrated a reduction in cellular infiltrate early in diabetic nephropathy of obese *db/db* mice by using an anti-c-fms monoclonal antibody AFS98 (39). This subsequently led to reductions in renal hyper-filtration, histological and gene expression evidence of tubular injury, inflammation and renal fibrosis. Despite these improvements, albuminuria was not affected, suggesting either the intervention was too late to prevent macrophage-mediated podocyte injury, or albuminuria occurred independent of macrophage accumulation.

Other animal model studies also support a key role for inflammation in the pathogenesis of diabetic kidney disease. For example, diabetic mice with a genetic deficiency of p38 mitogen-activated protein kinase (MAPK) signalling have reduced kidney inflammation associated with protection against injury and fibrosis (40). Some immunosuppressive therapies have also been found to attenuate inflammation and injury in DN, including mycophenolate mofetil (41), methotrexate (42), erythromycin (43) and colchicine (44). These studies have all indicated that reducing the inflammatory infiltrate, in particular macrophages, is protective against the development of DN.

1.4.1.2 Inflammation in glomerulonephritis

Inflammation is integral to the pathogenesis of GN. Multiple inflammatory cells have been implicated in GN, including macrophages (45-46), lymphocytes (47-48) and mast cells (49-50). A report of 83 human biopsies of GN found macrophages to be the predominant inflammatory cell in the kidney (51). Moreover, both glomerular and interstitial macrophage density correlated strongly with loss of renal function and histologic damage. In a series of macrophage inhibition studies, the pathogenic role of the macrophage was delineated. Macrophage depletion by anti-macrophage serum reduced the severity of anti-GBM GN of rabbits as determined by renal histology and proteinuria (52). Similar reductions in proteinuria were also seen in rat anti-GBM GN after total leukocyte suppression using systemic irradiation with kidney shielding (53).

However, macrophages also have important physiological roles such as phagocytosis, antigen presentation, and even reparative, anti-inflammatory properties (54). This made total body eradication of macrophage less attractive and supported a search for more specific macrophage inhibition. More recently, specific disruptions of macrophage recruitment by blocking colony-stimulating factor receptor c-fms, chemokines CX3CR1 and MCP-1 showed attenuation of renal injury (55-56) and reduced proteinuria in rodent models of GN (57). Furthermore, inhibition of leukocyte adhesion using antibodies to ICAM-1 has also shown promise in reducing macrophage infiltrate, proteinuria and crescent formation in a rat model of anti-GBM GN (58).

Downstream mediators of macrophage-induced inflammation have also been identified as potential therapeutic targets. Cytokines such as interleukin-1 (59), tumour necrosis factor-α (TNF-α) (60), macrophage migration inhibitory factor (61), or signalling pathways such as c-Jun amino terminal kinase (62), have all been shown to be instrumental for the inflammatory effects of macrophage, as their inhibition reduced renal injury and proteinuria in rodent models of GN (63). Moreover, reduction of osteopontin – a pro-inflammatory and pro-fibrotic phosphoglycoprotein – by genetic knock-out or targeting upstream cytokine interleukin-1, reduced renal injury in rat models of GN (59, 64-65). Collectively, these experiments illustrate the pathogenic role of macrophage-mediated inflammation in GN.

1.4.2 Role of fibrosis in progressive kidney disease

Renal fibrosis can be viewed as the end result of any sustained renal injury i.e. a common final pathway after repeated infections, chronic allograft rejection, radiation injury, hydrostatic pressure, inflammatory diseases such as GN and metabolic disorders such as DN. The progression of fibrosis results in the distortion of the delicate cellular architecture of the kidney and culminates in the loss of nephron function, leading to renal failure. At the histological level, it represents an accumulation of extracellular matrix (ECM) including collagen I, III, IV, fibronectin and reticular fibres produced by mesenchymal cells. When the balance between ECM production versus matrix remodelling and degradation is disturbed, pathological fibrosis follows. The exact determinants of this balance

is complex and appear to depend on: the type of insult; persistence of the insulting agent; depletion of endogenous anti-fibrotic factors (66-67); interactions with mononuclear cells such as macrophages (68-69) and CD4+ lymphocytes (70); and levels of cytokines such as transforming growth factor β-1 (TGF-β1) (71), platelet-derived growth factor (PDGF) (72) and connective tissue growth factor (CTGF) (73). These factors may explain why certain GN recover fully without fibrosis i.e. post infectious GN, while others progress to fulminate sclerosis i.e. focal segmental glomerulosclerosis (74).

1.4.2.1 Cells involved in renal fibrogenesis

At a cellular level, the cells responsible for the excessive deposition of ECM in the renal interstitium are myofibroblasts (Figure 1.1). They are characterised by having long processes, prominent rough endoplasmic reticulum, bundles of microfilament and expression of vimentin and cytoplasmic actin. In diseased tissue, myofibroblasts gain expression of desmin and α-smooth muscle actin (SMA) (75). As their name implies, they have both contractile properties similar to a vascular smooth muscle cell, as well as having the matrix secreting function of fibroblasts. Myofibroblasts are usually found in the intestinal villi (76) and the pulmonary interstitium (77). They are not usually seen in normal kidneys. However in pathologic kidneys, their numbers are increased and their density correlates with progressive renal failure (78-79). Not only are myofibroblasts important in renal fibrosis, they have also been implicated in other fibrosing diseases such as cirrhosis of the liver (80) and pulmonary fibrosis (81).

One of the key questions concerning renal fibrosis is the origin of myofibroblasts. The previously accepted notion that myofibroblasts are solely derived from activated local fibroblasts is no longer valid. The evidence suggests a multitude of origins to form a population of myofibroblasts in the kidney. Lin *et al.* described pericytes and perivascular fibroblasts as the predominant source of myofibroblasts in a mouse model of obstructive uropathy (82). Others have shown that a significant proportion of myofibroblasts derive from tubular epithelium via epithelial to mesenchymal transition (EMT) (83-85), a process where epithelial cells lose their epithelial adhesion molecules E-cadherin and zonula occludentes protein, and gain mesenchymal features including a fibroblast shape and expression of fibroblast-specific-protein (FSP). Furthermore, endothelial cells can also transition into activated fibroblasts via a similar process, endothelial-to-mesenchymal transition. This was demonstrated in three different mouse models of renal fibrosis: unilateral ureteric obstruction (UUO), DN, and a model of Alport syndrome with collagen IV α3 chain knockout. The proportion of activated fibroblasts with endothelial origin was determined by endothelial marker CD31 coexpression, or endothelial lineage tracing using *Tie2-Cre;R26R-stop-EYFP* transgenic mice (86). These experiments suggest that up to 50% of α-SMA expressing cells have endothelial origins. Other sources of myofibroblasts include bone marrow fibrocytes (85) and local proliferation of resident fibroblasts (87).

Intrinsic renal cells also play important roles in renal fibrosis – in particular glomerulosclerosis. As mentioned previously, mesangial expansion is one of the

earliest histological changes seen in diabetes. Its presence has also been correlated with progressive glomerulosclerosis (88-89). Mesangial cells have been shown to gain myofibroblastic features (90) and over-produce ECM in response to the diabetic environment (91-93). They also contribute to increased tissue oxidative stress with increased reactive oxygen species generation. Furthermore, mesangial cells contribute to renal injury by secreting pro-fibrotic factors (PDGF, TGF-β1) and pro-inflammatory cytokines that have a paracrine effect on glomerular cells such as podocytes and endothelial cells (89), and attract an inflammatory infiltrate including macrophages that further exacerbate glomerular injury. Conversely, injury to either endothelium (94) or podocytes (95) have been shown to induce mesangial expansion – thus illustrating the interdependence of intrinsic renal cells in response to injury. The role of other renal cells in fibrosis is further illustrated by evidence suggesting the binding of albumin to receptors on tubular cells and subsequent endocytosis can induce kidney fibrosis and inflammation (96).

1.4.2.2 Molecular mechanism of renal fibrosis

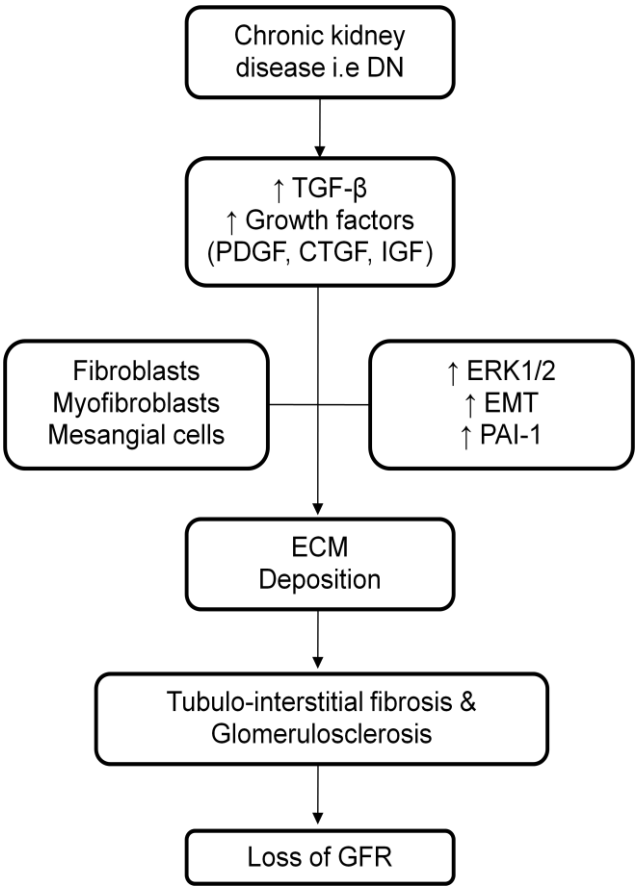
One of the quintessential cytokines that orchestrates fibrosis is TGF-β1. It is a multi-purpose cytokine with reported functions in: the inhibition of epithelial and endothelial proliferation; stimulation of leukocyte chemotaxis and ECM production (75). TGF-β1 exerts its effects by binding to type II receptor directly or indirectly via type III receptor. Type I receptor is then recruited and transphosphorylated, which then phosphorylates Smad 2 or 3 and ultimately

binds to Smad 4. The Smad complex is then moved in to the nucleus where it up-regulates gene transcription of pro-fibrotic factors (71, 97). There are also TGF-β1 independent pathways of inducing fibrosis (98). Several stimuli increase the production of TGF-β1 in diseased kidneys such as angiotensin II (99), PDGF (72), MCP-1, free radicals, glycated albumin, low density lipoprotein, leptin and high glucose (75). Functionally, TGF-β1 stimulates mesangial cells and myofibroblasts to produce ECM proteins (71) whilst reducing the degradation of ECM by modulating collagenase, heparinase, plasminogen activator inhibitor type 1 (PAI-1) and tissue inhibitor of metalloprotease. It has an autocrine effect to increase its own production by mesenchymal and infiltrating cells to potentiate the fibrosis response. Furthermore, TGF-β1 induces both epithelial and endothelial-to-mesenchymal transition (100).

Total inhibition of TGF-β1 to prevent fibrotic diseases has resulted in a lethal pro-inflammatory phenotype in mice (101). This is because TGF-β1 also has anti-inflammatory functions. Selective and sub-total inhibition of TGF-β1, have been shown to reduce renal fibrosis, although the results are variable (102). Given these limitations, it would seem appropriate to target more specific downstream signalling pathways including Smad7, PDGF (72, 103), CTGF (73, 104) and insulin-like growth factor (93) to attenuate the actions of TGF-β1 on fibrosis. Alternatively, TGF-β1 can be antagonised by increasing endogenous anti-fibrotic factors such as hepatocyte growth factor (67, 105), bone morphogenic protein-7 (86) and relaxin (106-107). These factors are reduced in

fibrotic kidneys, and increasing their levels can suppress development of renal injury (103, 107-108).

Figure 1.1 Mechanisms of renal fibrosis



1.4.2.3 Fibrosis in diabetic nephropathy and glomerulonephritis

In DN, one of the earliest pathological changes is the thickening of the GBM, which is the result of ECM accumulation. As fibrosis progresses, nodular glomerulosclerosis and tubulointerstitial fibrosis develop and are the defining features of advanced diabetic nephropathy (7, 89). Strategies to reduce the effect of fibrosis and improve the outcomes of experimental DN include:

blockade of TGF-β either by reducing kinase activity (109), at the receptor level (110), or SMAD signalling using N-acetyl-seryl-aspartyl-lysyl-proline (111); inhibition of osteopontin (112); inhibition of MKK3, which is the upstream kinase of the pro-inflammatory and pro-fibrotic p38MAPK (40); inhibition of PAI-1 (113); blockade of CTGF using antisense oligonucleotides (114); inhibition of transglutaminase enzyme which is important in stabilising elements of the ECM (115); and promoting activated protein C, the levels of which are depleted during DN and have been shown to increase renal fibrosis and apoptosis (94, 116). More recently, a clinical study using the anti-fibrotic agent pirfenidone has shown some promise in maintaining renal function in diabetic patients (117). This trial was based on experimental studies showing that pirfenidone, reduces mesangial expansion and ECM gene expression in mice with DN (118).

Anti-fibrotic therapies have also been shown to suppress the development of GN. For example, pirfenidone can reduce glomerulosclerosis and kidney fibrotic gene expression independent of changes to blood pressure in a model of anti-GBM GN (119). Similarly, treatment with neutralising anti-TGF-β monoclonal antibody inhibits renal fibrosis in an anti-Thy1 model of GN (120). Evidence also suggests that inhibiting the renin-angiotensin-aldosterone axis is a strategy for reducing kidney fibrosis in both DN and GN, and this will be reviewed later.

1.4.3 Role of apoptosis in progressive kidney disease

Apoptosis is a cellular process involved in normal physiological cell turnover and in cell depletion in response to pathological stress and injury. In diseased kidneys, apoptosis contributes to the depletion of podocytes and tubular cells, which leads to albuminuria and tubular dysfunction. When unopposed and persistent, apoptosis of these cells will lead to the eventual loss of renal function.

What differentiates apoptosis from necrosis – another mechanism of cell death – is mainly the lack of cellular swelling. Apoptosis is a genetically programmed process that causes cleavage of cytoskeletal proteins, resulting in the collapse of sub-cellular components, condensation of chromatin, karyorrhexis and culminating in cellular shrinkage. There are two major converging activating pathways of apoptosis: an extrinsic death-receptor pathway that is initiated by ligation of tumour necrosis factors; and the intrinsic mitochondrial pathway that pivots on the balance between pro and anti-apoptotic Bcl2 proteins. Either pathway will trigger a cascade of caspases 3, 6 and 7 that ultimately lead to proteolysis and apoptosis. These pathways are not mutually exclusive, as caspase 8 activation following death-receptor ligation has been found to activate the mitochondrial pathway of apoptosis (121).

1.4.3.1 Apoptosis in diabetic nephropathy

Diabetes mellitus creates a microenvironment where there is an increased presence of oxidative stress, inflammatory cytokines, activation of the angiotensin-aldosterone system, advanced glycosylation end products and glucose degradation products – all of which have pro-apoptotic properties. High glucose has been shown to promote the mitochondrial pathway of apoptosis in renal tubular cells by increasing levels of the pro-apoptotic molecule Bax, while down-regulating anti-apoptotic molecules (122). More recently, brain acid-soluble protein 1 (BASP1) has been isolated as an important protein for mitochondrial apoptosis in diabetic nephropathy. BASP1 is normally expressed in tubular cells and podocytes and has roles in regulating nuclear transcription, including suppression of Wilm's tumour-1 protein. In diabetic conditions, renal BASP1 expression and protein is increased, while small interfering RNA mediated blockade of BASP1 protected tubular cells from apoptosis (123).

To counter overwhelming apoptosis, anti-apoptotic Bcl2 family proteins such as Bcl2 and Bcl-xL act to oppose pro-apoptotic Bax and Bak. This balance of Bcl2 proteins dictates whether a cell survives or dies. Another mechanism of cell survival in diabetic nephropathy is by maintaining sufficient growth factors. It is accepted that growth factor starvation will lead to cell death and this has been linked to over-expression of Bim – a member of the pro-apoptotic Bcl2 family (124). Reductions in vascular endothelial growth factor (VEGF) and epidermal growth factor (EGF) expression are known to correlate with increased proteinuria in DN (125), suggesting that starvation of these cytokines may lead

to apoptosis. VEGF is normally expressed by the endothelium, including in the glomerulus, and is required to maintain vascular physiological functions. Multiple isoforms of VEGF are also expressed by glomerular epithelial cells, with roles in determining podocyte survival (126). Maintaining normal levels of EGF has also been shown to reduce tubulointerstitial apoptosis and improve renal function in human kidney diseases including DN (127).

Intrinsic pathway of apoptosis by ligation of the death-receptor also plays a major role in kidney disease. Ligands for the death-receptor include cytokines of the TNF superfamily. For example, ligation of TNF-α or Fas to its respective death receptors will trigger caspase 8, thus activating the apoptotic cascade. These ligands are up-regulated by high glucose and oxidative stress, and contribute to DN (128-129). A gene profiling study of human diabetic kidneys revealed that TNF-related apoptosis-inducing ligand (TRAIL) and death receptors, Fas and osteoprotegerin, were all highly expressed (130). TRAIL expression can be increased by pro-inflammatory cytokines such as interferon-γ (IFN-γ) or TNF-α and directly promote apoptosis of intrinsic renal cells (131). Furthermore, renal expression of TRAIL correlated with the degree of renal dysfunction, proteinuria and histological changes in DN (130).

1.4.3.2 Apoptosis in glomerulonephritis

Glomerulonephritis is an inflammatory condition that sees a dramatic elevation in the expression of pro-inflammatory cytokines such as TNF-α. There is also

evidence that GN shifts the balance between pro and anti-apoptotic proteins toward the former, by down-regulating Bcl2 in human IgA nephropathy (132). These findings suggest that both the intrinsic and extrinsic pathways of apoptosis are activated in GN. In fact, depending on the type of GN, apoptosis can either be beneficial or detrimental. During acute glomerular inflammation, apoptosis is required to clear infiltrating or non-viable cells in order to limit the extent of renal damage. This mechanism has been observed in human renal biopsy studies of post-streptococcal GN (133) as well as rat models of anti-GBM GN (134-135) where infiltrating neutrophils and macrophages underwent apoptosis to prevent overwhelming renal inflammation. Furthermore, apoptosis is important in reducing the extent of fibrosis by reducing cell proliferation in areas of glomerular scarring during the convalescent phase of nephritis (136).

In contrast, apoptosis also contributes to pathology during GN. Similar to DN, apoptosis contributes to podocyte and tubular cell depletion and subsequently leads to proteinuria and renal impairment. Furthermore, injury to podocytes and their reduction in number have been implicated as a mechanism for glomerulosclerosis (137). Mesangial cells have also been found to undergo apoptosis when co-cultured with activated macrophages harvested from rats with anti-GBM GN (138). Inhibition of apoptosis by modulating the balance of pro and anti-apoptotic molecules has been attempted for various kidney disease models (anti-GBM GN, UUO). In experimental GN, caspase inhibitors have been shown to suppress renal inflammation, fibrosis and proteinuria, but not loss of renal function (139-140).

1.4.4 Balancing physiology and pathology

It is important to understand that mechanisms facilitating the progression of kidney disease (i.e. inflammation, apoptosis and fibrosis) also have a role in normal physiology. One example is wound healing after soft tissue injury. Inflammation is required to recruit the necessary cells from the circulation to resist infection, and also to direct reparative processes such as scar formation, which requires ECM deposition from myofibroblasts. Therefore, targeting any of these disease mechanisms can be complex and may need to be limited

1.4.5 Molecules that regulate inflammation, apoptosis and fibrosis

Molecular mechanisms that facilitate inflammation, apoptosis and fibrosis have become attractive targets for therapeutic exploration. For example, some chemokines (i.e. osteopontin) have been shown to be both pro-inflammatory and fibrotic. Diabetic mice lacking the osteopontin gene develop less renal injury compared to wild type diabetic mice (112). Similarly, elements of the renin-angiotensin-aldosterone system have been shown to be potent inducers of inflammation, apoptosis and fibrosis (141).

1.5 Renin-angiotensin-aldosterone axis

The renin-angiotensin-aldosterone axis is a vital system that regulates salt, water and blood pressure. It probably allowed vertebrates to move out of the ocean and survive on land (142). In the setting of hypovolaemia or

hyponatraemia, a concerted neurohumoral stimulus is generated at the juxtaglomerular apparatus of the kidney, where baroreceptors, β-adrenergic nerves and the macular densa cells all play a role in renin release. Renin is an aspartyl-protease whose only known role is to act on the leucyl-leucine bond of rodents or leucyl-valine bond in humans of angiotensinogen to release angiotensin I (143). Angiotensin I is then cleaved by the angiotensin converting enzyme into a biologically active octapeptide, angiotensin II. This hormone is responsible for vasoconstriction, increasing sympathetic activity and aldosterone secretion – culminating in salt and water retention and elevation of the blood pressure.

1.5.1 Angiotensin II and the kidney

The roles of angiotensin II (AngII) on renal development and pathophysiology have been thoroughly examined. AngII is important in renal genesis and as such, its inhibition during fetal development causes teratogenicity. Physiologically, activation of angiotensin receptor AT_1 results in systemic vasoconstriction and subsequent elevation of blood pressure. It also causes intra-renal vasoconstriction, with a preference for the efferent arteriole, thus augmenting the intra-glomerular pressure and glomerular filtration. Angiotensin II is also implicated in pathology. It has pro-fibrotic effects on the heart, vasculature and the kidneys. Upon binding to the AT_1 receptor, AngII up regulates CTGF (144) and increases matrix production and collagen deposition in rat blood vessels. These fibrotic effects involve both TGF-β1-dependent and

independent mechanisms, since AngII can directly activate RhoA proteins (145-146) or Smad proteins to induce a fibrotic response (147).

Angiotensin II also has pro-inflammatory properties. Rats infused with AngII had increased expression of chemokines such as MCP-1 and regulated upon activation: normal T cell expressed/secreted (RANTES), leading to the development of a prominent leukocyte infiltrate in the glomerulus and interstitium (146). This effect was mediated by activation of nuclear factor-κB (NF-κB) pathways (148). Other *in vitro* and *in vivo* studies have shown AngII to enhance adhesion molecules including ICAM-1 (149) and vascular cell adhesion molecule type 1 expression (150). Moreover, ACEi have been shown to reduce infiltrating leukocytes and preserve renal function in multiple models of kidney disease (151).

1.5.2 Aldosterone breakthrough

Inhibition of AngII by means of ACEi or ARB has been accepted as first line therapy for diabetic and non-diabetic kidney diseases, as detailed earlier. However, multiple studies have shown patients who are on AngII inhibitors can develop high circulating aldosterone levels. This phenomenon has been called aldosterone breakthrough and can affect 10-53% of patients receiving AngII inhibitors (152-153). The mechanism has not been fully elucidated, but may be related to non-ACE pathways of AngII formation by chymases, which bypasses the effect of ACEi and lead to angiotensin escape-induced aldosterone

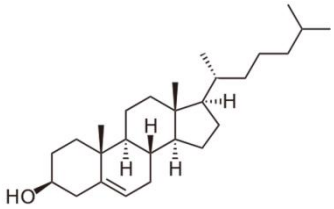
production (154). However, a systematic review of the existing literature found similar incidences of aldosterone breakthrough, regardless of whether ACEi or ARB usage (152). Regardless of the cause, aldosterone breakthrough has been associated with worse cardiac remodelling (155), impaired myocardial performance during stress (156), higher proteinuria in diabetes (153) and accelerated the loss of renal function (157-158).

1.5.3 Mineralocorticoids and their physiological role in the kidney

First identified over 50 years ago as the electrocortin hormone, aldosterone is the only physiological mineralocorticoid hormone in humans. It is synthesised by the aldosterone synthase enzyme CYP11B2 by converting corticosterone in the zona glomerulosa layer of the adrenal cortex (Table 1.4). The production of aldosterone is regulated by AngII, low serum potassium and the adrenocorticotropic hormone. The major organ targets of aldosterone are the kidney and the colon, where it exerts important physiological roles. The principal action of aldosterone is sodium and water reabsorption and potassium excretion in epithelial cells that line the distal convoluted tubule and collecting ducts of the kidney and also that of the distal colon. Aldosterone is vital for the survival of land living vertebrates to maintain hydration. Mice with mineralocorticoid receptor (MR) gene deficiency are mineralocorticoid unresponsive, which causes salt and water wasting and subsequent death 10 days after birth (159).

Table 1.4 Biosynthesis of aldosterone.

Cholesterol

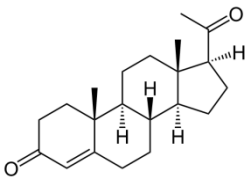


↓ *side chain cleavage enzyme*

Pregnenolone

↓ *3β-hydroxysteroid dehydrogenase*

Progesterone



↓ *21-hydroxylation*

11-deoxycorticosterone

↓ *11β-hydroxylase (CYP11B1)*

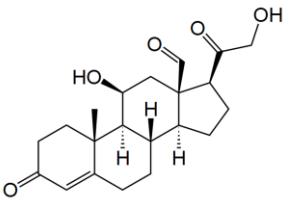
Corticosterone

↓ *aldosterone synthase (CYP11B2)*

11-hydroxy-corticosterone

↓ *aldosterone synthase (CYP11B2)*

Aldosterone



Adapted from Cortinovis et. al., *Ther Adv Cardiovasc Dis*, 2009. (160)

1.5.4 The mineralocorticoid receptor

The MR is a member of the steroid nuclear receptor family of ligand-dependent transcription factors (161-162). It contains 3 principal domains which are common to all receptors of this family: N-terminal domain; a central DNA-binding domain and a hinge region linking to the C-terminal ligand binding domain (LBD). The LBD of the human MR is structurally similar to the glucocorticoid, androgen and progesterone receptor. For this reason, agonists or antagonists of MR have a certain extent of cross reactivity with these receptors. When not occupied, the MR is in a transcriptionally inactive conformation coupled to its chaperones heat shock protein hsp90, hsp70, immunophilin FKBP-59 and cyclophilin CYP40. Upon aldosterone ligation, MR separates from its chaperone proteins, undergoes conformational change and translocates to the nucleus for transcription. In epithelial cells, this results in an increased expression of the apical epithelial sodium channel, the basolateral Na^+/K^+-ATPase pump, and intracellular kinases such as serum and glucocorticoid-regulated kinase 1 (Sgk1) and serine/threonine protein kinase (163). The end result is the promotion of salt and water conservation at specialised epithelial cells found in the renal distal tubule and the colon.

1.5.5 Non-genomic effects of the mineralocorticoid receptor

Rapid MR-mediated functions exist that cannot be explained by classic genomic mechanisms. These non-genomic actions have a rapid onset and offset within minutes, and are not blocked by transcription inhibitor actinomycin D or protein

synthesis inhibitor cycloheximide (164-165). Non-genomic effects involve activation of second messenger pathways and may even utilise a membrane receptor separate to MR (166). Rapid non-genomic actions have been identified in various organs including kidney, colon, vascular endothelium and the heart. From a renal perspective, physiological actions of aldosterone such as Na^+/K^+ handling or vasoconstriction have rapid response within minutes of an aldosterone infusion, suggesting activation of non-genomic pathways (167). Non-genomic MR activation has also been found to have deleterious effects on non-epithelial cells, which will be described later.

1.5.6 Ligands for the mineralocorticoid receptor

Ligands for the MR include aldosterone, cortisol, cortisone and deoxycorticosterone. In fact, the majority of MR *in vivo* is ligated by cortisol, due to the relative abundance of this circulating hormone compared to aldosterone. Fortunately cortisol has very weak potency to activate the MR, thus rendering the receptor inactive. It is only in epithelial cells where the enzyme 11*β*-hydroxysteroid dehydrogenase type 2 (11*β*-HSD2) is co-expressed that aldosterone can bind to MR. This is due to 11*β*-HSD2's ability to convert cortisol to cortisone, which has less MR affinity. More recently, the small molecule GTPase Rac1 has been identified as a novel non-ligand dependent activator of MR (168). Rac1, a member of the Rho family of GTPase, has been shown to activate MR and cause proteinuria, podocyte injury and subsequent glomerulosclerosis in *Arhgdia*-/- mice, which lack RHO-dissociation inhibitor-α.

Treatment of *Arhgdia*-/- mice with a Rac1 inhibitor or a MR antagonist significantly attenuated renal injury, thus confirming Rac1's interaction with MR (142).

1.5.7 Mineralocorticoid receptor antagonists

Existing MR antagonists available for clinical use include spironolactone and eplerenone. Eplerenone is a more specific antagonist of MR and lacks the adverse effects which spironolactone has on the endocrine system (e.g. gynaecomastia and menstrual irregularities). Other identified compounds such as RU 26752 and RU 28318 are also potent MR antagonists, but have only been tested in animal models and *in vitro* experiments (162). More recently, dihydropyridine calcium channel antagonists have been noted to have MR antagonist activity (169). This effect was mediated by a competitive binding to the MR LBD, as well as a reduction of co-activator SCR1. However, the concentration required to achieve adequate MR blockade was up to 40-fold the recommended dosage for usual antihypertensive use – thus limiting clinical use of dihydropyridines as MR antagonists. Mineralocorticoid receptor antagonists are clinically used as a diuretic, because they oppose salt and water reabsorption at the distal convoluted tubules and the collecting ducts of the nephron.

1.5.8 Mineralocorticoid and angiotensin II receptor cross-talk

More recently, studies have demonstrated an inter-dependence of the MR and angiotensin II receptor. Pathological effects of AngII on the heart and the kidney have been shown to be mediated by the MR, even in the absence of circulating aldosterone (170). The converse also holds true, as aldosterone-induced inflammatory cytokine expression was reduced by small-interfering RNA to the AngII receptor (171). These factors illustrate the incomplete renal protection afforded by AngII inhibition and support a role for adjunctive therapy with MR antagonists.

1.6 Mineralocorticoids and kidney disease

Recent studies have identified some of the pathological roles of MR activation on diseases affecting the heart, blood vessels and the kidneys. As alluded to earlier, mineralocorticoids such as aldosterone have pro-inflammatory, pro-fibrotic and pro-apoptotic properties. From the perspective of the kidneys, aldosterone not only regulates salt and water, it can also exert pathological effects through the MR in cells other than the tubular epithelial cells. The following section summarises the effects of aldosterone on non-epithelial renal cells which promote renal injury.

1.6.1 Mineralocorticoid excess and their impact on the kidney

In humans, observational studies have shown that patients with a condition of primary aldosterone excess such as Conn's syndrome have a significant association with proteinuria (172) and reduced renal function (173-174) compared to those with only essential hypertension. Furthermore, aldosterone excess has been associated with adverse cardiac remodelling and subsequent diastolic dysfunction (175), which was partially reversible after surgical resection of the adrenal tumour. Since then, several non-epithelial tissues have been found to express MR, including cardiomyocytes (176), vascular endothelial and smooth muscle cells (177), neurons of the hippocampus (178) and adipocytes (179). Research into non-epithelial MR activation has yielded much insight into the pro-inflammatory, pro-fibrotic and apoptotic properties of aldosterone.

1.6.2 Pathological role of MR signalling in kidney cells

Many cells that populate the kidney such as mesangial cells, podocytes, renal fibroblasts and macrophages have been shown to express MR (162). These cells even have the ability to generate aldosterone by expressing aldosterone synthetase, thus allowing them to activate their MR in an autocrine/paracrine manner, independent of circulating aldosterone. Furthermore, the concentration of aldosterone achieved by local synthesis may be much higher than that of the circulation (180-181). Therefore, an understanding of the cellular effects of MR activation is important in order to develop future treatment strategies.

1.6.2.1 Aldosterone and podocytes

Podocytes are specialised epithelial cells of the GBM that forms part of the apparatus required for glomerular filtration. Proteinuric diseases such as DN and membranous nephropathy result in podocyte damage, leading to loss of the filtration barrier, culminating in inappropriate loss of plasma proteins such as albumin. Proteinuria is not only a marker for glomerular disease, it is also associated with worse renal outcomes and cardiovascular morbidity. MR expression has been identified in podocytes, indicating that these cells are potential targets of aldosterone (182). Currently, there is little known about how podocytes respond to activation of their MR. In one animal model study involving aldosterone infusions, evidence of podocyte damage was identified by the loss of nephrin and podocin - structural proteins found only in podocytes. In this study, aldosterone-induced podocyte injury was associated with an increased generation of reactive oxygen species via the NADPH oxidase pathway and Sgk1 activation, suggesting that these pathways may be involved in the injury process (182).

Apoptosis appears to be a mechanism by which aldosterone causes podocyte loss. This was demonstrated in an experiment using cultured podocytes, where aldosterone treatment led to a dose and time dependent increase in apoptosis. This effect was mediated through an increase in pro-inflammatory p38MAPK signalling and the suppression of the survival signalling pathway phosphoinositide 3 kinase/Akt (183). *In vivo* evidence of aldosterone-induced podocyte loss has been seen in a model of renal injury caused by subtotal

nephrectomy. In this study (182), 5/6 nephrectomised rats developed podocyte loss, hypertrophy of remaining podocytes and proteinuria. These abnormalities were partially reversed with either quinapril or spironolactone alone, or abolished with combination therapy. These results also suggest that MR activation contributes to podocyte damage during hyperfiltration of the kidney injury.

Another potential mechanism by which aldosterone contributes to proteinuria is by reducing production of structural proteins that bind podocytes to the GBM. Integrin-αβ is a protein that serves this crucial purpose. Diabetic rats have reduced levels of integrin-αβ which correlate with podocyte loss. In contrast, diabetic rats treated with spironolactone have normal integrin-αβ expression and are protected from podocyte loss (184). A similar process of podocyte loss has been observed in diabetic patients (185). It is thought that podocytes lose adhesive properties, and gain a fibroblastic phenotype such as the expression of fibroblast-specific protein-1, through epithelial mesenchymal transition (185). Furthermore, aldosterone has also been implicated in glomerular hypertrophy and hyperfiltration in early DN, as spironolactone was able to attenuate glomerular surface area (184). Given that glomerular hypertrophy has also been linked to mechanical stress and subsequent podocyte loss (142), it provides indirect evidence that aldosterone can reduce podocyte numbers through mechanical stretch.

1.6.2.2 Aldosterone and renal fibroblasts

Several *in vitro* experiments in renal fibroblasts have confirmed the pro-fibrotic effects of aldosterone. Aldosterone has been shown to activate rat renal fibroblasts and promote the synthesis of collagen I, III and IV (186). This effect was mediated through the MR and rapid non-genomic activation of extracellular signal-regulated kinases (ERK1/2), a member of the MAPK signalling pathway. Furthermore, aldosterone increases production of PAI-1 and CTGF, which promote a pro-fibrotic environment by reducing matrix degradation (187) and enhancing ECM accumulation (188). Fibroblasts have also been shown to express aldosterone synthase, suggesting that aldosterone can be generated locally independent of the adrenal gland in an autocrine/paracrine manner (188).

1.6.2.3 Aldosterone and mesangial cells

Several experiments have highlighted the pathological role of aldosterone on mesangial cells of the kidney. An *in vitro* study has shown that aldosterone promotes cultured mesangial cell apoptosis at supra-physiological concentrations (189). In this study, apoptosis was markedly attenuated by administration of eplerenone, and also by a NADPH oxidase inhibitor. This suggests that mesangial apoptosis occurs in response to MR-induced oxidative stress. Furthermore, aldosterone promoted Bad de-phosphorylation, which reduced the availability of survival factors such as Bcl-2, resulting in an environment that favours apoptosis. In contrast, aldosterone at near

physiological levels can act as a mitogen. This was shown in cultured rat and human mesangial cells, where aldosterone exposure led to an increase in mesangial proliferation through activation of the MR and ERK1/2 (190-191).

Mesangial cells have also been shown to contribute to glomerular inflammation and fibrosis when exposed to aldosterone. This was demonstrated in cultured rat mesangial cells, where aldosterone stimulated NFκB activity via Sgk1 and led to enhanced expression of CTGF (192) and pro-inflammatory cytokines (31). Similar to renal fibroblasts, aldosterone also induced mesangial cell gene expression and protein synthesis of the pro-fibrotic cytokine PAI-1 (187, 193). Others have shown that aldosterone promotes fibronectin production in mesangial cells via activation of ERK1/2 (194) and TGF-β1/Smad2 pathways (195). Aldosterone can also alter the phenotype of cultured mesangial cells to exhibit myofibroblastic features (90). Much like EMT occurring in tubular epithelial cells of the kidney, mesangial cells acquire α-SMA, actin polymerisation and hypertrophy after exposure to aldosterone. Furthermore, aldosterone increases mesangial cell production of ECM such as collagen I, III and IV. These aldosterone-induced fibrotic effects were abolished by eplerenone, suggesting that they are dependent on MR activation (90). In addition, mesangial cells are capable of aldosterone synthesis, which indicates the possibility of autocrine/paracrine activation of MR (195).

1.6.2.4 Aldosterone and tubular cells

Tubular epithelial cells express MR (196) and excessive activation of tubular MR during disease can induce responses which facilitate inflammation, apoptosis and fibrosis. For example, proximal tubular cell (PTC) apoptosis was enhanced in a dose and time dependent manner in cultured human tubular cells treated with aldosterone (196). Aldosterone also increased tubular expression of the pro-apoptotic molecule Bad, and tubular production of reactive oxidative species. These effects were dependent on MR, as spironolactone prevented the apoptotic response. Another study by Fan *et al.* demonstrated aldosterone's effect on the senescence of human PTC (197). In this study, aldosterone up-regulated expression and staining of senescence-associated β-galactosidase and p53 via the MR. These changes led to reduced proliferation of cultured PTC, as p53 over-expression is associated with cell cycle arrest and apoptosis.

Similar to other intrinsic renal cells, tubular cells also contribute to renal inflammation and fibrosis after exposure to aldosterone. Aldosterone enhanced NFκB and Sgk1 signalling and up-regulated expression of the pro-inflammatory cytokines PAI-1, MCP-1 and IL-1β in principal cells from distal tubules (198). Human PTC has also been shown to undergo EMT and obtain myofibroblastic features through activation of the MR and ERK1/2 signalling (199). Furthermore, MR activation by aldosterone has been shown to increase CTGF expression and lead to enhanced collagen synthesis in PTC (200). Collectively, these experimental findings shed light on the pluripotent role of the tubular cell in both renal physiology and renal pathology.

1.6.2.5 Aldosterone and macrophages

Human mononuclear cells including monocytes/macrophages express the MR as well as aldosterone synthase (201). This suggests that mononuclear cells are also capable of activating MR through an autocrine/paracrine mechanism. Aldosterone-induced activation of MR increases expression of proinflammatory cytokines (TNFα, MCP-1 and PAI-1) in human monocytes (201-202). MR activation in these cells also increases protein expression of p22phox, a marker of NADPH derived oxidative stress (202). Similar findings have been identified in mouse macrophages. Thioglycolate-elicited macrophages from the peritoneum of wild type mice have been shown to have increased expression of classical M1 macrophage proinflammatory cytokines following MR activation (203). In contrast, macrophages isolated from the peritoneum of mice with myeloid MR deficiency, were found to have increased expression of M2 macrophage markers (eg. Ym1, Fizz1, Msr2) compared to wild type macrophages.

Table 1.5 Effects of MR activation in renal cells

Cell type	Species	Effect	Outcome	Reference
Fibroblast				
NRK-49F	Rat	↑ ERK1/2 mRNA ↑ Collagen protein	Increased collagen synthesis	(186)
NRK-49F	Rat	↑ PAI-1 protein ↑ TGF-β1 protein ↓ Matrix degradation	Reduced ECM degradation	(187)
NRK-49F	Rat	↑ NFκB activation ↑ Osteopontin protein ↑ Collagen mRNA	Promoted collagen synthesis	(204)
Primary	Rat	↑ SGK1 mRNA ↑ ROS (NADPH oxidase) ↑ CTGF mRNA	Increased CTGF	(188)
Podocyte				
MPC	Mouse	↑ ROS (mitochondrial) ↓ PPARγ co-activator	Enhanced oxidative stress	(205)
MPC	Mouse	↑ SGK1 mRNA ↑ ROS (NADPH oxidase) ↓ Nephrin, podocin mRNA	Enhanced oxidative stress and podocyte injury	(182)
Primary	Rat	↑ apoptosis staining ↓ PI3-k/Akt activity ↑ p38MAPK activation	Increased podocyte apoptosis	(183)
Primary	Rat	↓ Integrin-α3 protein	Loss of podocytes due to reduced adhesion	(184)
Mesangial cell				
RMC	Rat	↑ NFκB & SGK1 activation ↑ ICAM protein ↑ CTGF protein	Increased fibrosis and inflammatory potential	(192)
RMC	Rat	↑ Rho-kinase activity ↑ α-SMA mRNA ↑ Collagen mRNA	Facilitated myofibroblast trans-differentiation	(90)
RMC	Rat	↑ PAI-1 protein ↑ TGF-β1 protein ↓ Matrix degradation	Reduced ECM degradation	(187)
Primary	Human	↑ TUNEL apoptosis ↑ Bad activity	Enhanced apoptosis	(189)
RMC	Rat	↑ ROS (NADPH oxidase)	Increased oxidative stress	(206)

Cell type	Species	Effect	Outcome	Reference
Tubular cell				
HKC	Human	↑ ERK1/2 activity ↑ α-SMA mRNA ↑ Collagen mRNA	Promoted EMT and collagen synthesis	(199)
PTC	Human	↑ Bad activity ↑ ROS (mitochondrial)	Enhanced apoptosis and oxidative stress	(196)
PTC	Human	↑ β-galactosidase, p53 ↓ SIRTI	Increased cell senescence	(197)
Primary	Rat	↑ SGK1 mRNA ↑ NFκB activity ↑ MCP-1, IL-1β mRNA	Pro-inflammatory	(198)
Macrophage/Monocyte				
Primary	Human	↑ TNFα and MCP-1 protein	Pro-inflammatory	(201)
Primary	Human	↑ ROS (NADPH oxidase) ↑ PAI-1 protein	Enhanced oxidative stress	(202)
Primary	Mouse	↑ TNFα, IL-12 mRNA	Pro-inflammatory	(203)

1.6.3 MR antagonists in animal models of kidney disease

Activation of the MR by aldosterone or other ligands such as deoxycorticosterone in the setting of a high salt diet causes renal injury in rodents. This was demonstrated in uninephrectomised rats that were given deoxycorticosterone and salt. These rats developed interstitial fibrosis accompanied with a macrophage infiltrate and evidence of increased oxidative stress, while eplerenone treatment normalised collagen deposition (207). Similarly, rats administered aldosterone/salt also developed histological renal injury, as well as proteinuria and this was markedly attenuated by eplerenone treatment (208). Eplerenone has also been shown to reduce proteinuria and renal vascular injury without lowering blood pressure (209).

1.6.3.1 MR antagonists in experimental diabetic nephropathy

Several experimental studies have linked MR activation to the pathogenesis of nephropathy in both type 1 and type 2 diabetes mellitus. Otsuka Long-Evans Tokushima Fatty rats with type 2 diabetes developed albuminuria, glomerulosclerosis, increased expression of MCP-1, TGF-β1, as well as an ED-1 positive macrophage infiltrate. These changes, except for the ED-1 infiltrate, were abrogated by spironolactone treatment without lowering blood pressure (31, 210). Another model of type 2 diabetic nephropathy using obese *db/db* mice showed that eplerenone reduces not only the glomerular hypertrophy, mesangial expansion and albuminuria, but also the macrophage infiltrate (211). These changes were in association with a reduction in the expression of renal osteopontin but again, independent of blood pressure lowering. Antagonists of MR have also been efficacious in reducing the severity of type 1 diabetic nephropathy. In models of streptozotocin-induced type 1 diabetic nephropathy, MR antagonists have been shown to reduce albuminuria, glomerular ECM deposition, inflammatory infiltrates (211-212) and expression of cytokines TGF-β1, PAI-1 (213) and CTGF (200). Furthermore, in diabetic rats, spironolactone provided protection against podocyte apoptosis, through reducing caspase-3 and Bcl-2 activity (214).

In addition, the diabetic milieu could also enhance MR signalling by increasing renal MR (211) and aldosterone synthase (214). These changes could potentially amplify the deleterious effects of MR signalling in diabetic kidneys and support the use of MR antagonists.

1.6.3.2 MR antagonists in experimental glomerulonephritis

Given that MR signalling is associated with kidney macrophage accumulation, it is of no surprise that MR is involved in inflammatory diseases such as glomerulonephritis. In a study of murine lupus nephritis, NZB/W F_1 mice developed aggressive glomerulonephritis with typical features of human class IV nephritis such as leukocytic infiltrate, mesangial expansion and crescent formation, proteinuria and double stranded DNA autoantibodies (215). Treatment with spironolactone attenuated proteinuria, histological injury, crescent count, and expression of inflammatory and apoptotic genes such as IFN-γ, BLys and TRAIL. Again, these changes were blood pressure independent. Curiously, despite a reduction in the titres of autoantibodies to DNA, there were no changes to the glomerular deposition of immune complexes on immunofluorescence.

MR anatgonists have also been shown to exhibit reno-protective effects in anti-GBM GN. In mouse model of accelerated anti-GBM GN, eplerenone treatment attenuated albuminuria, glomerulosclerosis, mononuclear infiltration, as well as inflammatory cytokine expression MCP-1 and IL-6 (216). Further evidence of the pro-inflammatory, proliferative and fibrotic roles of MR signalling could be demonstrated in anti-thy 1.1 nephritis – a model of mesangial proliferative GN. Rats treated with MR antagonists had reduced renal inflammation with reduced macrophage infiltrate and MCP-1 expression (217). Furthermore, immunostaining of proliferating cells (Ki-67+) and fibrotic markers (TGF-β1, α-SMA) were also reduced by MR blockade (218).

1.6.4 MR antagonists in clinical practice

Two landmark trials in clinical medicine, the RALES (219) and EPHESUS (220) studies have demonstrated the benefits of MR blockade in patients with cardiovascular disease. The former investigated the outcomes of adding MR antagonist spironolactone to patients with advanced heart failure who were already receiving ACEi. It found that the addition of MR antagonist on top of ACEi conferred further cardiovascular mortality benefits. Similarly, the EPHESUS trial demonstrated the survival benefits of MR antagonist eplerenone in patients who had cardiac insufficiency as a result of acute myocardial infarction. These two trials demonstrate that despite blockade of AngII, aldosterone is still produced and can continue to exert its deleterious effects on the cardiovascular system.

In comparison, evidence supporting the use of MR antagonists in renal disease has largely revolved around the management of proteinuria (153, 221-222). In trials treating patients with kidney disease, MR antagonists have not been shown to improve mortality or preserve renal function – although these trials have not had the power or the length of follow up to demonstrate such findings. Due to this, a systematic review could only conclude that MR antagonists can be used for reducing proteinuria in renal diseases. This finding is not to be belittled. Proteinuria itself has been shown to be an independent cardiovascular disease and mortality risk factor (223), and has been correlated with worse renal outcomes (224). Put together, these clinical trials demonstrate the benefits of MR antagonism in cardiovascular and proteinuric kidney diseases in humans.

1.7 Animal Models of MR-Dependent Kidney Disease

Animal models have allowed rapid expansion of our understanding of the pathophysiology of MR activation in renal disease. They provide a unique opportunity to observe consequences of MR antagonism through different modes of injury to a whole organism, rather than in a single cell. Another advantage of animal models – in particular – mouse models of kidney disease is the ability to dissect out specific molecular pathways by means of targeted genetic deletion, which will be reviewed later. Three distinct murine models of kidney disease were adopted in this thesis to investigate the pathological role of MR: i) passive accelerated anti-GBM GN to assess acute kidney injury; ii) streptozotocin-induced DN to assess chronic kidney injury resulting from type 1 diabetes; and iii) unilateral ureteric obstruction – a model of acute interstitial renal fibrosis.

1.7.1 Anti-GBM Glomerulonephritis

The murine model of anti-GBM GN is an aggressive form of rapidly progressive glomerulonephritis. It has two distinct phases: the heterologous and autologous phases (225-226). During the heterologous phase, antibody raised against the GBM of the mouse is injected intravenously. This causes a linear deposition along the GBM that can be detected by immunofluorescence staining. These antibodies fix complement, and cause a dose-dependent effect on early GBM injury and albuminuria (226). The autologous phase is more dependent on the adaptive immune response, where antigen presentation cells recognise the

planted immunoglobulin and trigger an autoimmune response which includes generation of specific antibodies by plasma cells and recruitment of effector cells such as macrophages and lymphocytes. The net effect is further damage to the glomeruli, often with crescent formation, and also to the tubulointerstitium (225). This model can be further accelerated by priming the mice with immunoglobulin from the species used to develop anti-GBM antibodies.

Inflammation in mouse anti-GBM GN is characterised by an early transient glomerular neutrophil influx (days 0-1), followed by a progressive accumulation of glomerular and interstitial macrophages and T-cells, which subsides as kidneys become more fibrotic. Albuminuria appears at the onset of glomerular injury (day 1) and levels remain similar at days 7 and 14 of disease. Histological damage correlates with the progression of injury (inflammation, apoptosis, necrosis, cell proliferation, fibrosis) and leads to the development of glomerular crescents, tubular atrophy, sclerosis and loss of renal function at day 14 of disease. This model of murine GN is also strain dependent: C57BL/6 mice that exhibit a strong Th1-mediated immune response during anti-GBM GN develop more severe glomerular lesions, but less albuminuria, compared to BALB/c mice which have a more Th2-driven immune response (227-228).

1.7.2 Streptozotocin-Induced Diabetic Nephropathy

Streptozotocin is a chemical compound composed of glucosamine-nitrosurea that causes specific toxicity to the insulin producing β-cells of the pancreas

(229). The resultant exhaustion of β-cells leads to progressive hyperglycaemia, necessitating exogenous insulin treatment. Several pathological features seen in this model are similar to those found in human DN. For example, streptozotocin-induced diabetes causes early glomerular injury (macrophage infiltration, glomerular hypertrophy, podocyte damage, GBM thickening) which is associated with microalbuminuria. This disease progresses with the duration and severity of diabetes, resulting in worsening albuminuria, glomerulosclerosis, tubular atrophy, tubulointerstitial fibrosis, and declining glomerular function (7). However, most mouse strains used in these studies are not hypertensive, unlike many diabetic patients, and therefore, disease progresses more slowly in these strains and lacks some features associated with human DN such as nodular glomerulosclerosis. (229). High doses of streptozotocin can cause acute renal tubular toxicity, independent of the development of diabetes. This can confound the assessment of diabetic renal injury (230). This acute toxicity effect can be avoided by using a multiple low-dose regimen (55 mg/kg/day for 5 consecutive days), rather than a single high dose (>200 mg/kg) (229). Moreover, the degree of susceptibility to DN differs between strains of mice, i.e. DBA/2 > C57BL/6 > 129/Sv > BALB/c (231). These factors need to be considered in the planning of experiments in order to obtain consistent results.

1.7.3 Unilateral Ureteral Obstruction Model of Renal Fibrosis

Unilateral ureteral obstruction (UUO) is a surgically-induced model of acute interstitial kidney fibrosis. It is of particular value, as fibrosis is a common final

pathway following different renal insults. It mimics the pattern of renal injury observed after obstructive uropathy in humans, where the predominant histological finding is tubulointerstitial fibrosis (232). Following anaesthesia, a laparotomy is performed surgically and a ureter is ligated completely. The contralateral ureter is sparred and serves as an elegant control. Within 7 days, profound renal fibrosis is evident in the UUO kidney involving substantial accumulation of interstitial myofibroblasts, which is accompanied by a macrophage infiltrate, tubular atrophy and apoptosis. This model is frequently used for examining the mechanisms of kidney interstitial fibrosis and potential anti-fibrotic therapies (232).

1.8 Cell selective MR blockade in animal models

The vital role of epithelial MR function on salt and water reabsorption has been highlighted in studies of mice with MR gene deficiency (159). These mice had normal prenatal development, but all died 10 days post-partum from hypovolaemia secondary to overt salt and water wasting. Therefore, in order to study the specific effects of MR activation on non-epithelial cells, methods of conferring cell-type specific MR gene deficiency become necessary.

1.8.1 Cre-Lox technology

Cre is a site-specific recombinase that binds to specific sites known as loxP. Two loxP sites recombine in the presence of Cre, allowing DNA that is cloned

between two such sites to be removed by Cre-mediated recombination (54). By crossing mice with loxP sites around the MR gene (MR floxed mice) with other strains that express Cre under the control of a cell-specific promoter, it is possible to explore how MR activation in specific cell types contributes to mineralocorticoid-induced pathology.

1.8.2 Cell selective MR blockade in cardiovascular disease

In a model of myocardial infarction, mice with MR gene deletion in cardiomyocytes had reductions in adverse cardiac remodelling as determined by ventricular dilatation and extracellular matrix deposition (176). Importantly, cardiac contractility and function were substantially preserved post myocardial infarct (176). Other researchers have shown that mice with MR gene deletion in myeloid cells are protected from deoxycorticosterone and salt induced hypertension and cardiac fibrosis despite no reductions in macrophage infiltration (233). Similarly, mice with myeloid MR deficiency are protected from cardiac enlargement and remodelling in a model of cardiac fibrosis induced by N^G-nitro-L-arginine methyl ester and AngII (201). Myeloid MR deficiency has also been found to be neuro-protective. In a mouse study of focal ischaemic stroke, mice with myeloid MR deletion had a significant reduction in infarct volume and suppressed activation of macrophages/microglia in the ischaemic core (234). These studies elegantly demonstrate the cell specific contribution of MR in cardiac cells and macrophages to contribute to heart and brain injury.

However, the cell type specific roles of MR signalling in kidney disease remains to be investigated.

1.9 Aims and hypothesis

In order to reduce the burden of progressive kidney disease and minimise the complication of hyperkalaemia, an understanding of the relative importance of MR activation in renal cells is required. This may identify specific cellular targets for MR blockade, or novel signalling pathways to inhibit, thus reducing the pathological aspect of MR activation.

1.9.1 Research Aims

1. To examine the effects of aldosterone on the proliferation of cultured renal fibroblasts and to identify the molecular mechanisms involved.
2. To assess the relative importance of MR activation in macrophages and podocytes in a mouse model of acute kidney disease.
3. To determine the role of macrophage MR in the progression of chronic progressive kidney disease.

1.9.2 Research hypothesis

This thesis tests the following hypotheses:

1. Mineralocorticoid Receptor signalling promotes renal fibrosis by inducing the proliferation of kidney fibroblasts.

2. Mineralocorticoid receptor signalling in myeloid cells contributes to the development of acute inflammation and renal injury in anti-GBM glomerulonephritis.

3. Mineralocorticoid receptor signalling in podocytes contributes to the development of acute podocyte and glomerular injury and proteinuria in anti-GBM glomerulonephritis.

4. Mineralocorticoid receptor signalling in myeloid cells contributes to the development of chronic inflammation and renal injury in streptozotocin-induced type 1 diabetic nephropathy.

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Monash University

Declaration for Thesis Chapter 2

Declaration by candidate

In the case of Chapter 2, the nature and extent of my contribution to the work was the following:

Nature of contribution	Extent of contribution
Performed all NRK-49F cell culture work, and proliferation assays. Performed statistical analyses of the data. Prepared figures and the manuscript.	70%

The following co-authors contributed to the work. If co-authors are students at Monash University, the extent of their contribution in percentage terms must be stated:

Name	Nature of contribution	Signatures
David J. Nikolic-Paterson	Intellectual input	
Frank Y. Ma	Assistance with primary cell culture	
Greg H. Tesch	Project supervision Intellectual input	

No co-authors are students at Monash University, therefore percentage of contribution are omitted.

The undersigned hereby certify that the above declaration correctly reflects the nature and extent of the candidate's and co-authors' contributions to this work.

Candidate's Signature		**Date**
Main Supervisor's Signature		**Date**

*Note: Where the responsible author is not the candidate's main supervisor, the main supervisor should consult with the responsible author to agree on the respective contributions of the authors.

2 The effect of mineralocorticoid receptor activation on renal fibroblast proliferation

Aldosterone induces kidney fibroblast proliferation via activation of growth factor receptors and PI3K /MAPK signalling

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2.1 Abstract

***Background/Aims**:* The mineralocorticoid hormone, aldosterone, has pro-fibrotic properties which can cause kidney damage. The severity of kidney interstitial fibrosis is dependent on the accumulation of fibroblasts, which result largely from local proliferation; however, it is unknown whether aldosterone stimulates kidney fibroblast proliferation. Therefore, we examined the effects of aldosterone on the proliferation of cultured kidney fibroblasts.

***Methods**:* Uptake of ^{3}H-thymidine and cell number quantitation were used to determine the proliferative effects of aldosterone on a rat kidney fibroblast cell-line (NRK-49F cells) and interstitial fibroblasts extracted from the mouse kidneys after unilateral ureter obstruction. The role of different mitogenic signalling pathways in aldosterone-induced proliferation was assessed using specific inhibitors of receptors and kinases.

***Results**:* Physiological levels of aldosterone induced a doubling of proliferation of kidney fibroblasts ($p<0.0001$), which was inhibited by pre-treatment with the mineralocorticoid receptor (MR) antagonist, eplerenone. Aldosterone-induced fibroblast proliferation was dependent upon the kinase activity of growth factor receptors (PDGFR and EGFR). Notably, PDGF ligands were not involved in aldosterone-induced PDGFR activation, indicating receptor transactivation. Aldosterone-induced fibroblast proliferation also required signalling via PI3K, JNK and ERK pathways, but not TGF-β1R.

***Conclusion**:* Aldosterone ligation of MR in kidney fibroblasts results in rapid activation of growth factor receptors and induction of PI3K/MAPK signalling, which stimulates proliferation. This suggests that increased levels of aldosterone during disease may promote the severity of kidney fibrosis by inducing fibroblast proliferation.

2.2 Introduction

Increased levels of aldosterone promote fibrosis in the heart and kidneys (1); however, it is unclear whether these profibrotic effects are simply an indirect consequence of aldosterone-induced hypertension or a direct effect of aldosterone on fibroblasts via the mineralocorticoid receptor (MR).

Studies in hypertensive rats demonstrate that MR blockade with eplerenone, at doses that do not reduce blood pressure, can inhibit renal fibrosis in the presence or absence of diabetes (2-3). Eplerenone can also reduce renal fibrosis in diabetic mice which do not develop hypertension (4). These findings suggest that aldosterone can induce renal fibrosis in the absence of an effect on hypertension.

In vitro studies indicate that aldosterone can directly promote fibrotic responses. In kidney fibroblasts, physiological levels of aldosterone (10^{-9}M to 10^{-8}M) can enhance production of TGF-β1, PAI-1 and CTGF (5-6) and increase mRNA levels of collagen and ^{3}H-proline incorporation (7). These findings suggest that aldosterone may regulate matrix production by fibroblasts.

Aldosterone may also promote fibrosis by increasing fibroblast accumulation. In diseased kidneys, interstitial fibroblast number is a strong predictor of progression to end-stage renal failure (8). Aldosterone can induce epithelial to mesenchymal transition of cultured kidney tubular cells resulting in their

transformation into myofibroblasts (9). However, regardless of the origin of kidney interstitial fibroblasts, it is local proliferation of these fibroblasts that is a major factor driving their accumulation in the diseased kidney (10). Studies have reported that aldosterone can induce proliferation of cardiac myofibroblasts (11-12), and that a high level of aldosterone (10^{-7}M) can induce the proliferation of a kidney fibroblast cell-line (13). However, it is unknown whether physiological levels of aldosterone stimulate the proliferation of kidney fibroblasts, especially primary cells isolated from fibrotic kidneys. Therefore, this study was devised to examine whether kidney-derived fibroblasts proliferate in response to aldosterone and to determine the cell signalling mechanisms involved.

2.3 Methods

Rat kidney fibroblast cells

A fibroblast cell-line derived from normal rat kidney (NRK-49F) was obtained from the American Tissue Culture Collection (ATCC, Rockville, MD, USA).

Isolation of fibroblasts from fibrotic mouse kidneys

Interstitial fibroblasts were obtained from fibrotic mouse kidneys after 7 days of unilateral ureter obstruction (UUO) (10), in accordance with institutional ethics guidelines. These fibroblasts have stronger proliferative activity than those obtained from normal kidneys and more closely represent fibroblasts in diseased kidneys (14). Dissected kidneys were incubated in collagen-coated wells in 10% FCS/DMEM/penicillin/streptomycin. The primary outgrowth cells were trypsinised and subcultured for experiments. Primary cells were cultured with c-fms antibody (20 µg/ml AFS98) to deplete contaminating macrophages (15). After 2 weeks, the remaining kidney cells were found to be fibroblasts based on flow cytometry analysis (see results section).

Cell Proliferation Assays

Kidney fibroblasts were added to 96 well plates ($2.5x10^3$ cells/well) in 5% FCS/DMEM and, after overnight culture, the FCS was reduced to 0.5% for 24 h to render cells quiescent. After fasting, cells were incubated with and without stimuli for 24 h in DMEM containing 0.1% BSA and 1%

insulin/transferrin/selenium supplement (Invitrogen, Grand Island NY, USA). The stimuli used were aldosterone (Sigma) or rhPDGF-AB (10 ng/mL, Peprotech, Rocky Hill, NJ, USA). During the last 18 h of culture, ^{3}H-thymidine (0.5 µCi/well, Amersham, UK) was added to the medium. After incubation, cells were washed and lysed, and the lysate analysed with a β-counter. In some experiments, cell number was determined by adding a tetrazolium compound (MTS) to media and measuring its conversion to formazan (Celltiter, Promega, Madison, WI, USA).

Inhibition of Cell Signalling Pathways

To identify signalling mechanisms involved in aldosterone-induced proliferation, cells were pre-incubated with various inhibitors for 30 min before stimulation. Preliminary studies identified the minimal effective dose of these inhibitors and showed that these doses were not cytotoxic by measuring cellular release of lactate dehydrogenase (Cytotoxicity Detection Kit, Roche Diagnostics, Mannheim, Germany). The inhibitors used were: MR antagonist - eplerenone (Sigma); PDGFR kinase inhibitor - STI-571 (Novartis, Sydney, Australia); rmPDGFRα/Fc chimera and rmPDGFRβ/Fc chimera (R&D Systems, Minneapolis, MN, USA); EGFR kinase inhibitor - AG1478 (Calbiochem, La Jolla, CA, USA); PI3K inhibitor - LY294002 (Calbiochem); JNK inhibitor - SP600125 (Calbiochem); ERK inhibitor - UO126 (Calbiochem); and ALK5/TGF-β1R kinase inhibitor - SB431542 (Calbiochem).

Immunoprecipitation and Western Blots

NRK-49F cells were grown in 6 well plates to near confluency and then starved in 0.5%FCS/DMEM for 24 h. Cells were then incubated in DMEM with 10^{-4}M vanadate in the presence of either aldosterone (10^{-9}M or 10^{-8}M, Sigma) or rhPDGF-AB (10ng/mL, PeproTech) or no stimuli for 15, 30 or 60 min. After stimulation, cells were washed and placed in lysis buffer (1% Triton X-100, 50 mM Tris/HCl pH7.5, 150 mM NaCl, 100 mM NaF, 2 mM EGTA and phosphatase/protease inhibitor cocktail from Sigma).

For immunoprecipitation studies, cell lysates were incubated with rabbit anti-PDGF-Rβ antibody (sc-432, Santa Cruz Biotechnology, Santa Cruz, CA, USA). The lysate-antibody solution was then incubated with Protein-G Sepharose, and bound proteins were released in SDS-PAGE sample buffer. Proteins were separated by SDS-PAGE and electroblotted onto nitrocellulose membranes. After blocking with Odyssey buffer (LI-COR, Lincoln, NB, USA), membranes were incubated with mouse anti-phospho-tyrosine antibody (PY20, Upstate Biotechnology, Lake Placid, NY, USA) followed by donkey anti-mouse IRDye 800 (Rockland, Gilbertsville, PA, USA). Phosphorylated PDGFR was detected using the Odyssey Infrared Image Detection System (LI-COR). Blots were reprobed to determine total PDGFR levels by incubation with rabbit anti-PDGFRβ (sc-432, Santa Cruz) followed by goat anti-rabbit Alexa Fluor 680 (Invitrogen, Carlsbad, CA, USA) and LI-COR analysis.

For analysis of cell signalling pathway activation, proteins in cell lysates were separated by SDS-PAGE and electroblotted onto nitrocellulose membranes. After blocking in Odyssey buffer, membranes were incubated with rabbit primary antibodies (anti-phospho-ERK 1/2 [Thr202/204], anti-phospho-JNK 1/2 [Thr183/Thy185] or anti-phospho-Akt [Ser473]; all from Cell Signaling Technology, Beverly, MA, USA) followed by goat anti-rabbit Alexa Fluor 680 (Invitrogen, Carlsbad, CA, USA). Protein bands were detected using the Odyssey Infrared Detection System (LI-COR). Protein loading was determined using mouse anti-α-tubulin (Sigma) followed by donkey anti-mouse IRDye 800 (Rockland).

Real-time quantitative PCR analysis of PAI-1 mRNA levels in NRK-49F cells

NRK-49F cells were cultured for 8 h in media containing: no additions, TGF-β1 (10 µg/mL), or TGF-β1 + ALK5 inhibitor (5 µM SB431542). Total RNA was then extracted from NRK-49F cells using Trizol (Invitrogen) and reverse transcribed with random primers using the Superscript First-Strand Synthesis kit (Invitrogen). Real-time PCR was performed on a StepOne Real-Time PCR System (Applied Biosystems, Scoresby, VIC, Australia) with thermal cycling conditions of 37°C for 10 min, 95°C for 5 min, followed by 50 cycles of 95°C for 15 s, 60°C for 20 s, and 68°C for 20 s. The primer pairs and carboxyfluorescein-labelled minor groove binding probe used were: Pai-1 (Forward: CAA AAG GTC AGG ATC GAG GTA; Reverse: ATT GTC TCT GTC GGG TTG T; Probe: TTC TCT TTG TGG TTC GG). The relative amount of mRNA was calculated using

comparative Ct (ΔΔCt) method. All specific amplicons were normalized against 18S rRNA which was amplified in the same reaction as an internal control using commercial assay reagents (Applied Biosystems, Scoresby, VIC, Australia). Each of the primer/probe sets were pre-tested and determined to have equivalent PCR amplification efficiencies.

Statistical Analysis

Statistical differences were analysed by Student's t-test or one way ANOVA. Data were recorded as mean ± SEM and $p<0.05$ were considered significant. All analyses were performed using GraphPad Prism 5.0 (GraphPad software, San Diego, CA, USA). Each experiment was performed 3 times.

2.4 Results

Aldosterone induces renal fibroblast proliferation by MR signalling

Aldosterone at 10^{-9}M and 10^{-8}M increased ^{3}H-thymidine uptake in NRK-49F cells by approximately 2-fold; however, no mitogenic effect was seen at 10^{-7}M (Figure 2.1a). Aldosterone also increased the number of NRK-49F cells (Figure 2.1b). Similarly, fibroblasts extracted from obstructed mouse kidneys showed a 2-fold proliferative response to aldosterone at 10^{-8}M (Figure 2.1c,d). Pre-treatment of kidney fibroblasts with 10^{-6}M eplerenone prevented the proliferative effect of aldosterone without altering basal proliferation (Figure 2.1a-d). Flow cytometry analysis of the fibroblasts isolated from obstructed mouse kidneys shows that these cells were all myofibroblasts, with expression of fibroblast specific protein-1 and α-smooth muscle actin, but not cytokeratin C-18 (an epithelial marker) or CD68 (a macrophage marker) (Figure 2.1e).

Aldosterone-induced fibroblast proliferation requires activation of growth factor receptors

Pre-treatment of NRK-49F cells with an inhibitor of PDGFR kinase (0.5 μM STI-571) or EGFR kinase (0.25 μM AG1478) prevented the effect of 10^{-9}M aldosterone on fibroblast proliferation (Figure 2.2a,b), but did not influence basal proliferation. Immunoprecipitation of PDGFR-β from cell lysates of NRK-49F cells and subsequent western blotting of phosphorylated tyrosine showed that stimulation with 10^{-9}M aldosterone for 30 min increased phosphorylation of PDGFR-β (Figure 2.2c), suggesting that the receptor was rapidly activated by

aldosterone. The stimulatory effect of 10^{-9}M aldosterone on NRK-49F cell proliferation was unaffected by pre-treating cells with soluble PDGFR, which comprised PDGFRα/Fc and PDGFRβ/Fc fusion proteins (2 μM each). In contrast, the same pre-treatment suppressed the proliferation induced by 10 ng/ml rhPDGF-AB (Figure 2.2d), showing that the soluble PDGFR blocks ligand-mediated proliferation through the PDGF receptor.

Aldosterone-induced fibroblast proliferation involves activation of PI3K and MAPKs

The post-receptor signalling mechanisms involved in aldosterone-induced fibroblast proliferation were examined in NRK-49F cells. Stimulation of NRK-49F cells with 10^{-8}M aldosterone resulted in activation of Akt, JNK and ERK signalling (Figure 2.3a). We then examined whether specific inhibitors of these kinases could inhibit aldosterone-induced kidney fibroblast proliferation. The proliferative effect of 10^{-9}M aldosterone on NRK-49F cells was prevented by pre-treatment with inhibitors of PI3K (3 μM LY294032), JNK (1 μM SP600125) and ERK (1 μM UO126), and the doses of inhibitors used had no effect on basal proliferation (Figure 2.3b-d). Since aldosterone also induces TGF-β1 production by NRK-49F cells, we examined whether signalling via the TGF-βR1 receptor is involved in this proliferative response. Treatment with an inhibitor of TGF-βR1/ALK5 (5 μM SB431542) did not prevent aldosterone-induced fibroblast proliferation (Figure 2.3a), although this inhibitor suppressed a TGF-β1-induced increase of PAI-1 mRNA level at 8 h in NRK-49F cells (PAI-1/18s: no treatment 1.0±0.1, TGF-β1 14.2±2.6, TGF-β1+ALK5 1.1±0.1).

Figure 2.1 Aldosterone induces renal fibroblast proliferation via the mineralocorticoid receptor

(a) Dose-response effect of aldosterone on ^{3}H-thymidine uptake in NRK-49F cells at 24 h and impact of eplerenone blockade. (b) Effect of aldosterone and eplerenone blockade on the number of NRK-49F cells. (c) Analysis of aldosterone-induced ^{3}H-thymidine uptake and eplerenone blockade in primary fibroblasts extracted from mouse UUO kidneys. (d) Effect of aldosterone and eplerenone blockade on the number of mouse UUO kidney fibroblasts. (e) Flow cytometry analysis of fibroblasts isolated from mouse UUO kidneys. Compared to labelling with negative control (NC) antibodies, fibroblasts from UUO kidneys had strong expression of fibroblast specific protein-1 (FSP-1) and α-smooth muscle actin (α-SMA), but did not express cytokeratin C-18 or CD68. Graphed data = mean ± SEM; n = 12 (a,c); n = 6 (b,d).

Figure 2.1

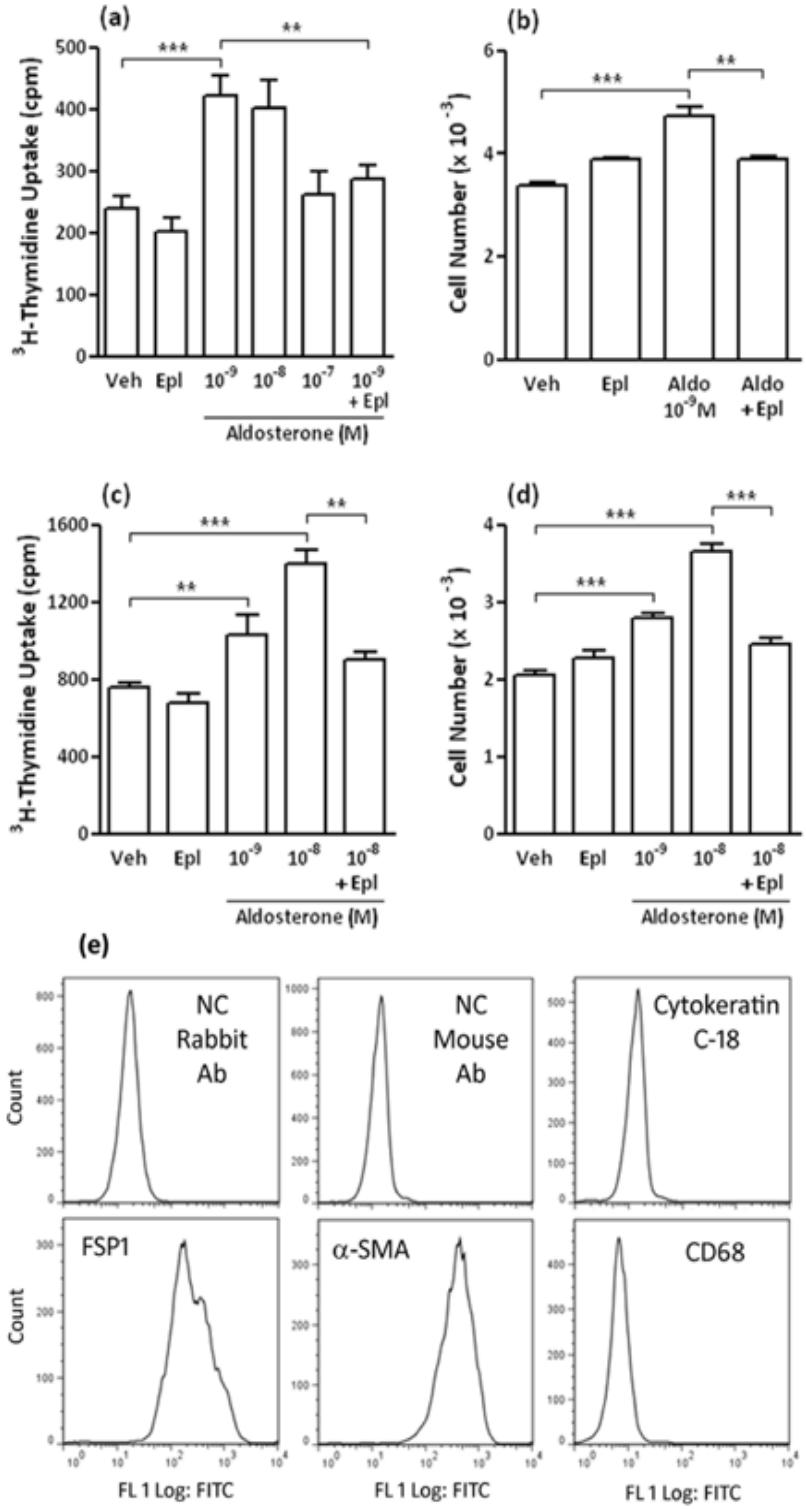

(a)
^{3}H-Thymidine Uptake (cpm)
Veh
Epl
10^{-9}
10^{-8}
10^{-7}
10^{-9} + Epl
Aldosterone (M)
(b)
Cell Number (x 10^{-3})
Veh
Epl
Aldo 10^{-9}M
Aldo + Epl
(c)
^{3}H-Thymidine Uptake (cpm)
Veh
Epl
10^{-9}
10^{-8}
10^{-8} + Epl
Aldosterone (M)
(d)
Cell Number (x 10^{-3})
Veh
Epl
10^{-9}
10^{-8}
10^{-8} + Epl
Aldosterone (M)
(e)
NC Rabbit Ab
NC Mouse Ab
Cytokeratin C-18
FSP1
α-SMA
CD68
Count
FL 1 Log: FITC

Figure 2.2 Aldosterone-induced kidney fibroblast proliferation requires activation of PDGF and EGF receptors

Pre-treatment with inhibitors of (a) the PDGFR (PDGFRi, STI571) and (b) the EGFR (EGFRi, AG1478) prevented aldosterone-induced proliferation of NRK-49F fibroblasts. (c) A western blot of PDGFR-β immunoprecipitate from NRK-49F cell lysate showed that aldosterone increases tyrosine phosphorylation of the PDGFR-β in these cells. (d) Pretreatment with soluble PDGFR (sPDGFR) was unable to prevent aldosterone-induced proliferation of NRK-49F fibroblasts. Graphed data = mean ± SEM; n= 9 (a,b), n=12 (d).

Figure 2.2

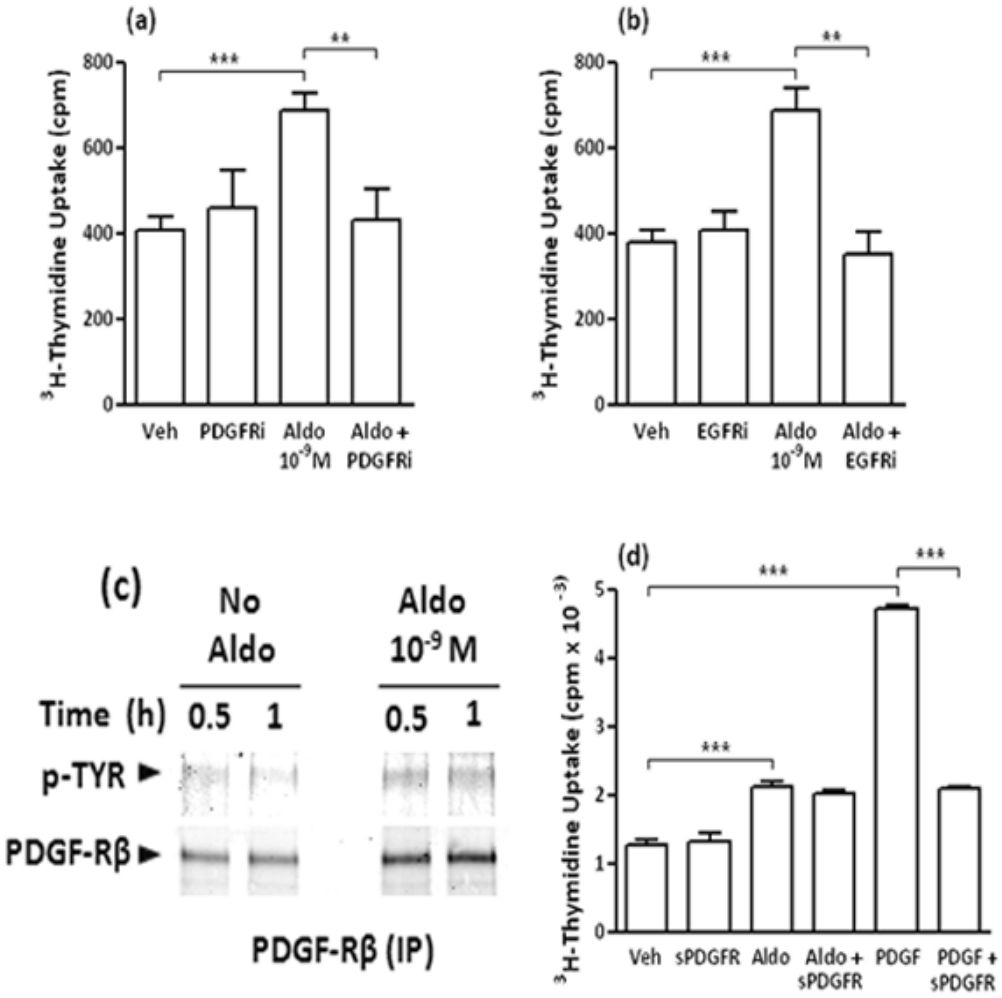
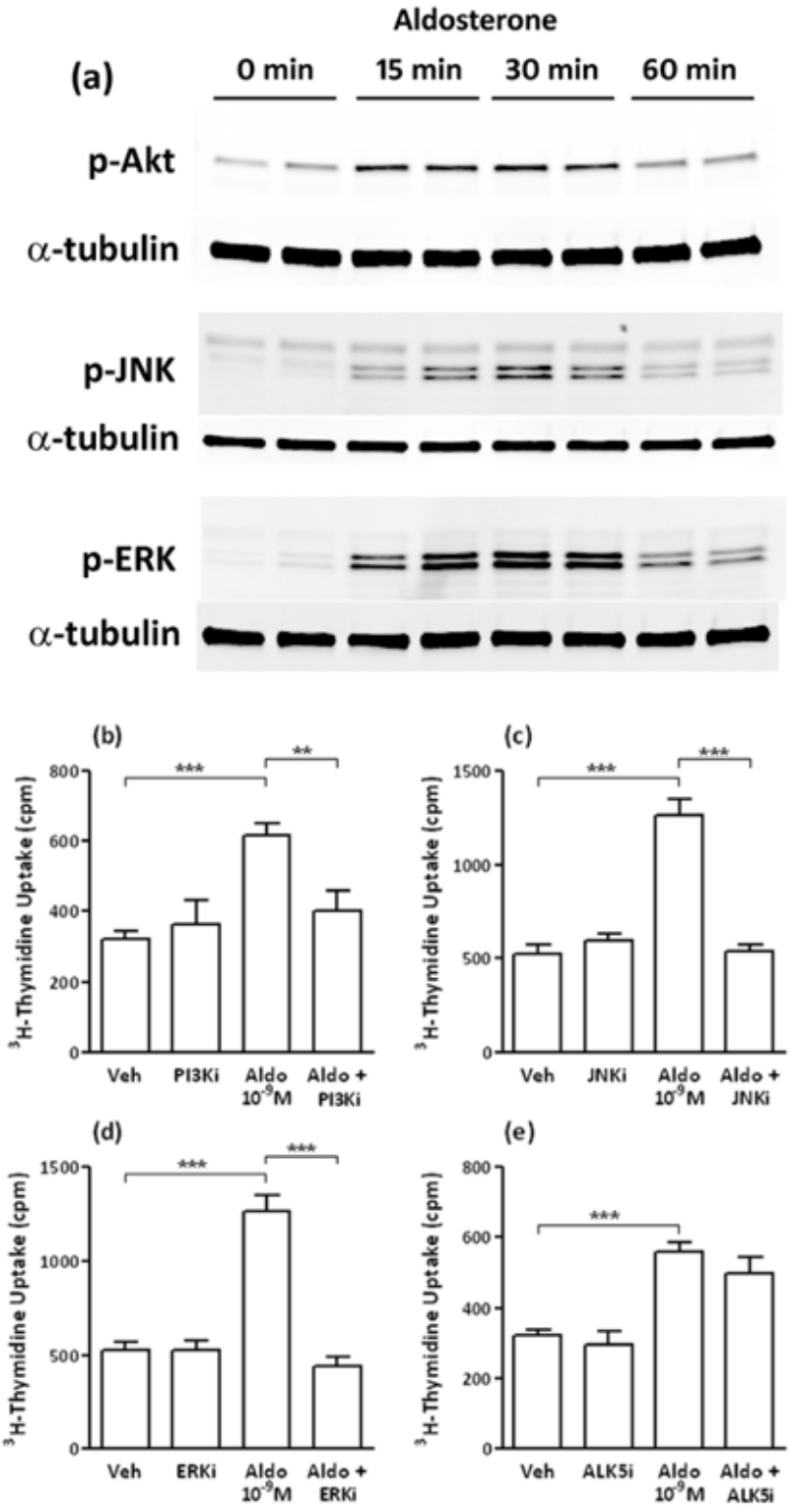

(a)
^{3}H-Thymidine Uptake (cpm)
800
600
400
200
0
***
**
Veh
PDGFRi
Aldo 10^{-9}M
Aldo + PDGFRi
(b)
^{3}H-Thymidine Uptake (cpm)
800
600
400
200
0
***
**
Veh
EGFRi
Aldo 10^{-9}M
Aldo + EGFRi
(c)
No Aldo
Aldo 10^{-9} M
Time (h) 0.5 1 0.5 1
p-TYR ►
PDGF-Rβ ►
PDGF-Rβ (IP)
(d)
^{3}H-Thymidine Uptake (cpm x 10^{-3})
5
4
3
2
1
0
***
***
Veh
sPDGFR
Aldo
Aldo + sPDGFR
PDGF
PDGF + sPDGFR

Figure 2.3 Aldosterone-induced fibroblast proliferation involves activation of PI3K/Akt, JNK and ERK signalling pathways

Western blotting of NRK-49F cell lysates demonstrates (a) aldosterone-induced phosphorylation of Akt, JNK1/2 and ERK1/. α-tubulin levels are shown as loading controls. Pretreatment with inhibitors of (b) PI3K (PI3Ki, LY294002), (c) JNK (JNKi, SP600125) and (d) ERK (ERKi, UO126) prevented aldosterone-induced proliferation of NRK-49F fibroblasts. In comparison, an inhibitor of (e) TGF-βR1/ALK5 (ALK5i, SB431542) did not suppress aldosterone-induced fibroblast proliferation. Data = mean ± SEM; n= 12 (b); n = 9 (c-e).

Figure 2.3


Aldosterone
(a)
0 min
15 min
30 min
60 min
p-Akt
α-tubulin
p-JNK
α-tubulin
p-ERK
α-tubulin
(b)
(c)
(d)
(e)
^{3}H-Thymidine Uptake (cpm)
Veh
PI3Ki
Aldo 10^{-9}M
Aldo + PI3Ki
JNKi
Aldo + JNKi
ERKi
Aldo + ERKi
ALK5i
Aldo + ALK5i
***
**

2.5 Discussion

Our study has shown that physiological levels of aldosterone induce proliferation of a kidney fibroblast cell-line and fibroblasts extracted from fibrotic mouse kidneys, demonstrating a capacity for increased levels of aldosterone to promote fibroblast accumulation in diseased kidneys. This finding is directly relevant to kidney disease since previous research has reported elevated aldosterone levels in plasma and urine of patients with chronic kidney disease (1) and increased MR levels in diabetic kidneys (4, 16).

Aldosterone-induced proliferation of kidney fibroblasts was found to be dependent on the activation of specific receptors. Firstly, eplerenone prevented the proliferative response to aldosterone, indicating that this effect was mediated by the established cytoplasmic MR. Secondly, aldosterone induced proliferation required activation of two growth factor receptors, PDGFR and EGFR. Interestingly, the PDGFR-mediated response was ligand-independent, indicating that activated cytoplasmic MR was inducing transactivation of the PDGFR. It is also likely that the induction of EGFR signalling in kidney fibroblasts is dependent on transactivation, since aldosterone binding of MR facilitates transactivation of EGFR in other kidney cell types (17-18). Furthermore, it is possible that transactivation of PDGFR and EGFR may be linked by heterodimerisation of these receptors, which occurs in vascular smooth muscle cells (VSMC) resulting in cross-talk (19). Indeed, studies with

VSMC have identified that aldosterone can stimulate transactivation of both the PDGFR and EGFR in the presence of low levels of angiotensin II (20).

This study has revealed critical post receptor signalling mechanisms which facilitate aldosterone-induced proliferation of kidney fibroblasts. Aldosterone induced rapid phosphorylation of Akt, JNK and ERK, and blockade of PI3K, JNK or ERK could prevent the aldosterone-induced proliferative response. Our finding is supported by studies of Müller glial cells in which ATP stimulates transactivation of the PDGFR and EGFR and subsequent activation of PI3K and ERK1/2, resulting in proliferation (21). Aldosterone can also induce proliferation of cardiac myofibroblasts via activation of the Ras/Raf/MEK1/2/ERK1/2 pathway (11) and proliferation of mesangial cells by a pathway dependent on activation of EGFR, PI3K and ERK1/2 (18). Our discovery that JNK signalling is also required for aldosterone-induced renal fibroblast proliferation is supported by studies showing that PDGF-BB induced proliferation of mesangial cells and dermal fibroblasts is JNK dependent (22-23).

Previous work shows that aldosterone increases TGF-β1 mRNA levels in NRK-49F cells (5), and TGF-β1 can stimulate proliferation of NRK-49F cells (24) and fibroblasts isolated from renal biopsies (25). However, our results indicate that the proliferative effects of aldosterone on kidney fibroblasts are rapid and probably precede TGF-β1 production. Furthermore, TGF-β1R blockade did not

alter aldosterone-induced proliferation in NRK-49F cells. Therefore, aldosterone does not appear to induce fibroblast proliferation by producing TGF-β1.

Aldosterone promotes fibrosis in animal models of hypertensive kidney disease, glomerulonephritis and diabetic nephropathy (3-4, 26), suggesting that aldosterone-induced fibroblast proliferation may be an important pathomechanism in chronic kidney diseases. Our study demonstrates that aldosterone directly induces the proliferation of cultured kidney fibroblasts via MR-mediated activation of growth factor receptors and downstream signalling through PI3K/Akt, ERK and JNK pathways (Figure 2.4).

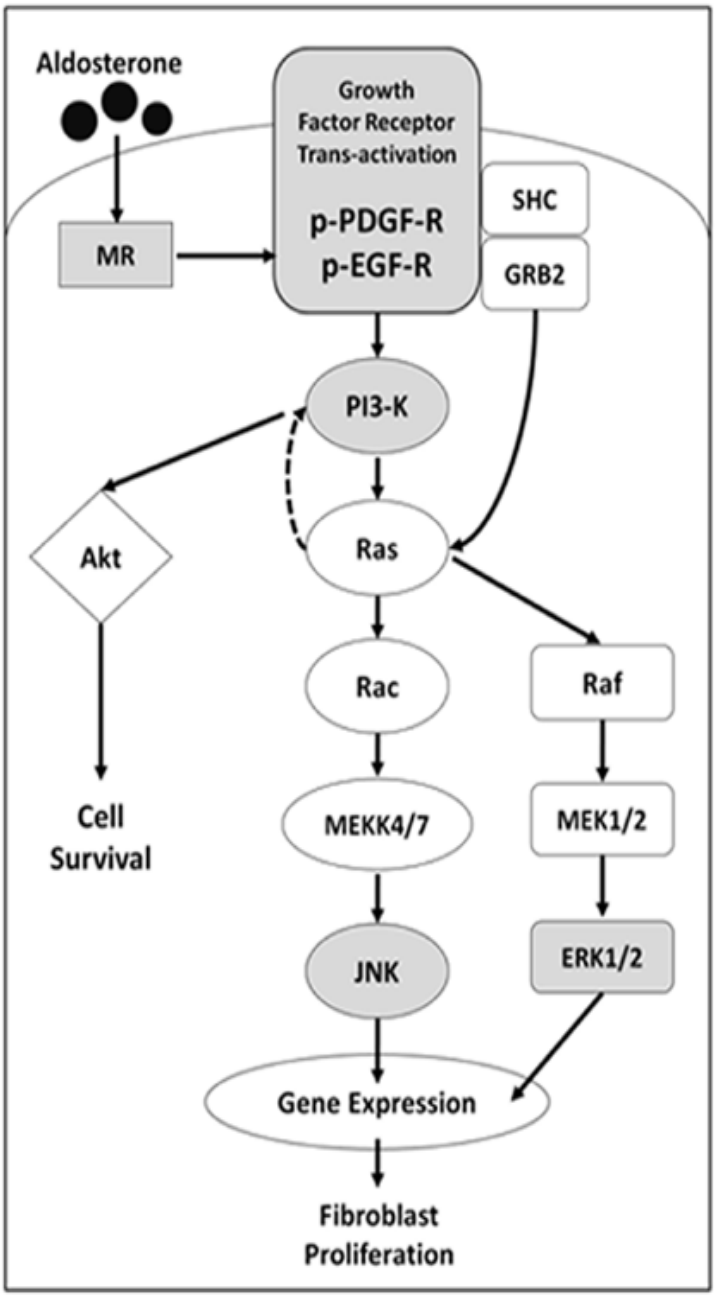
Disclosure Statement

The authors have no conflicts of interest. This study was supported by the Jacquot Foundation and the National Health and Medical Research Council of Australia.

Figure 2.4 Proposed mechanism of aldosterone-induced kidney fibroblast proliferation

Aldosterone binds with cytoplasmic mineralocorticoid receptors resulting in the transactivation of growth factor receptors, which facilitate activation of PI3K/Akt and Ras signalling. Activation of Ras induces JNK signalling via Rac and MEKK4/7 and ERK1/2 signalling via Raf and MEK1/2, which stimulates expression of genes that promote fibroblast proliferation.

Figure 2.4


Aldosterone
Growth
Factor Receptor
Trans-activation
p-PDGF-R
p-EGF-R
SHC
GRB2
MR
PI3-K
Akt
Ras
Rac
Raf
Cell
Survival
MEKK4/7
MEK1/2
JNK
ERK1/2
Gene Expression
Fibroblast
Proliferation

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Monash University

Declaration for Thesis Chapter 3

Declaration by candidate

In the case of Chapter 3, the nature and extent of my contribution to the work was the following:

Nature of contribution	Extent of contribution
Maintained animals throughout disease. Cut tissue sections and performed all immunohistochemistry staining. Prepared figures and the manuscript.	65%

The following co-authors contributed to the work. If co-authors are students at Monash University, the extent of their contribution in percentage terms must be stated:

Name	Nature of contribution	Signatures
David J. Nikolic-Paterson	Intellectual input	
Yingjie Han	Assistance in setting up animal model	
Elyce Ozols	Establishment of mice colonies Genotyping mice	
Frank Y. Ma	Assistance with macrophage isolation	
Morag J. Young	Intellectual input Establishment of mice colonies	
Greg H. Tesch	Project supervision Intellectual input	

No co-authors are students at Monash University, therefore percentage of contribution are omitted.

The undersigned hereby certify that the above declaration correctly reflects the nature and extent of the candidate's and co-authors' contributions to this work.

Candidate's Signature		**Date**
Main Supervisor's Signature		**Date**

3 The roles of myeloid and podocyte MR in glomerulonephritis

Mineralocorticoid Receptor Activation in Macrophages Contributes to Progressive Kidney Disease

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3.1 Abstract

Background/Aims: Clinical and experimental studies have shown that mineralocorticoid receptor (MR) antagonists substantially reduce kidney injury. However, to fully understand the benefits of this therapy, it is important to identify the specific cellular targets and mechanisms by which MR antagonists protect against kidney injury. This study used conditional gene deletion of MR signaling in myeloid cells ($MR^{flox/flox}$ $LysM^{Cre}$ mice = MyMRKO) or podocytes ($MR^{flox/flox}$ Pod^{Cre} = PodMRKO) to establish the role of MR in these cells types in the development of mouse glomerulonephritis.

Method: Accelerated anti-GBM glomerulonephritis was examined in groups of mice: MyMRKO; PodMRKO; wild type littermates (WT); and WT mice receiving eplerenone (100 mg/kg/bid, EPL-treated).

Results: At day 15 of disease, WT mice had glomerular crescents (37±5%), severe proteinuria and a 6-fold increase in serum cystatin-C. MyMRKO, PodMRKO and EPL-treated mice with glomerulonephritis displayed similar proteinuria to these disease controls. However, MyMRKO and EPL groups had a 35% reduction in serum cystatin-C levels and reduced crescent numbers compared to WT, whereas PodMRKO mice were not protected. The kidney protection seen in MyMRKO mice was associated with reductions in macrophage accumulation, gene expression of M1 proinflammatory markers

(TNF-*α*, iNOS, MMP-12), tubular damage and renal fibrosis, which was similar in EPL-treated mice.

Conclusion*:* In conclusion, MR signaling in macrophages, but not podocytes, contributes to the progression of renal injury in mouse glomerulonephritis. Selective targeting of MR in macrophages may be able to provide the therapeutic benefit seen with current systemic MR antagonists without the associated problems of hyperkalemia.

3.2 Introduction

Mineralocorticoid receptor (MR) antagonists (eplerenone, spironolactone) are known to inhibit renal and cardiovascular disease (CVD) by direct blockade of MR in tissues and by reducing hypertension (1). They can also suppress the development of kidney damage in animal models of glomerulonephritis and diabetic nephropathy without affecting blood pressure (2-6). In addition, MR antagonists provide added protection against proteinuria and loss of renal function when used with standard anti-hypertensive therapies in treating patients with diabetic and non-diabetic chronic kidney disease (CKD) (7-9).

The clinical use of MR antagonists is currently limited by the development of hyperkalemia due to the importance of the MR in tubular regulation of salt balance (10). This consequence of MR blockade in the distal tubule is most evident during renal impairment and can require either a reduction in the dosage of MR antagonist or its withdrawal as a therapy (7-8). The specific renal cell types which are targeted by MR antagonists to reduce injury during kidney disease have not been clearly identified. Establishing the identity of these cells is an important step towards developing more selective inhibitors of MR signaling which do not interfere with tubular cell function.

Animal studies demonstrate that the protection afforded by MR antagonists in glomerulonephritis and diabetic nephropathy is associated with reductions in renal inflammation, proteinuria and glomerular injury (2-3, 5, 11). These studies

also link MR blockade to specific disease mechanisms such as proinflammatory cytokine production, leukocyte recruitment and podocyte injury. This suggests that the major pathological effects of MR signaling may occur in podocytes and inflammatory cells.

Recent *in vitro* studies have suggested that MR signaling can induce apoptosis in podocytes and oxidative stress in macrophages (12-13), which supports a role for MR signaling in these cell types in kidney disease. In addition, an MR-deficiency in myeloid cells has been shown to be protective against cardiovascular injury and ischemic cerebral infarcts by reducing inflammation and fibrosis (14-16). However, no *in vivo* studies have identified whether MR signaling in podocytes or macrophages is specifically important to the development of kidney disease.

In the current study, we have created mice with a selective genetic deficiency of MR in myeloid cells or podocytes, and have used these strains to evaluate the hypothesis that MR signaling in macrophages or podocytes is required for the development of renal injury in a normotensive model of progressive glomerulonephritis.

3.3 Methods

Animal Model

Conditional gene deletion of MR was performed in C57BL/6 mice using the Cre-LoxP system. Mice with homozygous floxed MR gene were crossed with littermates expressing Cre recombinase under the control of either a Lysozyme M promoter (14), to create $MR^{flox/flox}$ $LysM^{cre/-}$ (MyMRKO) mice lacking MR in mature myeloid cells (neutrophils, macrophages), or a Podocin promoter (17), to create $MR^{flox/flox}$ $Pod^{cre/-}$ (PodMRKO) mice lacking MR in podocytes. $MR^{flox/flox}$ littermates were used as WT controls. The presence of the MR wild type and MR floxed alleles, and the Cre transgene were determined by PCR analysis of genomic DNA from tail tips. The genotyping details are presented in Table 3.1.

Table 3.1 Genotyping PCR primer sequences

Target	Forward Primers	Reverse Primers
MR	TTCTTTCCCCAGCTCTACCTTTACGA	AGCAAGAGACAACTGCAGCGTTTTA ATGTGGAATGTGTGCGAGGCCAGAG
Cre	ATGTCCAATTTACTGACCG	TTCACTGGTTATGCGGCG

For experimentation, 12 week old female mice (n=8) were pre-immunised subcutaneously with 1 mg sheep IgG in Freund's complete adjuvant. Four days later, glomerulonephritis was induced by intravenous injection of sheep anti-mouse GBM serum (10 µl/g). Groups were killed after 3 h of disease, to

examine the initial inflammatory response, or after 15 days of disease, to examine the progression of renal damage. To compare conditional MR gene deletion with systemic MR blockade, groups of diseased WT mice were given eplerenone (100 mg/kg/bid) by gavage, beginning 2 h before disease induction. All animal experiments were performed in accordance with the guidelines of the Monash Medical Centre Animal Ethics Committee.

Hematology and Biochemical Analysis

Urine was collected from mice housed in metabolic cages. At day 15, heparinised blood and serum were collected from anaesthetised mice. White blood cell counts were determined by a hematology analyser (Cell-Dyn 3700, Abbott Laboratories, North Chicago, IL). Urine albumin levels were assessed by ELISA (Bethyl Laboratories, Montgomery, TX). Urine creatinine levels were determined by the Jaffe rate reaction method. ELISAs were used to measure serum levels of cystatin-C (BioVendor, Karasek, Czech Republic) and Ig (18). Levels of K+ or Na+ in plasma or urine were determined by indirect potentiometry using ion selective electrodes.

Antibodies

Antibodies used in this study were: anti-MR (2136, a gift from Prof. Celso Gomez-Sanchez, University of Mississippi); anti-CD68 (FA-11, Serotec, Oxford, UK); anti-Ly6g (Abcam, Cambridge, UK); anti-CD3 (KT3, Abcam); anti-CD41 (Serotec); anti-alpha-smooth muscle actin (α-SMA) (1A4, Sigma, St. Louis,

MO); anti-cleaved caspase 3 (Cell Signaling, Beverly, MA); anti-Wilm's Tumor antigen 1 (WT-1) (Santa Cruz Biotechnology, Santa Cruz, CA); anti-Cre (Novagen, Madison, WI); fluorescein-conjugated anti-CD11b (BD Biosciences, San Jose, CA); fluorescein conjugated sheep F(ab)2 fragment to mouse Ig (Silenus, Melbourne, Australia); and fluorescein conjugated goat F(ab)2 fragment to mouse C3 (MP Biomedicals, Solon, OH).

Isolation of Kidney Macrophages

Kidneys were decapsulated, diced, and incubated with 1 mg/ml collagenase (Sigma) and 0.1 mg/ml DNase I (Invitrogen, Carlsbad, CA) in Dulbecco's Modified Eagle Medium at 37˚C for 30 min with shaking. Following erythrocyte lysis, the cells were filtered (70 µm) and incubated with fluorescein-conjugated CD11b antibody for 30 min at 4 ˚C before fluorescence activated cell sorting (MoFlo XDP cell sorter, Beckman Coulter, Brea, CA). Approximately 1.5-2.5 x 10^6 CD11b+/propidium iodide- cells were isolated from two pooled diseased kidneys or four pooled normal kidneys.

Real-time PCR

Total RNA was extracted from whole kidney, magnetic-bead isolated glomeruli (19), or isolated kidney macrophages using Trizol (Invitrogen) and reverse transcribed with random primers using the Superscript First-Strand Synthesis kit (Invitrogen). Real-time PCR analysis was performed as previously described (20). The Taqman probe and primer sequences are listed in Table 3.2.

Table 3.2 qPCR probe and primer sequences

Target	Probe or Reference	Forward Primer	Reverse Primer
MR	TTTGAGGAGATGAGGAC	GGTTTTGCTGCTACTGAG	TGTTGGGACACTTGGTGA
CD68	CACAGTTTCTCCCACCA	CATGGGAATGCCACAATTTCT	ACAGTGGAGGATCTTGGACTA
TNFα	TCACCCACACCGTCAG	GGCTGCCCCGACTACGT	TTTCTCCTGGTATGAGATAGCAATC
iNOS/NOS2	TCCCTCCCCTCTCTCC	TCTCTGCTCTCAGCTCCAA	ACTACTAAATCTCTCTCCTCTCC
CCL2	ACAACCACCTCAAGCAC	GACCCGTAAATCTGAAGCTAA	CACACTGGTCACTCCTACAGAA
MMP-12	AGTCCAGCCACCAACA	CACAACAGTGGGAGAGAAAA	AGCTTGAATACCAGATGGGATG
Arginase-1	GGAAGGAAGAAAAGGC	GCAAGGTGATGGAAGAGA	TCGACATCAAAGCTCAGGTGAA
CD206	GTTTGGTTGGATTGAGG	GACAGATATGAACAAGCATTCC	TGAACATCTGAGAGTCCTGTCC
CD163	GGTTTCTTTGTTGTGG	TGGTTCTTCTTGGAGGTG	GCCAGTCTCAGTTCCTTCTT
CCR7	TGCTGGTGGTGGCTCTC	GCCTTCCTGTGTGATTTCT	TGGTATTCTCGCCGATGTAGT
CD3e	AGGAACCAGTGTAGAG	GCCGAGAACATTGAATACAAAG	TGGTGTGTAGCAGACGTAGTAG
CCL5	SYBR Green	ATATGGCTCGGACACCACTC	TCCTTCGAGTGACAAACACG
IL-12β (p40)	CCATTCCTACTTCTCC	CTTCTTCATCAGGACATCAT	CCTCCTCTGTCTCCTTCATCTT
Granzyme B	CTATGTGGCTGGTTGG	CAAGAGGACTAGAGCTGTGA	GCGTGTTTGAGTATTTGCCCAT
Collagen I	GTGGTGTGGTCGGTCT	GATCTCCTGGTGCTGATG	GAAGCCTCTTTCTCCTCTCTGA
Fibronectin	GGAGAGACAGGAGGAA	GCGCTATTACAGAATCACCTA	TTGATGGTGGCTGTGGACTT
PAI-1	TTCTCTTTGTGGTTCGG	CAAAAGGTCAGGATCGAGGTA	ATTGTCTCTGTCGGGTTGT
TGF-β1	ACAACCAACACAACCC	GGACACACAGTACAGCAA	GACCCACGTAGTAGACGAT

Histology and Immunostaining

Formalin-fixed kidney sections (2 μm) were stained with periodic acid Schiff's (PAS) reagent to identify kidney structure and crescent formation, and with hematoxylin to distinguish cell nuclei. The percentage of glomeruli with crescents was determined by microscopy. Direct immunofluorescence staining for glomerular Ig and C3 was performed on 4 μm ethanol-fixed kidney sections and slides were mounted with DABCO medium (Sigma). Glomerular deposition was assessed by performing serial 2-fold dilutions of antibody (1:200-1:25600), on sections and determining the dilution at which specific staining disappears by fluorescence microscopy. Immunoperoxidase staining for leukocytes (CD68, CD3, Ly6g) was performed on 2% paraformaldehyde-lysine-periodate (PLP)-fixed kidney cryostat sections (5 μm). Immunoperoxidase staining for MR, α-SMA, activated-caspase 3, WT-1 and Cre was performed on 10% formalin-fixed sections (4 μm). For detection of activated-caspase 3, WT-1 and Cre, tissue sections were microwave treated (1000 W) for 12 min in 400 ml sodium citrate buffer pH6.0 prior to immunostaining. For immunoperoxidase staining, tissue sections were incubated with 20% normal rabbit serum for 30 min and then overnight at 4°C with 2-5 μg/ml of primary antibody in 1% BSA. Sections labelled with leukocyte antibodies were then incubated for 20 min each with 0.6% hydrogen peroxide followed by avidin and biotin block (Vector Laboratories, Burlingame, CA, USA). After washing in PBS, these sections were incubated with biotinylated rabbit antibody (anti-rat IgG, Zymed, San Francisco, CA, USA) for 1 hr followed by ABC peroxidase solution (Vector) for 1 hr and developed with 3,3-diaminobenzidine (DAB, Sigma) to produce a brown color.

Sections labelled with antibodies to MR, α-SMA, activated-caspase 3, WT-1 and Cre were incubated sequentially with 0.6% hydrogen peroxide for 20 min, peroxidise-conjugated secondary antibodies (swine anti-rabbit Ig, rabbit anti-mouse Ig from Dako) for 45 min and peroxidise-conjugated anti-peroxidase (PAP) complexes (rabbit PAP, mouse PAP from Dako) for 45 min, and developed with DAB.

Glomerular leukocytes and WT-1+ cells were counted at high magnification (x400) in 20 hilar glomerular tuft cross-sections per animal. Interstitial leukocytes and activated caspase-3+ cells were counted in 25 consecutive high magnification (x 400) interstitial fields (representing 30-40% of kidney cortex in the cross-section) and expressed as cells/mm^2. Immunostaining of α-SMA was assessed as percent area stained within the glomerular tuft or renal cortex (excluding glomeruli and vessels) by computer image analysis software (Image Pro Plus, Media Cybernetics, CA, USA). All scoring was performed on blinded slides.

Statistical Analysis

Statistical differences between two groups were analysed by t-test and differences between multiple groups were assessed by ANOVA with post hoc analysis using Tukey's multiple comparison test. Data was analysed using Graph Prism 5.0 (GraphPad Software, San Diego, CA) and was recorded as the mean ± standard error with $p<0.05$ defined as significant.

3.4 Results

Mice with myeloid MR deficiency have normal leukocyte levels and antibody responses

Deletion of MR in macrophages from $MR^{flox/flox}$ $LysM^{Cre}$ mice (MyMRKO) has been previously reported by our group (14). MyMRKO mice have normal circulating levels of white blood cells (wild type-WT 5.2±0.4 x 10^9/L vs MyMRKO 5.0±0.4 x 10^9/L) with a similar proportion of monocytes (WT 1.4±1.0% vs MyMRKO 1.3±0.9%) and neutrophils (WT 9.6±0.2% vs MyMRKO 10.9±2.0%). During the development of glomerulonephritis (GN), MyMRKO mice produced an equivalent humoral immune response to WT mice, which included equal levels of circulating anti-sheep Ig and glomerular deposition of mouse Ig and C3 (Figure 3.1).

Myeloid MR deficiency protects mice from declining renal function in anti-GBM glomerulonephritis

Pre-experimental analysis showed that urinary protein excretion in MyMRKO mice is equivalent to that seen in normal mice (Figure 3.2A). Induction of GN in WT mice resulted in the development of proteinuria at day 1 of disease, which remained elevated at days 7 and 14 (Figure 3.2A). Administration of eplerenone to WT mice with GN did not affect the development of proteinuria. However, proteinuria was reduced by 40% in MyMRKO compared to WT mice at day 1 of disease, but was not different at days 7 or 14 of disease.

Serum levels of cystatin-C were the same in WT and MyMRKO mice without kidney disease, indicating that MyMRKO mice had normal renal function (Figure 3.2B). WT mice had a 6-fold increase in the serum level of cystatin-C at day 15 of anti-GBM GN, which was reduced by approximately 35% in both MyMRKO mice and eplerenone-treated WT mice.

Myeloid MR deficiency reduces kidney damage in anti-GBM glomerulonephritis

At day 15 of GN, wild type mice had severe histological kidney damage which included crescents in 37% of glomeruli (Figure 3.3,Table 3.3). In comparison, MyMRKO mice and eplerenone-treated WT mice had significantly reduced crescent formation with levels of 26% and 11% of glomeruli, respectively. Immunostaining analysis identified increased glomerular accumulation of myofibroblasts in diseased compared to normal WT mice which was diminished in MyMRKO and eplerenone-treated WT mice (Figure 3.3, Table 3.3).

Table 3.3 Kidney damage in MyMRKO mice

Analysis	*WT*	*MyMRKO*	*Anti-GBM glomerulonephritis*		
			WT	*MyMRKO*	*WT + EPL*
Crescents (%)	0	0	37±2	26±1[c]	11±2[c,e]
Apoptotic Cells/mm^2	0.5±0.2	0.4±0.2	6.0±0.5	2.9±0.2[c]	3.7±0.4[b]
Glomerular Myofibroblasts (% area)	0.5±0.1	0.4±0.1	12.2±1.0	4.4±0.4[c]	5.6±0.5[c]
Interstitial Myofibroblasts (% area)	0.4±0.1	0.2±0.1	5.3±0.8	1.2±0.2[c]	2.4±0.5[b,d]
Collagen I mRNA/18s	1.1±0.3	2.6±0.2	38±4	16±5[b]	16±2[c]
Fibronectin mRNA/18s	1.1±0.2	2.2±0.2	27±3	17±3[a]	18±3[a]
PAI-1 mRNA/18s	1.2±0.2	2.1±0.4	35±11	18±4	18±2
TGF-β1 mRNA/18s	1.2±0.4	1.3±0.3	4.4±0.6	3.7±0.4	4.9±0.6

Data = mean ± standard error.

[a]$p<0.05$, [b]$p<0.01$, [c]$p<0.001$ vs WT with disease. [d]$p<0.05$, [e]$p<0.001$ vs MyMRKO with disease.

Figure 3.1 Antibody and complement activity in anti-GBM glomerulonephritis are unaltered by deficiency of MR signaling

(A) Serum levels of mouse anti-sheep Ig are equivalent in WT, MyMRKO and eplerenone-treated mice with glomerulonephritis. (B) Antibody titration analysis showed that glomerular deposition of mouse Ig and C3 are the same in WT, MyMRKO and eplerenone-treated mice with glomerulonephritis. (C) Immunofluorescence staining shows extensive glomerular deposition of mouse Ig and C3 after the development of anti-GBM glomerulonephritis, which was similar in WT, MyMRKO and eplerenone-treated mice (Magnification x 400). Data (A, B) = mean ± SEM; n=8.

Figure 3.1

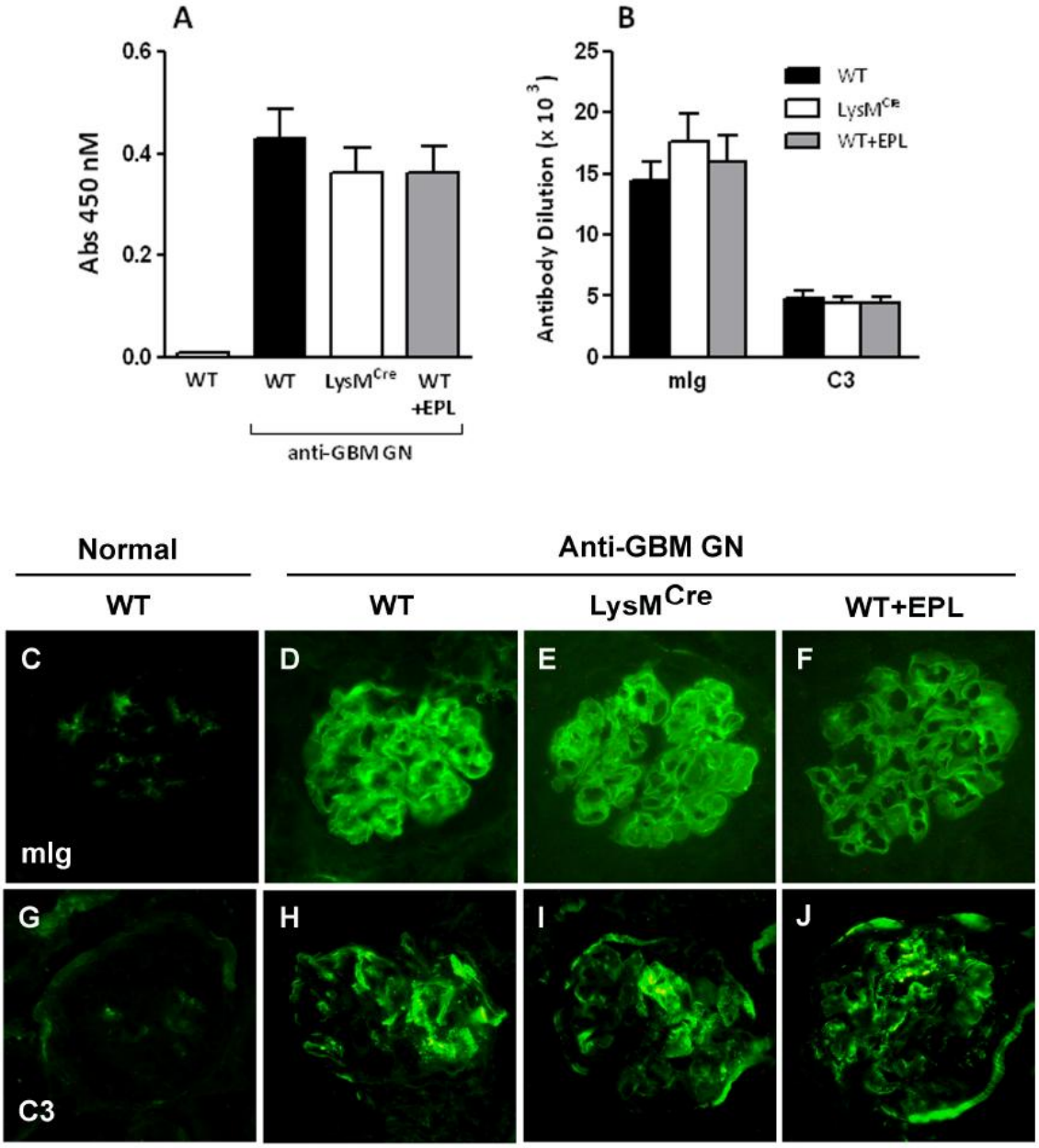

A
Abs 450 nM
0.6
0.4
0.2
0.0
WT
WT
LysM^Cre
WT +EPL
anti-GBM GN
B
Antibody Dilution (x 10^3)
25
20
15
10
5
0
WT
LysM^Cre
WT+EPL
mIg
C3
Normal
Anti-GBM GN
WT
WT
LysM^Cre
WT+EPL
C
D
E
F
mIg
G
H
I
J
C3

Figure 3.2 Myeloid MR deficiency protects mice from loss of renal function in anti-GBM glomerulonephritis

(A) Albuminuria was reduced in MyMRKO mice compared to WT and eplerenone-treated mice at day 1 of anti-GBM disease. However, albuminuria was similar in each of these groups at days 7 and 14 of disease. (B) At day 15 of anti-GBM disease, there was a 6-fold increase in serum levels of cystatin-C in WT mice, which was reduced by 35% in MyMRKO and eplerenone-treated groups. Data = mean ± SEM; n=8. *$p<0.05$ vs WT with disease.

Figure 3.2

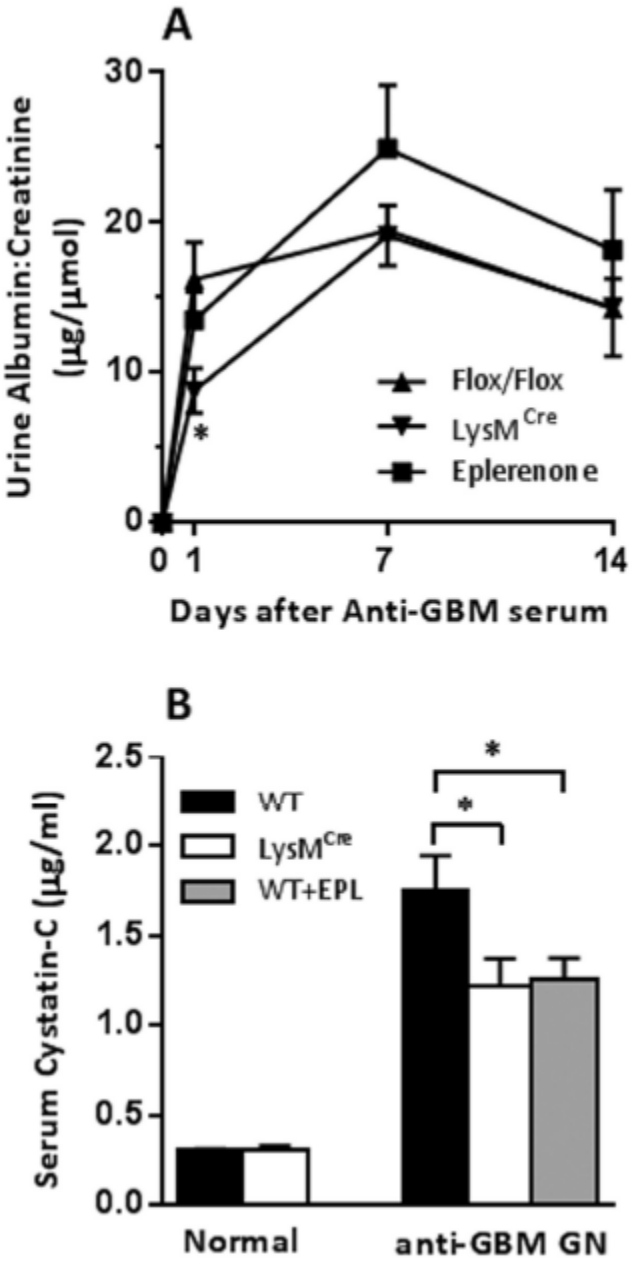
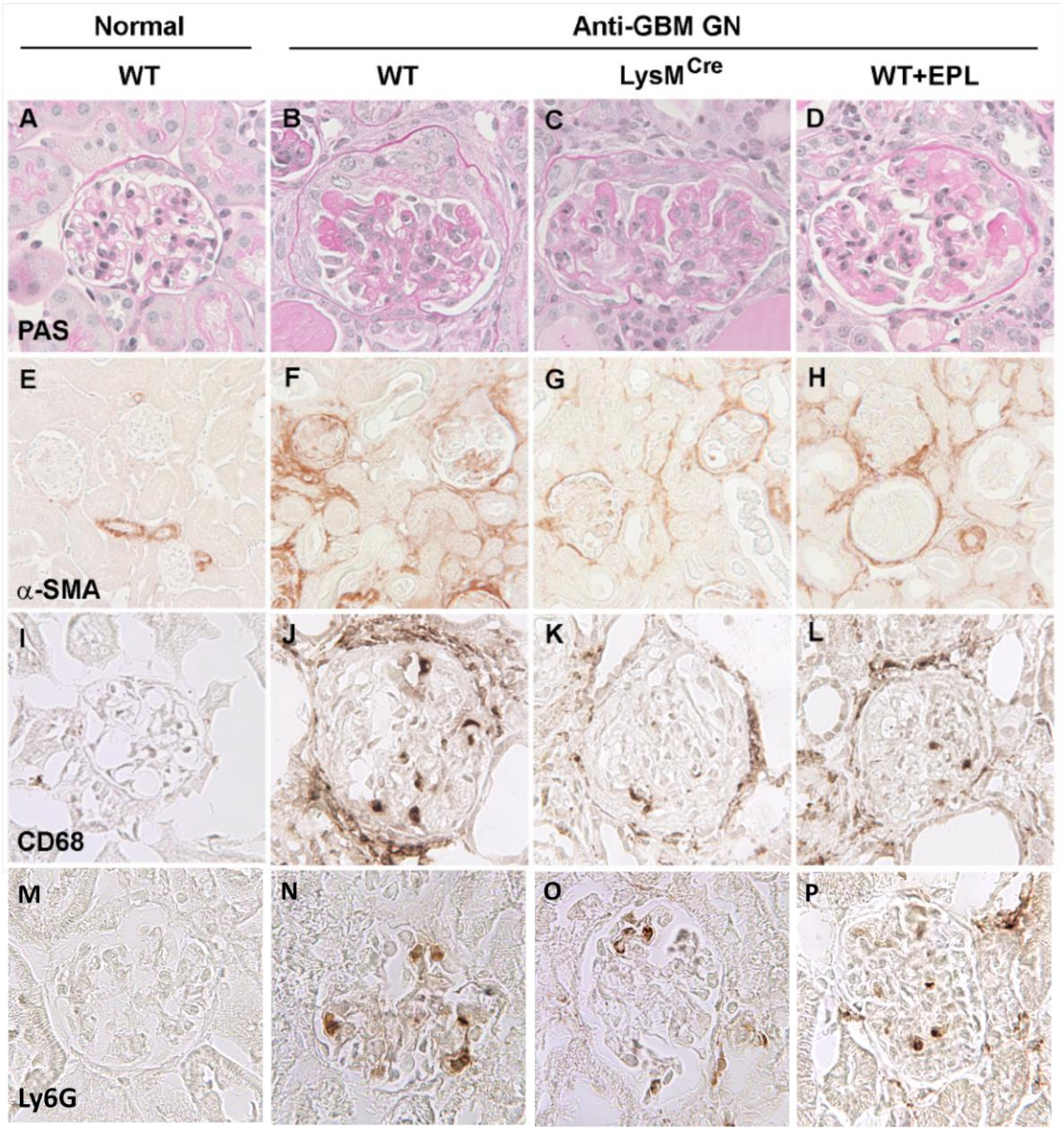

A
Urine Albumin:Creatinine (µg/µmol)
30
20
10
0
0 1 7 14
Days after Anti-GBM serum
Flox/Flox
LysM Cre
Eplerenone
*
B
Serum Cystatin-C (µg/ml)
2.5
2.0
1.5
1.0
0.5
0.0
WT
LysM Cre
WT+EPL
*
*
Normal
anti-GBM GN

Figure 3 Myeloid MR deficiency reduces kidney damage in anti-GBM glomerulonephritis

Histological staining with PAS and hematoxylin shows the kidney structure of (A) a normal mouse. In comparison, there is severe damage to glomeruli (crescent formation, sclerosis) and tubules (dilatation, atrophy) in (B) a WT mouse at day 15 of anti-GBM disease, which is attenuated in (C) a MyMRKO mouse and (D) an eplerenone-treated WT mouse. Immunostaining of α-smooth muscle actin was only found in the vasculature in (E) a normal mouse kidney. However, significant expression of α-smooth muscle actin was seen in myofibroblasts accumulating in glomerular, periglomerular and tubulointerstitial regions in (F) a WT mouse at day 15 of anti-GBM disease, which is reduced in (G) a MyMRKO mouse and (H) an eplerenone-treated WT mouse. Immunostaining identified small numbers of resident CD68+ macrophages in (I) a normal mouse kidney. In contrast, there were many infiltrating macrophages in glomeruli and the interstitium of (J) a WT mouse at day 15 of anti-GBM disease, which is reduced in (K) a MyMRKO mouse and (L) an eplerenone-treated WT mouse. At 3 h post disease induction, neutrophil infiltration was observed in WT mice with anti-GBM disease (N), which is reduced in a MyMRKO mouse (O) and with eplerenone treatment (P). Magnification: (A-D, I-P) x400; (E-H) x250.

Figure 3.3


Normal
Anti-GBM GN
WT
WT
LysM Cre
WT+EPL
A
B
C
D
PAS
E
F
G
H
α-SMA
I
J
K
L
CD68
M
N
O
P
Ly6G

Extensive damage was also found in the tubulointerstitial area of the renal cortex in mice with GN. Apoptotic cells, identified by immunostaining for activated caspase-3, were mostly located in the tubulointerstitium in diseased WT mice; however, their numbers were decreased in MyMRKO mice and eplerenone-treated WT mice (Table 3.3). Similarly, periglomerular and peritubular myofibroblasts were frequently observed in WT mice with GN, and their presence was substantially reduced in MyMRKO mice and eplerenone-treated WT mice (Figure 3.3, Table 3.3). Quantitative PCR analysis of whole kidney identified a 30-fold increase in gene expression of collagen I, fibronectin and PAI-1 in WT mice with GN (Table 3.3). The expression of these profibrotic genes was about 50% lower in both MyMRKO mice and eplerenone-treated WT mice. In comparison, the elevated levels of TGF-β1 mRNA seen in the diseased kidneys of WT mice was not reduced in either MyMRKO mice or EPL-treated mice with GN (Table 3.3); however, TGF-β1 activity was not examined.

Myeloid MR deficiency reduces renal inflammation in anti-GBM glomerulonephritis

Because MyMRKO mice showed protection from proteinuria at day 1 of GN, we examined these mice at 3 h after disease induction to identify whether the early glomerular inflammatory response was affected. Immunostaining demonstrated that Ly6g-positive neutrophils were scarce in the glomeruli of mice without disease (WT 0.22±0.05 vs MyMRKO 0.21±0.15 cells/gcs). In comparison, there was a marked influx of neutrophils in glomeruli at 3 h of disease in WT mice (3.5±1.1 cells/gcs) which was reduced by 40% in MyMRKO mice (2.1±0.7

cells/gcs, $p<0.05$) and eplerenone-treated mice (1.9±0.5 cells/gcs, $p<0.05$). At 3 h of disease, glomerular macrophages were not significantly increased and glomerular CD41+ platelets were increased 2-fold but were not different between WT and MyMRKO strains (data not shown).

At day 15 of GN, neutrophils had returned to normal levels in the glomeruli of WT mice and were rarely detected in the tubulointerstitium (data not shown). In contrast, the glomerular and interstitial numbers of macrophages and T-cells were substantially increased in these mice (Table 3.4). Compared to the WT mice, MyMRKO mice had a 60-70% reduction in the accumulation of glomerular and interstitial macrophages at day 15, whereas eplerenone-treated WT mice were less protected from macrophage infiltration (Figure 3.3, Table 3.4). At day 15, glomerular T-cells were not reduced in MyMRKO mice or by eplerenone treatment; however, there was a 30% reduction in the number of interstitial T-cells in both these groups (Table 3.4).

PCR analysis of WT diseased kidneys at day 15 identified a marked increase in the gene expression of proinflammatory cytokines (TNF-α, iNOS, MMP-12) associated with a macrophage M1 phenotype, which was reduced in MyMRKO mice and to a lesser extent by eplerenone treatment (Table 3.4). In comparison, gene expression of markers of a M2 macrophage phenotype (Arginase-1, CD206) were increased in diseased WT kidneys but the expression levels were unchanged in MyMRKO mice or by eplerenone treatment. Further PCR analysis

found that the gene expression of T-cell-related molecules (CD3e, Granzyme B, CCL5) were moderately increased in WT kidneys at day 15 of GN; however, only CCL5 expression was reduced in the MyMRKO mice and eplerenone-treated mice (Table 3.4).

Table 3.4 Kidney inflammation in MyMRKO mice

			Anti-GBM glomerulonephritis		
Analysis	***WT***	***MyMRKO***	***WT***	***MyMRKO***	***WT + EPL***
Glomerular CD68 (cells/gcs)	0.15±0.01	0.22±0.04	2.0±0.1	0.9±0.1[c]	1.4±0.1[b,d]
Glomerular CD3 (cells/gcs)	0.4±0.1	0.4±0.1	1.4±0.1	1.2±0.1	1.2±0.1
Glomerular Ly6G (cells/gcs)	0.09±0.03	0.17±0.05	0.12±0.02	0.10±0.01	0.17±0.07
Interstitial CD68 (% area)	0.7±0.1	0.7±0.1	5.2±0.6	2.1±0.2[c]	4.4±0.6[e]
Interstitial CD3 (cells/mm^2)	7±1	8±2	150±10	103±11[b]	103±13[a]
TNF-α mRNA/18s	1.2±0.2	1.0±0.2	10.1±1.3	4.1±1.0[b]	9.0±1.2
NOS2 mRNA/18s	1.1±0.2	0.7±0.1	3.4±0.5	1.3±0.3[b]	1.6±0.2[b]
CCL2 mRNA/18s	1.3±0.4	1.6±0.6	10.0±1.6	4.0±1.4[a]	7.2±1.3
MMP12 mRNA/18s	1.1±0.2	0.6±0.1	26±5	7.7±3.1[b]	7.9±1.5[b]
Arginase-1 mRNA/18s	1.3±0.3	1.0±0.3	33±7	25±10	40±14
CD206 mRNA/18s	1.2±0.3	0.9±0.2	58±11	32±10	35±5
CD3e mRNA/18s	1.2±0.4	1.7±0.6	4.2±0.7	2.6±1.0	2.1±0.8
IL-12β mRNA/18s	0.8±0.1	1.0±0.2	4.9±1.9	4.0±1.4	3.5±1.3
Granzyme B mRNA/18s	1.1±0.3	0.6±0.1	3.6±0.6	2.8±1.3	3.0±0.6
CCL5 mRNA/18s	1.0±0.1	0.7±0.1	2.9±0.4	1.3±0.4[b]	1.2±0.3[b]

WT = wild type, gcs = glomerular cross-section. Data = mean ± standard error. [a]p<0.05, [b]p<0.01, [c]p<0.001 vs WT with disease. [d]p<0.05, [e]p<0.01 vs MyMRKO with disease.

Additional phenotyping studies were performed on CD11b+ macrophages isolated from normal WT kidneys and kidneys obtained from WT or MyMRKO mice at day 15 of disease. Quantitative PCR analysis showed that macrophage expression of CCL2, CCR2, NOS2 and CD163 were not different between these groups. However, in diseased kidneys there was a significant increase of some M1 (IL-12, MMP-12) and M2 markers (Arginase-1, CD206, CCR7) and a small decrease in TNF-α (a M1 marker), which was not different between WT and MyMRKO mice (Table 3.5).

Table 3.5 Kidney macrophage phenotype

PCR Analysis	***WT***	***Anti-GBM glomerulonephritis***	
		WT	***MyMRKO***
CD68 mRNA/18s	1.00±0.02	0.90±0.07	1.09±0.15
CCR2 mRNA/18s	1.00±0.05	0.92±0.07	1.06±0.06
CCL2 mRNA/18s	1.00±0.08	1.17±0.16	1.04±0.10
TNF-α mRNA/18s	1.00±0.02	0.59±0.08[b]	0.68±0.04[a]
NOS2 mRNA/18s	1.00±0.11	1.18±0.21	0.88±0.20
IL-12β mRNA/18s	1.00±0.04	3.28±0.26[c]	3.42±0.07[c]
MMP12 mRNA/18s	1.02±0.10	4.79±0.19[c]	5.49±0.72[c]
Arginase-1 mRNA/18s	1.00±0.25	109±9[c]	99±15[b]
CD206 mRNA/18s	1.00±0.03	2.41±0.15[c]	2.47±0.06[c]
CD163 mRNA/18s	1.00±0.08	1.26±0.23	1.07±0.06
CCR7 mRNA/18s	1.00±0.10	4.40±0.49[b]	4.44±0.44[b]

WT = wild type, MyMRKO = Myeloid MR deficiency.

Data = mean ± standard error. n = 4.

[a]$p<0.01$, [b]$p<0.001$, [c]$p<0.0001$vs WT without disease by ANOVA

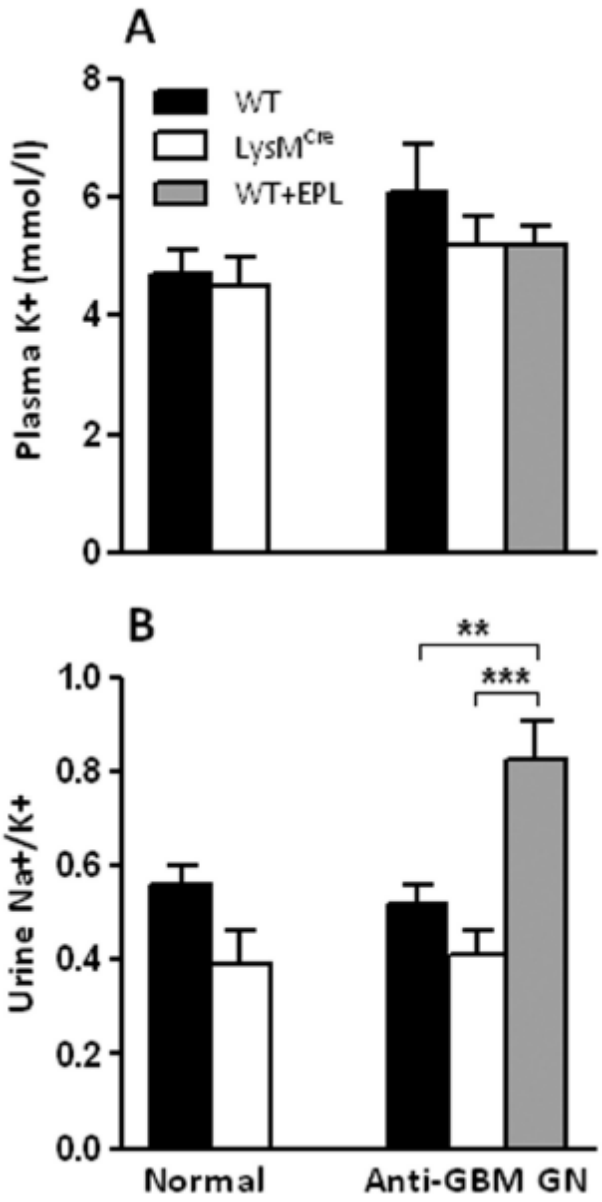
Myeloid MR deficiency does not affect tubular regulation of salt balance

Analysis of serum K+ levels found no evidence of hyperkalemia in MyMRKO mice or in mice treated with eplerenone (Figure 3.4A). However, when we assessed urine levels of Na+ and K+, we found that eplerenone treatment significantly increased the Na+/K+ in urine, whereas urine Na+/K+ remained similar in WT and MyMRKO mice with and without GN (Figure 3.4B).

Figure 3.4 Myeloid MR deficiency does not alter salt balance

(A) Plasma levels of K+ were similar in mice with and without anti-GBM glomerulonephritis, and were not affected by myeloid MR deficiency or eplerenone-treatment. (B) Urine levels of Na+/K+ were equivalent in WT and MyMRKO mice with and without disease. In contrast, urine Na+/K+ was significantly elevated in eplerenone-treated WT mice, indicating that tubular regulation of salt balance had been affected. Data = mean ± SEM; n=8; **p<0.01, ***p<0.001.

Figure 3.4


A
Plasma K+ (mmol/l)
8
6
4
2
0
WT
LysM^Cre
WT+EPL
B
Urine Na+/K+
1.0
0.8
0.6
0.4
0.2
0.0
**
***
Normal
Anti-GBM GN

Mice with MR deficiency in podocytes have normal kidney development

Deletion of MR in podocytes in $MR^{flox/flox}$ $Pod^{cre/-}$ (PodMRKO) mice was confirmed by immunostaining in kidney sections and by a 50% reduction in MR gene expression in isolated glomeruli (Figure 3.5). MR immunostaining was readily detected in the podocytes of WT mice but was absent in the podocytes of PodMRKO mice. Additional immunohistochemistry analysis showed that PodMRKO mice had the same number of WT-1+ podocytes (10.0±0.7cells/gcs) as normal WT mice (10.0±0.7cells/gcs) and that their kidney structure was normal (not shown).

Podocyte MR deficiency does not prevent loss of renal function in anti-GBM glomerulonephritis

Urine protein excretion and renal function were identical in WT and PodMRKO mice without disease (Figure 3.6). Induction of anti-GBM GN resulted in the development of proteinuria in WT and PodMRKO mice which was equivalent at days 1, 7 and 14 (Figure 3.6A). Similarly, both WT and PodMRKO mice had a 6-fold increase in the serum levels of cystatin-C at day 15 of GN, indicating that these strains had equal loss of renal function (Figure 3.6B).

Podocyte MR deficiency does not prevent renal damage in anti-GBM glomerulonephritis

PAS staining showed that the severity of kidney damage in PodMRKO mice was similar to WT mice at day 15 of GN (Figure 3.7). PodMRKO mice with GN had a high proportion of glomeruli with crescents which was not different to WT mice (Table 3.6). Immunostaining of WT-1 revealed that podocyte numbers were similarly reduced in WT mice and PodMRKO mice at day 15 of GN compared to mice without disease (Table 3.6). In addition, immunostaining demonstrated that the kidney accumulation of myofibroblasts and apoptotic cells were equivalent in WT mice and PodMRKO mice with GN (Table 3.6).

Table 3.6 Kidney damage in PodMRKO mice

Analysis	***WT***	***PodMRKO***	***Anti-GBM glomerulonephritis***	
			WT	***PodMRKO***
Crescents (%)	0	0	37±2	37±5
WT-1+ Podocytes/gcs	10.0±0.3	10.0±0.3	3.9±0.2	4.3±0.2
Apoptotic Cells/mm^2	0.5±0.2	0.5±0.2	6.0±0.5	5.1±0.6
Glomerular Myofibroblasts (% area)	0.5±0.1	0.5±0.1	12.5±1.0	11.6±1.2
Interstitial Myofibroblasts (% area)	0.4±0.1	0.4±0.1	5.3±0.8	6.8±0.8

Data = mean ± standard error.

Figure 3.5 MR expression by podocytes is absent in PodMRKO mice

(A) Immunostaining of Cre-recombinase in the podocytes of a $MR^{flox/flox}$/$Podocin^{Cre/-}$ (PodMRKO) mouse. Immunostaining of serial sections shows that PodMRKO mice have (B) normal glomerular expression of the podocyte marker WT-1 (arrows), and that their podocytes are (C) deficient in MR expression. MR expression is sparred in other glomerular cell such as the mesangial cells (C). In contrast, MR immunostaining is observed in (D) the podocytes of a wild type mouse (arrows). Graph (E) shows that MR gene expression is reduced by 50% in the glomeruli of PodMRKO compared to wild type mice. Magnification (A-D) x400.

Figure 3.5

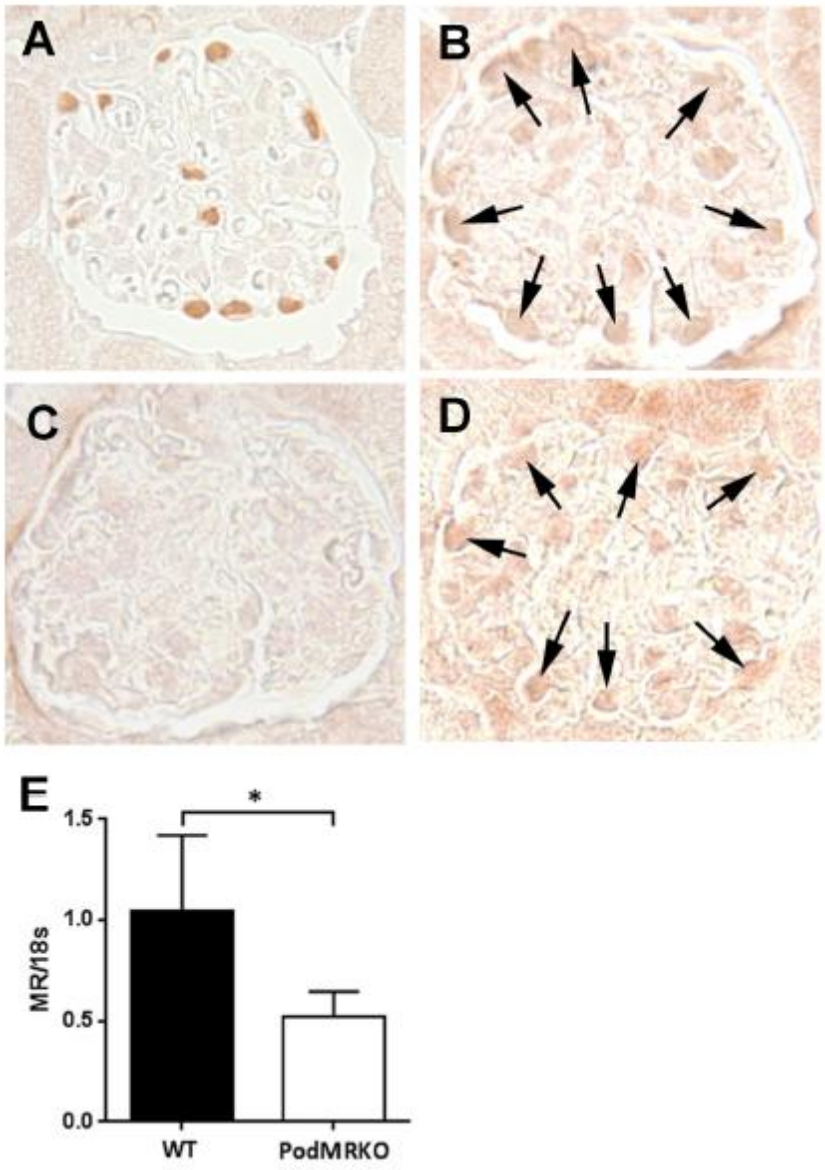


Figure 3.6 Podocyte MR deficiency does not prevent loss of renal function in anti-GBM glomerulonephritis

(A) Albuminuria was equivalent in PodMRKO mice compared to WT mice at days 1, 7 and 14 of anti-GBM disease. (B) At day 15 of anti-GBM disease, the serum levels of cystatin-C were equally elevated in PodMRKO and WT mice, indicating a similar decline in renal function. Data = mean ± SEM; n=8; ***p<0.001.

Figure 3.6

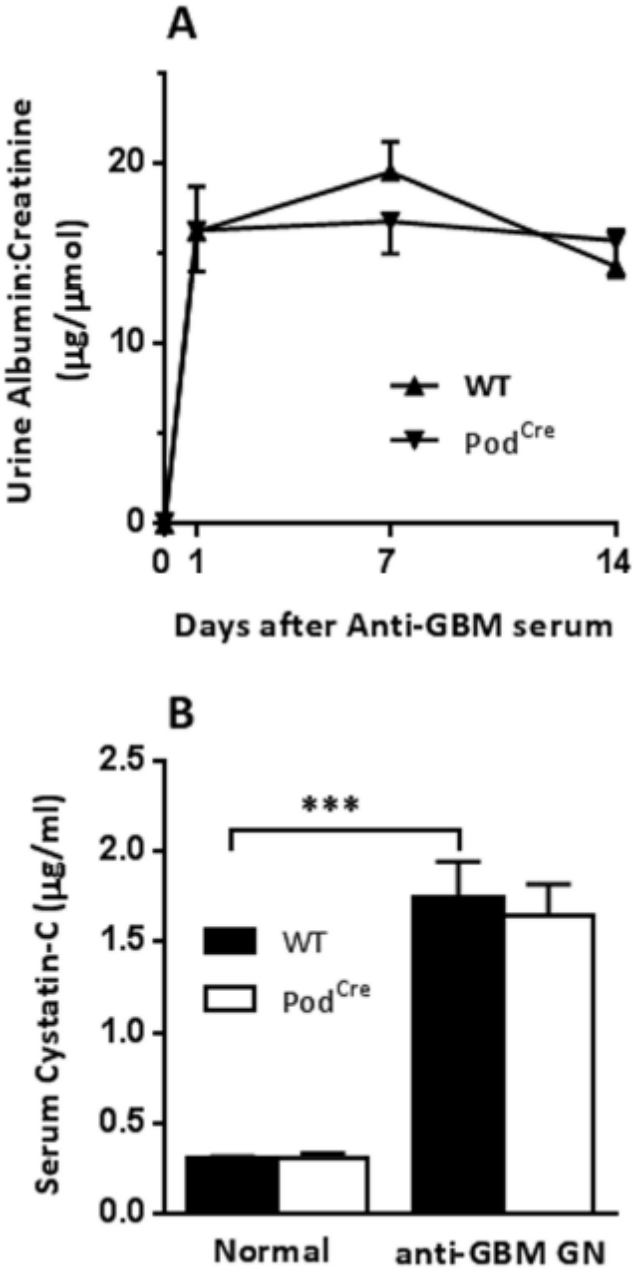
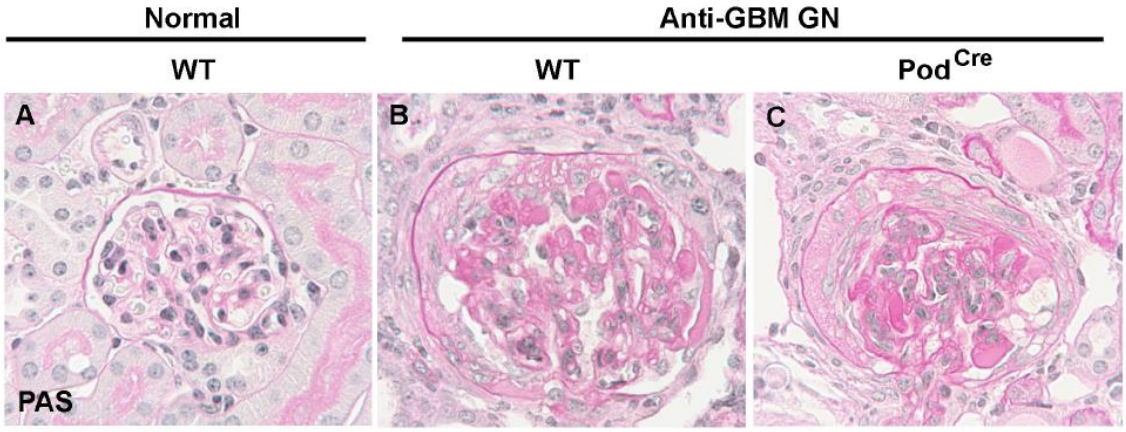

A
Urine Albumin:Creatinine (µg/µmol)
20
10
0
0 1 7 14
WT
Pod^Cre
Days after Anti-GBM serum
B
Serum Cystatin-C (µg/ml)
2.5
2.0
1.5
1.0
0.5
0.0
***
WT
Pod^Cre
Normal
anti-GBM GN

Figure 3.7 Podocyte MR deficiency provides no protection against kidney damage in anti-GBM glomerulonephritis

Histological staining with PAS and hematoxylin shows the kidney structure of (A) a normal mouse. In comparison, there is severe damage to glomeruli (crescent formation, sclerosis) and tubules (dilatation, atrophy) in (B) a WT mouse at day 15 of anti-GBM disease, which is similar in (C) a PodMRKO mouse. Magnification x400.

Figure 3.7



3.5 Discussion

Our study has demonstrated a pathological role for MR signaling in macrophages, but not podocytes, in a model of rapidly progressive glomerulonephritis. This is the first report that *in vivo* targeting of MR signaling in myeloid cells can reduce renal injury. Furthermore, we have shown that blockade of MR signaling in macrophages can achieve a level of renal protection which is comparable to systemic MR blockade with eplerenone.

MR signaling was found to play a role in the initial immune response in anti-GBM glomerulonephritis. In this model, the rapid glomerular influx of neutrophils at 3 h of disease was reduced by 40% in both MyMRKO mice and WT mice treated with eplerenone. Notably, MyMRKO mice showed a similar reduction in proteinuria at day 1; however, this protection was not seen with eplerenone-treatment, suggesting that a more complete inhibition of MR in myeloid cells is required for this effect. Previous research has demonstrated that a transient infiltration of neutrophils at the onset of anti-GBM disease is responsible for the induction of proteinuria but not the progression of injury (21). In addition, we observed no increase in glomerular macrophages at this stage of disease. Therefore, our findings suggest that MR signaling in neutrophils may contribute to the induction of proteinuria in this model.

At day 15 of GN, when a neutrophil infiltrate was no longer present, deficiency of MR signaling was closely associated with suppression of the macrophage-

dependent inflammatory response. Compared to WT mice, glomerular and interstitial macrophages and gene expression of macrophage-related proinflammatory cytokines were markedly reduced in MyMRKO mice and to a similar or lesser extent in eplerenone-treated WT mice. In contrast, T-cell accumulation and activity appeared to be less affected by deficiency of MR signaling. In anti-GBM disease, MyMRKO and EPL-treated mice had reduced kidney gene expression of the T-cell chemokine CCL5, but the levels of other T-cell-related genes (CD3ε, IL-12β and Granzyme B) remained similar to WT mice. In addition, our study found that myeloid MR deficiency had no impact on the development of antibody responses, indicating that antigen presentation was not altered. These findings suggest that the effect of MR blockade on inflammation is primarily through macrophage MR signaling during the progression of anti-GBM GN.

Myeloid MR deficiency had specific anti-inflammatory effects in anti-GBM GN, including reducing macrophage recruitment and inhibiting the kidney production of proinflammatory cytokines associated with a M1 macrophage phenotype. In comparison, studies in models of CVD and cerebral ischemia have shown that MyMRKO mice have reduced levels of M1 cytokines and that this can occur with and without a reduction in macrophage numbers (15-16, 22). Notably, some of these *in vivo* studies and experiments using cultured cells have indicated that a deficiency of MR signaling in macrophages can alter macrophage gene expression towards a M2 alternatively activated phenotype (15-16). Our PCR analysis of CD11b+ macrophages isolated from WT and

MyMRKO kidneys at day 15 of disease found no difference in the M1/M2 phenotype. Thus, the difference in expression of most of these markers in the diseased kidneys is likely due to changes in macrophage numbers rather than in their phenotype. In addition, macrophages from diseased kidneys showed a significant increase of mRNA levels of some M1 (IL-12, MMP-12) and M2 markers (Arginase-1, CD206, CCR7), a small decrease in TNF-α (an M1 marker), and no change a number of other markers (CCL2, CCR2, NOS2 and CD163) compared to macrophages from normal kidneys. This mixed macrophage phenotype is consistent with our recent time course study of rat anti-GBM disease which showed a transition from an M1 phenotype during acute inflammation to a more M2 phenotype during the fibrotic phase (23). However, these findings do not rule out the possibility that MR deficiency may affect the kidney macrophage phenotype at an earlier stage of disease. Furthermore, it is possible that deletion of MR occurs in some dendritic cells in MyMRKO mice, which may contribute to protection against anti-GBM GN.

Kidney damage in anti-GBM GN was markedly reduced by myeloid MR deficiency. The development of glomerular crescents, the accumulation of myofibroblasts and the gene expression of profibrotic molecules (collagen I, fibronectin, PAI-1) were all decreased in MyMRKO mice compared to WT mice. This suppression of profibrotic molecule gene expression indicates a reduction in TGF-β1 activity, although TGF-β1 mRNA levels were not affected by MR deficiency/blockade. Eplerenone-treated mice had a similar reduction in myofibroblasts and matrix gene expression but a greater reduction in crescents

than MyMRKO mice. This indicates that eplerenone has additional anti-proliferative or anti-fibrotic effects on parietal epithelial cells to those caused by blockade of myeloid MR. Our investigation also found that MyMRKO mice and eplerenone-treated mice were equally protected against declining renal function, which suggests that macrophage MR signaling is responsible for the injury-dependent loss of renal function in this model. These findings are comparable to studies in CVD which show that systemic MR blockade can reduce histological lesions and fibrosis in the absence of an effect on hypertension (24).

Protection from renal damage in MyMRKO mice and eplerenone-treated mice occurred without any reduction in proteinuria at days 7 and 14. In contrast, eplerenone treatment of a mild form of anti-GBM disease has previously been shown to reduce proteinuria in mice at these time-points (2). The different outcome of our study may be due to the rapid development of proteinuria in our model which plateaus at day 1, rather than developing progressively over 14 days, and involves mechanisms of injury which cannot be suppressed by MR blockade.

Normal regulation of salt balance appears to be unaffected by myeloid MR deficiency. In our study, hyperkalemia was not detected in the serum of MyMRKO or eplerenone-treated mice. However, urine levels of Na+/K+ were increased in eplerenone-treated mice, but not in MyMRKO mice, suggesting

that systemic MR blockade was causing tubular dysfunction (25). Therefore, selective blockade of MR signaling in macrophages has potential for treating kidney and cardiovascular disease, without disturbing salt regulation by tubules.

In contrast to MyMRKO mice, PodMRKO mice had no protection against renal injury in anti-GBM GN. Wild type and PodMRKO mice had equivalent proteinuria during the progression of disease and a similar decline in renal function. In addition, glomerular and tubulointerstitial damage was the same in both strains. This indicates that MR signaling in podocytes is not required for the progression of anti-GBM GN. Previous studies have identified that aldosterone can induce MR-mediated podocyte injury in cultured cells (26) and when infused into uninephrectomized rats on a high salt diet (27). Furthermore, systemic MR blockade reduces podocyte loss or damage in diabetic rats (11-12). Therefore, our data indicates that podocyte damage in this rapidly progressive model is mediated by activated myeloid cells rather than a direct effect of MR signaling on podocytes. It remains to be determined whether this also applies to chronic glomerular disease.

In conclusion, our study has identified an important role for macrophage MR signaling in the development of glomerulonephritis. Deletion of MR in macrophages was effective in suppressing the progression of glomerulonephritis, suggesting that MR blockade could be targeted specifically to macrophages to avoid the deleterious consequences of systemic MR

antagonism on tubular regulation of salt balance. However, further testing is required to establish the role of macrophage MR signaling in chronic forms of kidney disease, such as diabetic nephropathy.

Acknowledgements

We thank Professor Susan Quaggin (Samuel Lunenfeld Research Institute, Mount Sinai Hospital, Toronto, Ontario, Canada) for generously providing Podocin^{Cre} mice. We also thank Julie Ho for her technical support. This work was supported by the Victorian Government's Operational Infrastructure Support Program, a grant from Prince Henry's Institute of Medical Research and a PhD scholarship from the National Health and Medical Research Council of Australia (Louis Huang).

Disclosures

The authors have no competing financial interests.

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Monash University

Declaration for Thesis Chapter 4

Declaration by candidate

In the case of Chapter 4, the nature and extent of my contribution to the work was the following:

Nature of contribution	Extent of contribution
Maintained animals throughout disease. Cut tissue sections and performed all immunohistochemistry staining. Prepared figures and the manuscript.	70%

The following co-authors contributed to the work. If co-authors are students at Monash University, the extent of their contribution in percentage terms must be stated:

Name	Nature of contribution	Signatures
David J. Nikolic-Paterson	Intellectual input	
Elyce Ozols	Establishment of mice colonies Genotyping of mice	
Greg H. Tesch	Project supervision Intellectual input	

No co-authors are students at Monash University, therefore percentage of contribution are omitted.

The undersigned hereby certify that the above declaration correctly reflects the nature and extent of the candidate's and co-authors' contributions to this work.

Candidate's Signature		**Date**
Main Supervisor's Signature		**Date**

*Note: Where the responsible author is not the candidate's main supervisor, the main supervisor should consult with the responsible author to agree on the respective contributions of the authors.

4 The role of myeloid MR in diabetic nephropathy

Role of macrophage mineralocorticoid receptor in early mouse streptozotocin induced-diabetic nephropathy

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4.1 Abstract

***Background/Aims**:* Current treatments for diabetic nephropathy (DN) are insufficient to curb the increasing prevalence of end stage renal failure from DN. Both macrophage accumulation and mineralocorticoid receptor (MR) activation have been implicated in the pathogenesis of DN. Given that macrophages also express MR, we examined the contributions of macrophage MR in a model of DN from type 1 diabetes mellitus.

***Methods**:* Streptozotocin induced type 1 diabetes was induced in groups of (n=10) mice with selective MR deletions in myeloid cells (KO) and wild type (WT) counterparts. Mice with progressive hyperglycaemia (n=8 in each group) were followed for 22 weeks, before being sacrificed for analyses of renal injury.

***Results**:* Both groups of mice developed equivalent diabetes as measured by fasting glucose and HbA_{1C}. At 22 weeks, WT mice developed mild renal injury with 26.6% increase of cystatin-C and microalbuminuria 73.8 ± 12.0 mg/mmol, which were not different to KO mice. Knockout mice had reduced glomerular infiltration of CD68+ macrophages and also significantly attenuated glomerular hypertrophy compared to WT mice (2438 ± 64 μm^2 vs. 3458 ± 174 μm^2, $p<0.0001$). Knockout mice also had reduced tubular apoptosis. Despite this, interstitial macrophage infiltration and its inflammatory phenotype, myofibroblast accumulation and collagen IV deposition were not affected by myeloid MR deletion.

***Conclusion**:* Macrophage MR mediates glomerular hypertrophy during diabetic nephropathy. Our results also suggest that macrophage MR has a limited role in renal inflammation and fibrosis during early diabetic nephropathy. Given the reproducible benefits of systemic MR antagonists in diabetic nephropathy, our study suggests that investigating targeted MR gene deletion in other renal cells, such as podocytes, mesangial cells, or fibroblasts, may be warranted.

4.2 Introduction

Diabetic nephropathy (DN) has overtaken glomerulonephritis and hypertensive nephrosclerosis to become the most common cause of end stage renal failure in the developed world. It is characterised by glomerular basement membrane thickening, mesangial matrix expansion, glomerulosclerosis, which leads to albuminuria and ultimately the loss of renal function. Current therapies directed at glycaemic and blood pressure control with agents such as angiotensin converting enzyme inhibitors or angiotensin receptor antagonists have slowed the progression of DN. Despite this, up to 40% of diabetic patients still develop progression of their nephropathy (1). These factors prompt an urgent improvement in the understanding of the mechanisms of DN so that novel therapies can be developed.

One of the emerging paradigms of DN treatment involves targeting inflammatory cells such as the macrophage. Experimental studies have shown macrophages to accumulate in the kidney during diabetes mellitus (2-3). Furthermore, the severity of DN can be ameliorated by the inhibition of monocyte recruitment (4-5) or by reducing macrophage proliferation and survival (6). Inhibition of osteopontin and protein kinase C have also been shown to reduce kidney macrophage accrual and improve renal outcomes (7). Recent human trials using Bardoxolone methyl, an anti-inflammatory/oxidative stress therapy, have shown some success with reducing renal injury (8); although concerns over safety have abruptly stopped further investigation.

Another important mechanism involved in the accumulation of inflammatory cells is the activation of the mineralocorticoid receptor (MR). The MR is historically known for salt and water reabsorption in the distal convoluted tubules of the kidney, as well as the epithelial cells of the colon. However, there is increasing recognition of the pathological roles of MR activation in tissues such as the heart, vasculature and the kidneys. Rodent models of DN have consistently shown MR antagonists such as eplerenone and spironolactone to offer renal protection (9-12). Furthermore, macrophage infiltration and key inflammatory cytokines in the kidney were reduced by MR inhibition (10-11). In addition to modulating inflammation, MR antagonists have also been shown to reduce glomerular matrix accumulation, interstitial fibrosis and podocyte apoptosis.

Despite these benefits, MR antagonists can cause salt handling disturbance and result in hyperkalaemia. Spironolactone can also cause gynaecomastia due to non-specific antagonism of androgen receptors. These adverse effects often limit the use of MR antagonists in patients with existing kidney impairment. One potential solution to avoid these side effects is to target MR signalling in selective cells. This will ensure that the beneficial aspects of MR antagonism are maintained, while undesirable MR blockade in tubules is avoided. Cell selective MR inhibition has been achieved using conditional gene deletion technology: mice with deletions of MR in cardiomyocytes in a model of myocardial infarction were protected from progressive cardiac remodelling (13); MR deletion in macrophage conferred protection in mouse models of cerebral

vascular disease (14) and deoxycorticosterone/salt induced cardiac fibrosis (15). Given that both macrophages and MR activation play important roles in DN, I hypothesised that MR signalling in macrophages is important in the pathogenesis of DN.

4.3 Methods

Animal Model

Conditional gene deletion of MR was performed in C57BL/6 mice using the Cre-LoxP system. Mice with homozygous floxed MR gene were crossed with littermates expressing Cre recombinase under the control of the Lysozyme M promoter (16), to create $MR^{flox/flox}$ $LysM^{Cre/-}$ (KO) mice that lack MR in mature myeloid cells such as neutrophils and macrophages. $MR^{flox/flox}$ littermates were used as wild-type (WT) controls. For genotyping, presence of the $MR^{flox/flox}$ and Cre transgenes were determined by PCR analysis of genomic DNA from tail tips (MR: forward primer TTCTTTCCCCAGCTCTACCTTTACGA, reverse primer AGCAAGAGACAACTGCAGCGTTTTAATGTGGAATGTGTGCGAGGCCAGAG; Cre: forward primer ATGTCCAATTTACTGACCG, reverse primer TTCACTGGTTATGCGGCG).

To induce diabetes, 12 week old male mice (n=10) were given intra-peritoneal streptozotocin (STZ) for 5 consecutive days at a dose of 55 mg/kg/day. Two weeks after STZ induction, mice (n=8) with significantly elevated fasting blood glucose >16 mmol/L were used for experimentation and followed for 22 weeks. Equal proportion of mice in either strains developed diabetes and the degree of hyperglycaemia was also comparable. Aged matched non-diabetic WT and KO mice (n=8) served as controls.

Mice were housed in cages and maintained on a normal diet at the Monash Medical Centre Animal Facility (MMCAF Clayton, Australia). Morning fasting blood glucose via tail vein sampling and fasting weights were obtained weekly, after withholding chow for 4 hours. Random morning blood glucose and weight were obtained three times per week in the first 11 weeks of the experiment, and increased to daily for the remainder of the experiment. Mice with morning glucose levels >25 mmol/L or losing excessive weight were given protaphane insulin subcutaneously as well as mash to maintain condition. Urine collections were performed at weeks 8, 12, 16 and 21 to assess urine albumin excretion. Glycated haemoglobin (HbA_{1C}) was determined from blood samples at weeks 14 and 22 as a measure of chronic glycaemic control.

At week 22, mice were anaesthetised with inhaled isoflurane prior to killing by cardiac puncture. Serum and plasma were collected and stored in -20°C. Kidneys and spleen were collected and fixed in 10% (vol./vol.) neutral buffered formalin, 2% (wt./vol.) paraformaldehyde-lysine-periodate (PLP), or snap frozen and stored at -80°C. All animal experiments were performed in accordance with the guidelines of the Monash Medical Centre Animal Ethics Committee.

Biochemical Analysis

Urine was collected from mice housed in metabolic cages for 8 hours. A 24 hour collection was deemed to be inappropriate due to the condition of the mice. Plasma cystatin-C and urine albumin were determined by ELISA kits (BioVendor, Karasek, Czech Republic; Bethyl Laboratories, Montgomery, TX,

USA, respectively). Urine creatinine was determined by the Jaffe rate reaction method at the Department of Biochemistry at Monash Medical Centre (Clayton, VIC, Australia). Albuminuria was expressed as urine albumin creatinine ratio (ACR) to correct for hydration. HbA_1c was determined by Haemoglobin A_{1C} Reagent Kit (Siemens Healthcare Diagnostics, Tarrytown, NY, USA).

Antibodies

Antibodies used in this study were: rat anti-CD68 (FA-11, Serotec, Oxford, UK); rat anti-CD169 (MCA884, Serotec, Oxford, UK); anti-CD4 (supernatant, a gift from A/Prof. David Nikolic-Paterson); mouse anti-α-smooth muscle actin (α-SMA) (1A4, Sigma, St. Louis, MO); rabbit anti-cleaved-caspase 3 (Cell Signaling, Beverly, MA); mouse anti-proliferating cell nuclear antigen (PCNA) (M0879, Dako, Carpinteria, CA, USA); goat anti-collagen IV (Southern Biotechnology, Birmingham, AL, USA).

Histology and Immunostaining

Formalin-fixed kidney sections (2 μm) were stained with periodic acid Schiff's (PAS) reagent to identify kidney structure, and with hematoxylin to distinguish cell nuclei. Glomeruli size and hypercellularity was determined by Image-Pro Plus (Media Cybernetics, CA, USA) and microscopy respectively. Immunoperoxidase staining for leukocytes (CD68, CD3, Ly6g) was performed on 2% PLP-fixed kidney cryostat sections (5 μm). Immunoperoxidase staining for collagen IV, α-SMA, PCNA and cleaved-caspase 3 was performed on 10%

formalin-fixed sections (4 μm). For detection of cleaved-caspase 3 and PCNA, tissue sections were microwave treated (1000 W) for 12 min in 400 ml sodium citrate buffer pH 6.0 prior to immunostaining. Antigen retrieval of collagen IV utilised a pressure cooker at HIGH setting for 20 min and full pressure for 5 min. For immunoperoxidase staining, tissue sections were incubated with 20% normal rabbit serum for 30 min and then overnight at 4°C with 2-5 μg/ml of primary antibody in 1% BSA. Sections labelled with leukocyte antibodies were then incubated for 20 min each with 0.3% hydrogen peroxide followed by avidin and biotin block (Vector Laboratories, Burlingame, CA, USA). After washing in PBS, these sections were incubated with biotinylated rabbit antibody (anti-rat IgG, Zymed, San Francisco, CA, USA) for 1 h, followed by ABC peroxidase solution (Vector) for 1 h, and developed with 3,3-diaminobenzidine (DAB, Sigma) to produce a brown colour. Sections labelled with antibodies to collagen IV, α-SMA, PCNA and cleaved-caspase 3 were incubated sequentially with 0.6% hydrogen peroxide for 10 min, peroxidise-conjugated secondary antibodies (goat anti-mouse Ig, rabbit anti-mouse Ig from Dako) for 45 min and peroxidise-conjugated anti-peroxidase (PAP) complexes (rabbit PAP, rat PAP, mouse PAP from Dako) for 45 min, and developed with DAB.

Glomerular cellularity and leukocytes were counted at high magnification (x400) in 20-30 hilar glomerular tuft cross-sections per animal. Interstitial leukocytes, cleaved-caspase 3+ and PCNA+ cells were counted in 25 consecutive high magnification (x 400) interstitial fields (representing 30-40% of kidney cortex in the cross-section) and expressed as cells/mm^2. Immunostaining of α-SMA and

collagen IV were assessed as percent area stained within the glomerular tuft or renal cortex (excluding glomeruli and vessels) by computer image analysis software (Image Pro Plus, Media Cybernetics, CA, USA). All scoring was performed on blinded slides.

Real-time PCR

Total RNA was extracted from whole kidney using Trizol (Invitrogen) and reverse transcribed with random primers using the Superscript First-Strand Synthesis kit (Invitrogen). Real-time PCR was performed (Rotor-Gene 3000 system; Corbett Research, Sydney, NSW, Australia) with thermal cycling conditions of 37°C for 10 min and 95°C for 5 min, followed by 50 cycles at 95°C for 15 s, 60°C for 20 s and 68°C for 20 s. The relative amount of mRNA was calculated using comparative Ct (ΔΔCt) method. All specific amplicons were normalised against 18s rRNA, which was amplified in the same reaction as an internal control using commercial assay reagents (Applied Biosystems, Scoresby, VIC, Australia). Each of the primer/probe sets was pretested and determined to have equivalent PCR amplification efficiencies. The Taqman probe and primer sequences are listed in Table 4.1.

Table 4.1 Real-Time RT-PCR probe and primer sequences

Target	Probe	Forward Primer	Reverse Primer
Arginase-1	GGAAGGAAGAAAAGGC	GCAAGGTGATGGAAGAGA	TCGACATCAAAGCTCAGGTGAA
MCP-1	ACAACCACCTCAAGCAC	GACCCGTAAATCTGAAGCTAA	CACACTGGTCACTCCTACAGAA
CD68	CACAGTTTCTCCCACCA	CATGGGAATGCCACAATTTCT	ACAGTGGAGGATCTTGGACTA
Fizz-1	GTTCCCTTCTCATCTGC	CCAGGATGCCAACTTTGAATAG	GTTCCTTGACCTTATTCTCCAC
IDO	TGGTCTGGTCTCTGTG	CAGTCCGTGAGTTTGTCATTT	TACTATTGCGAGGTGGAACTTT
iNOS	TCCCTCCCCTCTCTCC	TCTCTGCTCTCAGCTCCAA	ACTACTAAATCTCTCTCCTCTCC
MR	TTTGAGGAGATGAGGAC	GGTTTTGCTGCTACTGAG	TGTTGGGACACTTGGTGA
TNFα	TCACCCACACCGTCAG	GGCTGCCCCGACTACGT	TTTCTCCTGGTATGAGATAGCAATC

Statistical Analysis

Statistical differences between two groups were analysed by t-test and differences between multiple groups were assessed by ANOVA with post hoc analysis using Tukey's multiple comparison test. Data was analysed using Graph Prism 5.0 (GraphPad Software, San Diego, CA) and was recorded as the mean ± standard error with $p<0.05$ defined as significant.

4.4 Results

Myeloid MR deletion does not affect the development of streptozotocin induced diabetes mellitus

Hyperglycaemia as determined by fasting blood glucose developed progressively and equally between the WT and KO mice 2 weeks post STZ induction (Figure 4.1a). Despite weight loss early in the disease process, all mice maintained their weights for the duration of the experiment (Figure 4.1b). The extent of diabetes mellitus was confirmed by plasma HbA_{1C} at weeks 14 and 22 (Figure 4.1c).

Myeloid MR deletion had no effect on albuminuria and renal function during early DN

By week 8, both WT and KO mice developed early renal injury as determined by elevated urine ACR (73.8 ± 12.0 and 65.0 ± 8.0 mg/mmol respectively, *p*=ns) (Figure 4.2a). Loss of renal function also occurred in both groups of mice at the end of the study (Figure 4.2d). However, no protection was seen in the KO mice. There was a reduction in ACR towards the end of the study, which affected predominantly urinary albumin excretion (Figure 4.2b) rather than creatinine (Figure 4.2c). To explain this reduction, serum albumin was determined using ELISA. This showed hypoalbuminaemia in both groups of mice, and helps to explain the pattern of albuminuria (Figure 4.2c).

Glomerular hypercellularity and hypertrophy were reduced in KO mice

Diabetes induced glomerular changes such as hypercellularity (41.6 ± 1.3 cells/gcs) and hypertrophy (3458 ± 174 μm^2) in the WT mice (Figure 4.3b). These parameters were significantly reduced in the KO mice (30.0 ± 0.9 cells/gcs and 2438 ± 64 μm^2 respectively) (Figure 4.3d,e). This suggests that myeloid MR signalling may be involved in inflammatory cell recruitment and compensatory glomerulomegaly.

Leukocyte infiltration and activation were not affected by MyMRKO during early DN

To explore the increased cell count in the glomeruli, detection of leukocytes were performed. Immunostaining for macrophages using CD68 marker identified an increase in glomerular and interstitial macrophages during DN (Figure 4.4b). There was a significant reduction in macrophage infiltration in the glomeruli, but not the interstitium, in the KO mice (Figure 4.4c, Table 4.2). Despite this, the proportion of activated CD169+ macrophage to total CD68+ macrophage was not different between the WT and KO mice (Figure 4.4d-f, Table 4.2). Analysis of CD169+ cells also showed that the majority of macrophages detected in the glomeruli were not activated. There was also the presence of a small but significant lymphocyte infiltrate in the kidney, which was not affected by myeloid MR deletion (Table 4.2).

Table 4.2. Kidney inflammatory infiltrates

	Normal	DN WT	DN KO
Glomerular			
CD68+ (cells/gcs)	0.15 ± 0.01	3.64 ± 0.62[b]	1.54 ± 0.35[a,d]
CD169+ (cell/gcs)	0.02 ± 0.01	0.61 ± 0.18[a]	0.40 ± 0.13[a]
% activated mΦ	11.3 ± 6.9	15.2 ± 2.5	22.8 ± 4.4
CD4+ (cell/gcs)	0.05 ± 0.01	0.13 ± 0.10	0.14 ± 0.11
Interstitium			
CD68+ (cells/mm^2)	36.2 ± 1.5	68.5 ± 5.3[b]	72.6 ± 8.2[a]
CD169+ (cells/mm^2)	1.7 ± 0.5	58.4 ± 6.7[c]	52.6 ± 8.6[b]
% activated mΦ	4.9 ± 1.6	82.6 ± 8.6[c]	71.1 ± 11.8[a]
CD4+ (cells/mm^2)	14.0 ± 1.1	27.2 ± 2.5[a]	26.0 ± 2.7[a]

gcs = glomerular cross section, mΦ = macrophage

Data are means ± SEM, n=8

[a]p<0.01, [b]p<0.001, [c]p<0.0001 vs. normal mice,

[d]p<0.05 vs. DN WT mice.

Kidney expression of typical macrophage inflammatory cytokines (M1) including TNF-α, iNOS and MCP-1, were increased during DN (Figure 4.5a-c). Given that others have found MR signalling to polarise macrophage phenotype, alternative macrophage phenotype markers (M2) were sought. Gene expression of M2 markers such as Arg-1, IDO and FIZZ-1 were not reduced during DN (Figure 4.5d-f). Furthermore, myeloid MR deletion did not alter either M1 or M2 gene expression during DN (Figure 4.5a-f). Expression of lymphocyte phenotype markers and function were less up-regulated during DN and also were not affected in the kidneys of the KO mice (data not shown).

Myeloid MR deletion reduced tubular injury but not renal myofibroblast and collagen accumulation

Advanced DN is characterised by renal fibrosis. To assess this, immunostaining for collagen IV and α-SMA+ myofibroblasts were sought for the glomeruli and the interstitium respectively. Compared to the WT mice, there was an increase in glomerular collagen, as well as interstitial myofibroblast accumulation during early DN (Table 4.3). Myeloid MR deletion did not have an effect in reducing myofibroblast or matrix accumulation (data not represented in a figure due to overall mild changes). Diabetes mellitus also caused tubulo-interstitial injury as determined by an increase of cleaved caspase-3+ and PCNA+ cells (Table 4.3). These changes were attenuated in the KO mice, suggesting a role for macrophage MR in mediating tubular apoptosis.

Figure 4.1 Development of diabetes mellitus

Streptozotocin treated WT and KO mice developed equivalent levels of fasting hyperglycaemia (Figure 4.1a) and glycated haemoglobin - HbA_{1C} (Figure 4.1c). Despite a drop in fasting weight during the onset of diabetes, all mice maintained their weights when the level of diabetes remained relatively stable after week 2 (Figure 4.1b).

Figure 4.1

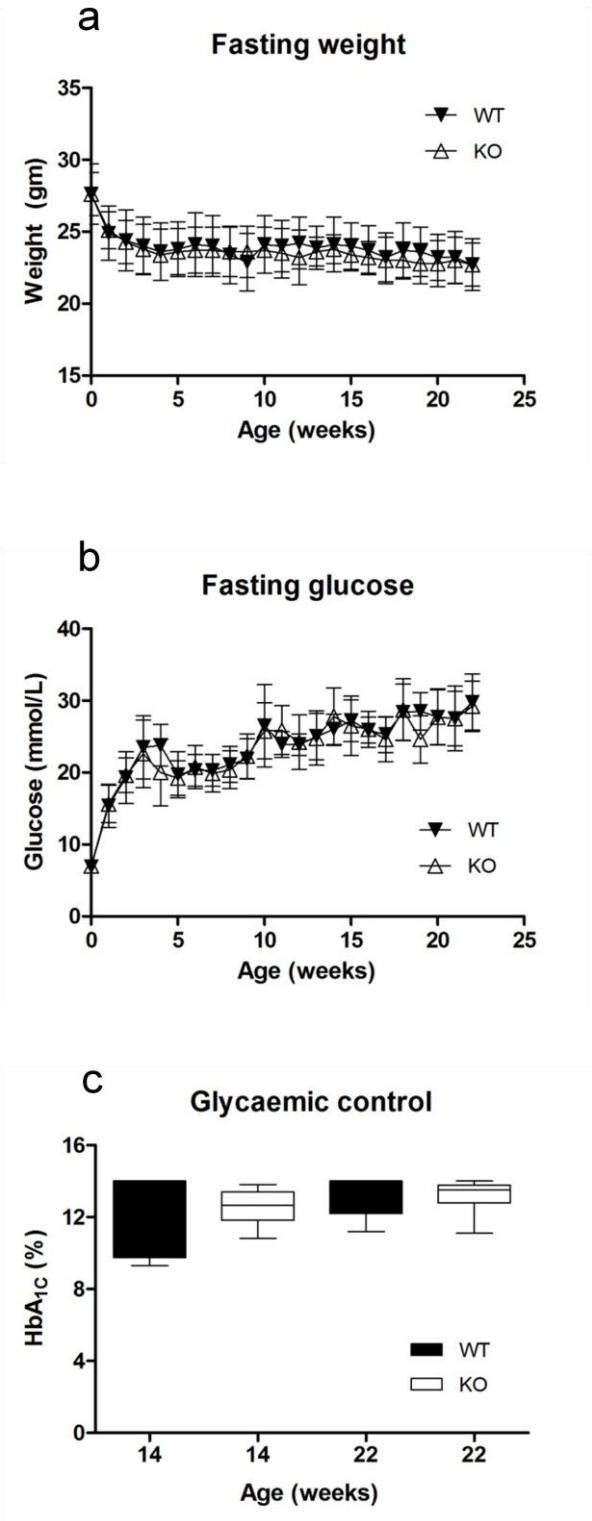
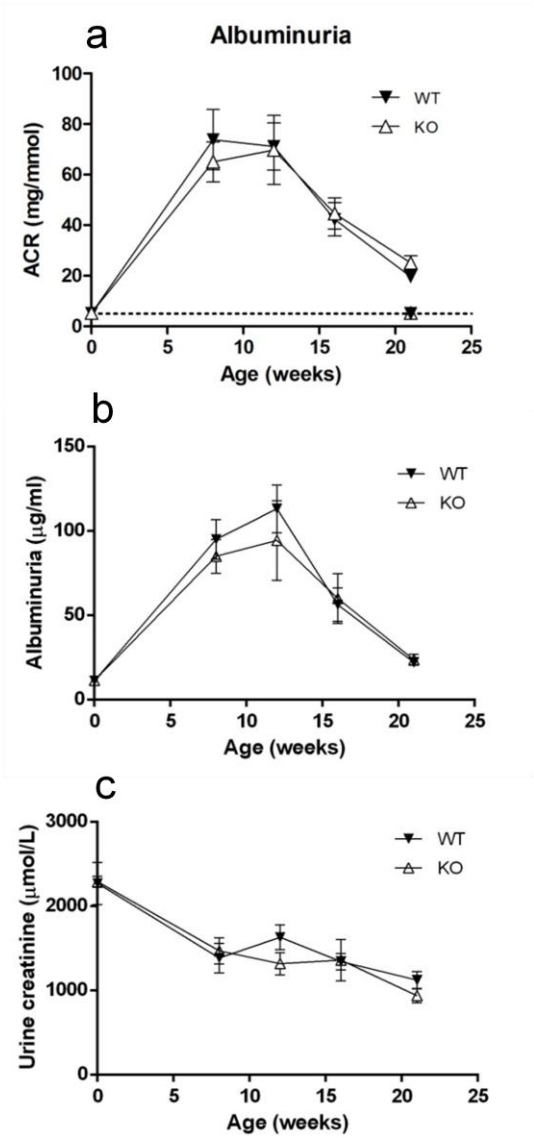

a
Fasting weight
Weight (gm)
Age (weeks)
WT
KO
b
Fasting glucose
Glucose (mmol/L)
Age (weeks)
WT
KO
c
Glycaemic control
HbA1C (%)
Age (weeks)
14
14
22
22
WT
KO

Figure 4.2 Albuminuria and loss of renal function

Early diabetic nephropathy manifested as albuminuria with an elevated urine albumin to creatinine ratio - ACR (Figure 4.2a), which was mainly driven by a reduction in albumin excretion (Figure 4.2b) rather than changes in urine creatinine excretion (Figure 4.2c). Loss of renal function was identified by serum levels of cystatin-C (Figure 4.2d). Analysis of serum albumin levels found moderate hypoalbuminaemia in the diabetic mice (Figure 4.2e). The development of albuminuria, renal dysfunction and hypoalbuminaemia in diabetic mice were unaffected by deficiency of myeloid MR.

Figure 4.2


a
Albuminuria
ACR (mg/mmol)
Age (weeks)
WT
KO
b
Albuminuria (μg/ml)
Age (weeks)
WT
KO
c
Urine creatinine (μmol/L)
Age (weeks)
WT
KO

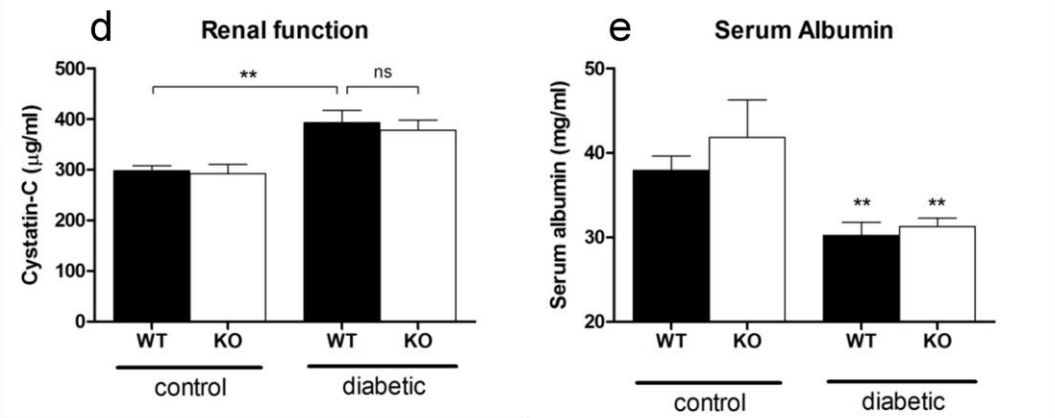

d
Renal function
Cystatin-C (μg/ml)
**
ns
WT
KO
WT
KO
control
diabetic
e
Serum Albumin
Serum albumin (mg/ml)
**
**
WT
KO
WT
KO
control
diabetic

Figure 4.3 Glomerular changes during diabetic nephropathy

Diabetes induced glomerular damage which was identified by PAS staining. This damage included glomerular hypercellularity and hypertrophy in the diabetic WT mice (Figure 4.3b), both of which were markedly reduced in KO mice (Figure 4.3d,e).

Figure 4.3

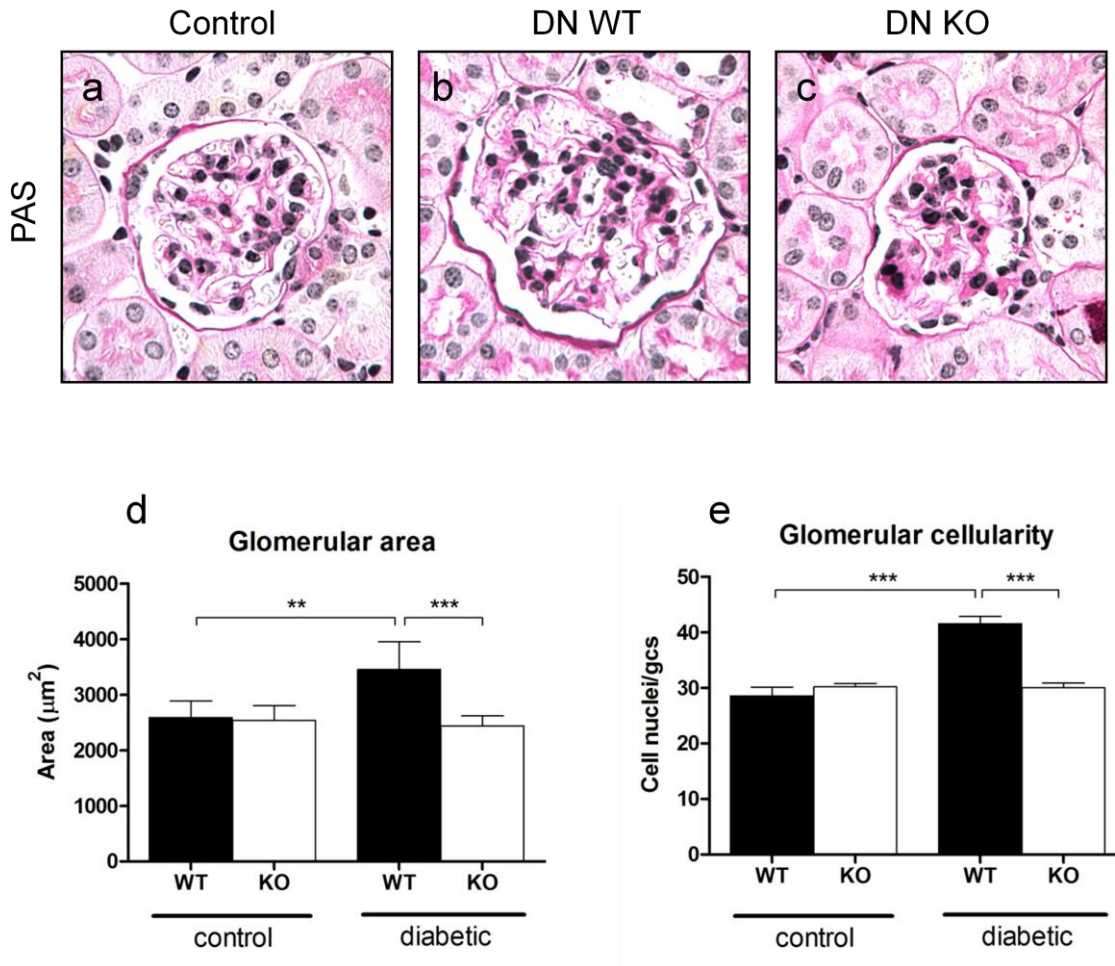

Control
DN WT
DN KO
PAS
a
b
c
d
Glomerular area
Area (μm²)
5000
4000
3000
2000
1000
0
**
***
WT
KO
WT
KO
control
diabetic
e
Glomerular cellularity
Cell nuclei/gcs
50
40
30
20
10
0
***
***
WT
KO
WT
KO
control
diabetic

Figure 4.4 Kidney inflammatory cell infiltration

Diabetic nephropathy is associated with an inflammatory infiltrate that consists of predominantly macrophages and lymphocytes (b,e,h). Myeloid MR deletion reduced the glomerular CD68+ macrophage infiltrate (b-c), but did not affect their activation in terms of CD169 expression (e-f). Lymphocytic infiltration was also not affected in the KO mice (g-i).

Figure 4.4

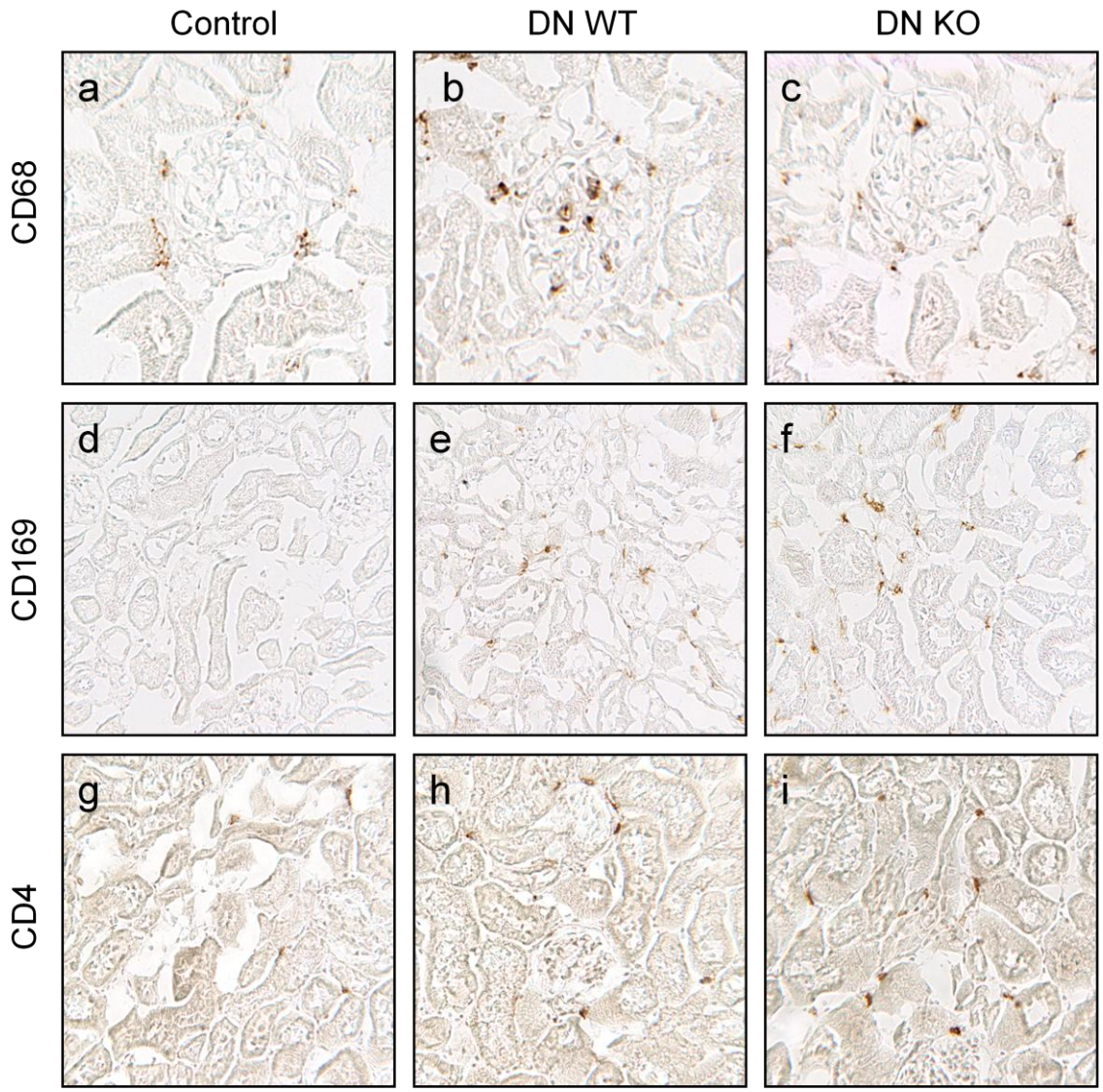


Figure 4.5 Inflammatory response in diabetic kidneys

Development of DN produced an inflammatory response which resulted in increased kidney gene expression of proinflammatory cytokines (TNF-α, MCP-1 and iNOS) which are typically associated with a classically activated macrophage M1 phenotype (Figure 4.5a-c). In contrast, kidney gene expression of inflammatory markers associated with the alternate macrophage M2 phenotype (Arg-1, FIZZ-1 and IDO) were mostly unaltered (Figure 4.5d-f). Deletion of myeloid MR did not affect the gene expression of these inflammatory markers in diabetic or non-diabetic kidneys (Figure 4.5a-f).

Figure 4.5

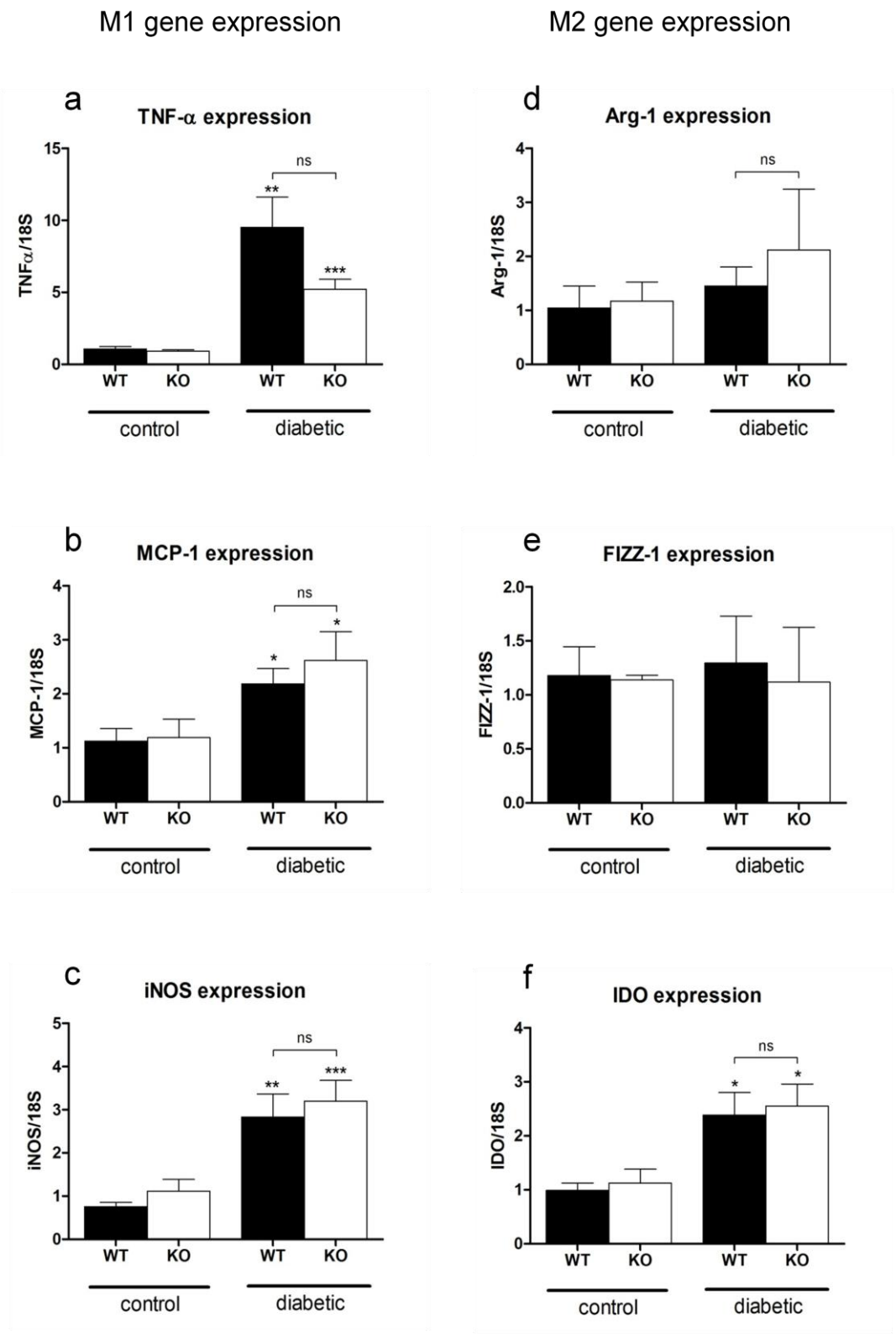

M1 gene expression
M2 gene expression
a
TNF-α expression
TNFα/18S
ns
**
***
WT
KO
WT
KO
control
diabetic
d
Arg-1 expression
Arg-1/18S
ns
WT
KO
WT
KO
control
diabetic
b
MCP-1 expression
MCP-1/18S
ns
*
*
WT
KO
WT
KO
control
diabetic
e
FIZZ-1 expression
FIZZ-1/18S
WT
KO
WT
KO
control
diabetic
c
iNOS expression
iNOS/18S
ns
**
***
WT
KO
WT
KO
control
diabetic
f
IDO expression
IDO/18S
ns
*
*
WT
KO
WT
KO
control
diabetic

Table 4.3 Histologic assessment of renal fibrosis and tubular injury

	Normal	DN WT	DN KO
Fibrosis			
Collagen IV (% gcs)	6.1 ± 1.0	12.9 ± 1.0[c]	12.9 ± 0.8[c]
α-SMA (% area)	0.33 ± 0.04	2.34 ± 0.22[d]	2.19 ± 0.32[c]
Tubular injury			
Proliferation (cells/mm^2)	15.9 ± 1.6	76.3 ± 6.7[d]	47.7 ± 5.4[b,f]
Apoptosis (cells/mm^2)	0.5 ± 0.2	1.9 ± 0.3[a]	0.9 ± 0.1[e]

gcs = glomerular cross section, SMA = smooth muscle actin.

Data are means ± SEM, n=8.

[a]$p<0.05$, [b]$p<0.01$, [c]$p<0.001$, [d]$p<0.0001$ vs. normal mice.

[e]$p<0.05$, [f]$p<0.01$ vs. DN WT mice.

4.5 Discussion

This study demonstrates a limited role for myeloid MR during the early phase of DN and has identified some disease mechanisms that are influenced by myeloid MR signalling in diabetic kidneys. Although the KO mice used in this study have deletion of MR in macrophages, neutrophils and some dendritic cells, the kidney leukocyte infiltrate during diabetes results from an innate immune response that does not appear to involve neutrophils or dendritic cells. Therefore, the results obtained in KO mice in this study relate mainly to the role of macrophage MR signalling in diabetic kidneys.

Macrophage MR signalling did not affect the development of diabetes, as both strains of mice had equally progressive hyperglycaemia. This was later confirmed on plasma HbA_{1C} testing. Diabetes led to early kidney damage including glomerular inflammation, glomerular hypercellularity, glomerular hypertrophy, loss of renal function and microalbuminuria in the WT mice. Diabetic KO mice had reduced levels of inflammation, cellularity and hypertrophy in glomeruli – a result that has been demonstrated in studies utilising the systemic MR antagonist spironolactone (11). Therefore, we have shown for the first time, that macrophage MR signalling is important in mediating compensatory glomerular enlargement. The exact mechanism of this effect cannot be elucidated by the current study; however, it appears that increased numbers of glomerular macrophages in WT versus KO mice may be contributing to this response. Previous reports have shown that cytokine

production and oxidative stress can be induced by MR signalling in renal cells (17-18). As such, MR deletion in macrophages may have prevented macrophages from producing factors that stimulate glomerular cell proliferation or hypertrophy.

In diabetic mice, a decline in renal function was identified in by measuring serum levels of cystatin-C, which showed no obvious protection in the KO mice. However, given that there was evidence of compensatory glomerulomegaly and hyperfiltration only in the WT mice, it is possible that any protective effect afforded by the myeloid MR deletion may be masked.

Another area where KO mice showed protection during DN was in the tubulointerstitial compartment. MR is known to play a role in tubular apoptosis (19) and interstitial fibrosis (20). In our study, DN in the WT mice caused an increase of tubular apoptosis and proliferation, which were both attenuated in the KO mice. However, macrophage infiltration in the interstitium was not reduced in the KO mice, suggesting a role for myeloid MR in mediating tubular injury, or increased tubular cell turnover in DN. In contrast, there was no difference in the accumulation of interstitial α-SMA+ myofibroblasts, which is a predictor for progressive renal fibrosis. Furthermore, glomerular deposition of collagen IV was not different between the groups of mice with DN. One explanation for this disparate finding is that our disease model represents an

early phase of DN, where the extent of renal injury has not progressed to significant renal fibrosis.

Previous publications using systemic MR antagonists have shown a reduction in renal inflammation in rodent models of DN for both type 1 and type 2 diabetes (10-11). In our study, it was an unexpected finding that overall renal inflammation was not modulated by macrophage MR deletion. Macrophage MR deletion did have a small effect in reducing glomerular macrophage infiltration; however this was not observed in the interstitium, where the vast majority of macrophages were found. Furthermore, the state of activation of existing macrophages as determined by CD169 immunostaining, as well as gene expression of classic macrophage inflammatory cytokines TNF-α, iNOS and MCP-1, showed no difference between WT and KO mice. One possible explanation for this observation is the variability of the Cre mediated deletion of MR gene. Unlike aggressive murine models of sepsis or crescentic glomerulonephritis, where the lysozyme gene is highly active and hence leads to a near-complete Cre mediated MR gene deletion (21), a chronic disease such as DN may not provide efficient deletion of the MR gene. Time may also be an important factor. If the disease was allowed to progress further, the increased activity of inflammatory macrophages may lead to a higher degree of Cre mediated MR gene deletion.

Another important factor to consider is the relative importance of MR signalling in other intrinsic renal cells. The MR has been identified in mesangial cells (22) and also epithelial cells such as the podocyte (23). Activation of MR in the kidney has been shown to increase glomerular inflammation and matrix deposition leading to glomerulosclerosis and even interstitial fibrosis (24). Our results suggest that MR signalling in macrophages may only play a limited role in the pathogenesis of mouse DN. Therefore, it is possible that MR inhibition in either podocytes or mesangial cells may provide more protection during DN.

Despite progressive hyperglycaemia, albuminuria reduced in both groups of mice towards the end of the study. This was another unexpected finding. The results remained unchanged when albuminuria was corrected for hydration by using the albumin to creatinine ratio. Several factors could contribute to this finding: i) reduction in renal function could potentially reduce the excretion of albumin; ii) due to chronic disease, hypoalbuminaemia developed, thus reducing available circulating albumin for excretion; iii) the degree of albuminuria was small i.e. microalbuminuria, therefore even subtle changes of renal function or serum albumin could significantly reduce albuminuria; and lastly iv) STZ may transiently cause tubular injury in the early phase of this disease model, which may reduce the reabsorption of filtered albumin, hence manifest as higher initial albuminuria. Our results also showed that there was no difference in albuminuria between the WT and KO mice during DN. This adds to the existing body of evidence that suggest macrophages have a limited role in the development and progression of proteinuria in kidney disease (6).

Our study is limited by an overall mild severity of disease, which made detection of any benefits conferred by targeted MR deletion difficult. One possibility for future studies would be to extend the duration of DN, or use a DN model which incorporates hypertension. Given that up to 70% of patients with DN have concomitant hypertension (25), it would be more clinically relevant to examine the effects of targeted MR deletion in an experimental model which involves hypertension. Furthermore, the inclusion of a treatment control group using systemic MR antagonist eplerenone would provide more meaningful comparisons between targeted versus systemic MR blockade. Unfortunately, we did not have the resources to provide long term drug therapy. Lastly, in order to ensure adequate selective Cre mediated deletion of the MR gene in macrophages, a different promoter such as CD68 or CD11b may be more appropriate in a disease model such as DN.

In conclusion, this study identifies a role for macrophage MR in mediating glomerular hypertrophy and hypercellularity during diabetic nephropathy. It also suggests that macrophage MR has a limited role in renal inflammation and fibrosis during early diabetic nephropathy. Given the reproducible benefits of systemic MR antagonists in diabetic nephropathy, our study suggests that investigating targeted MR gene deletion in other renal cells, such as podocytes, mesangial cells, or fibroblasts, may be warranted.

4.6 References

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5 Discussion

5.1 Introduction

The research in this thesis has focussed on examining the cell-type specific roles of MR signalling in fibroblasts, macrophages and podocytes in the development of acute and chronic renal injury. Collectively, these results have demonstrated the pathological effects of MR signalling in these renal cells, leading to kidney inflammation and fibrosis. This chapter will summarise the major findings, discuss the impact of these findings and identify potential directions for further study.

5.2 MR signalling in fibroblasts

5.2.1 Aldosterone-induced proliferation of renal fibroblasts

Work in Chapter 2 of this thesis has demonstrated that MR signalling can cause proliferation of rat and mouse kidney fibroblasts and particularly those from fibrotic mouse kidneys. Aldosterone-induced fibroblast proliferation represents a new mechanism by which MR signalling can activate fibroblasts and promote a fibrotic response. Our finding is significant because fibroblast proliferation has recently been recognised as the predominant source of myofibroblast accumulation in the diseased kidney (1). This may have relevance to a number of kidney diseases including DN (2) and obstructive uropathy (3), which feature myofibroblast proliferation and in which progression is dependent on renin-angiotensin-aldosterone activity. In addition, this finding suggests that other

renal cells that have myofibroblast-like properties (e.g. activated mesangial cells) may also proliferate in response to aldosterone. Indeed, the receptor and cell signalling pathways induced by MR activation in fibroblasts appear to be similar to those that are seen in mesangial cells (4).

5.2.2 Cellular pathways mediating MR responses in kidney fibroblasts

Identifying the intracellular mechanisms which mediate aldosterone-induced fibroblast proliferation has provided important insight into the MR signalling pathway. My work has demonstrated that MR-mediated fibroblast proliferation occurs by a non-genomic pathway involving rapid ligand-independent activation of growth factor receptors (PDGFR, EFGR) and specific signalling kinases (Akt, ERK, JNK). Elements of this pathway are also activated in other kidney cells following stimulation with aldosterone. For example, mesangial cells exposed to aldosterone had increased proliferation via activation of MR and ERK (4); while aldosterone treatment to podocytes promoted apoptosis by reducing survival factor PI3k/Akt (5). If rapidly activated non-genomic pathways are critical to the pathological effects of MR signalling, then studies like this one will help to identify downstream mediators of MR signalling which could be targeted therapeutically.

5.2.3 Downstream mediators of fibroblast MR signalling as potential therapeutic targets

As systemic blockade of TGF-β and MR can cause significant adverse effects, the identification of signalling pathways downstream of MR may provide potential therapeutic targets. My results demonstrate a reduction in MR-mediated renal fibroblast proliferation by inhibiting the activity of PDGF and EGF receptors. Given that these pathways are shared in mesangial cells (4), blockade of these growth factor receptors may be beneficial in conditions such as obstructive uropathy, or mesangio-proliferative glomerulopathies such as IgA nephropathy (6). In fact, blockade of PDGF or its tyrosine kinase signalling pathways have been shown to reduce fibrotic gene expression by down-regulating subsequent ERK, Akt phosphorylation in hepatic stellate cells (7). Similar pathways involving growth factor receptor activation and PI3K/Akt signalling are also implicated in scleroderma (8), as well as idiopathic pulmonary fibrosis (9) – systemic diseases characterised by intense fibrosis that have little therapeutic options at present. These results demonstrate a common, pro-fibrotic signalling pathway used by fibroblast-like cells in different organs. Inhibition of these pathways may provide benefits not only in renal fibrosis, but also in liver and pulmonary fibrosis.

My findings have also demonstrated that JNK signalling mediates the pro-proliferative effects of MR activation in renal fibroblasts. This result supports previous suggestions that JNK signalling promotes pro-fibrotic responses in kidney disease, which has been observed in other renal cells such as tubular

epithelial cells (10) and mesangial cells (11), as well as animal models of renal fibrosis (12). Inhibition of JNK signalling in a rat UUO model of obstructive uropathy significantly reduced matrix deposition and myofibroblast accumulation (13). JNK signalling was also identified in myofibroblasts during the late, fibrotic stages of rat anti-GBM GN (14). In contrast, JNK inhibition did not appear to affect glomerular or tubulointerstitial fibrosis during early stages of type 1 diabetes and hypertension (15). Put together, these experimental findings suggest both ERK and JNK signalling are potential targets in reducing renal fibrosis during certain renal diseases.

5.2.4 Identifying the importance of fibroblast MR signalling in progressive kidney diseases

What remains to be determined is whether MR signalling in fibroblasts is a substantial component of the pathological effect of MR signalling in progressive kidney disease. Specific gene deletion of MR signalling in fibroblasts is now possible with the availability of mice expressing Cre under the control of a fibroblast specific promoter (e.g. pro-α1(I) collagen), which would target collagen I producing cells such as the fibroblast and myofibroblast. This could also be conditionally activated in adult mice by tomoxifen (e.g. pro-α1(I) collagen^{Cre}/ERT). Once fibroblast selective MR KO mice have been developed, it will be important to investigate the role of fibroblast MR in appropriate models of progressive kidney fibrosis including the UUO model of obstructive uropathy, and the adriamycin-induced model of focal and segmental glomerulosclerosis.

The role of fibroblast MR may also be elucidated by using these mice in late stages of murine DN, and that of anti-GBM GN.

5.3 MR signalling in macrophages

Macrophages are important cells in the pathogenesis of kidney diseases including DN and GN. Findings from our experiments in Chapter 2 showed that macrophage MR had a significant role in promoting macrophage accumulation in the kidney and subsequent renal injury during acute GN. Although previous studies have shown MR activation to be proinflammatory and pro-fibrotic, our findings are the first to demonstrate the contribution of macrophage MR signalling in kidney inflammation during acute GN. Results from Chapter 4 of this thesis suggest that macrophage MR contributes significantly toward glomerulomegaly during diabetes – a novel finding that may have implications in understanding the cellular mechanisms of reactive glomerular change.

5.3.1 Macrophage MR signalling is a major contributor to injury in acute GN

Findings from Chapter 3 of my thesis looked at the contribution of MR signalling in myeloid cells (neutrophils and macrophages) in a model of rapidly progressive GN. Mice with MR gene deletion in myeloid cells had reduced levels of renal inflammation, crescent formation, tubular damage and interstitial fibrosis, which resulted in diminished albuminuria during the early stage of

disease, and protection from loss of renal function later on. In the advanced stages of anti-GBM GN, which are neutrophil-independent, myeloid MR signalling played a critical role in orchestrating macrophage accumulation in the kidney – both in the glomeruli, as well as the interstitium. These results demonstrate that macrophage MR signalling can promote renal injury by increasing proinflammatory cytokine expression, glomerular crescent formation, myofibroblast accumulation, ECM deposition and tubular apoptosis. This finding is the first to identify a significant contribution of macrophage MR signalling in acute inflammatory kidney disease.

Systemic treatment with the MR antagonist eplerenone produced similar results to mice deficient in myeloid MR in a mouse model of anti-GBM GN in terms of reductions in renal inflammation, fibrosis and apoptosis. However the degree of benefit was less pronounced with eplerenone treatment in terms of renal macrophage and myofibroblast accumulation. The difference between drug treatment and gene deletion was an interesting observation. One might expect the effects of macrophage MR gene deletion to sit between the effects of WT and eplerenone treated mice. There are multiple explanations which could explain these findings. Firstly, the effect of drug treatment is very dependent on its serum levels, which varies according to bioavailability, half-life and dosage. The troughs and peaks of plasma eplerenone, even in steady state, could affect the degree of MR antagonism. In comparison, once MR is lost in myeloid cells in MyMRKO mice, the MR deficiency is maintained. My analysis of kidney macrophages in mice with anti-GBM GN, showed a reduction in macrophage

MR gene expression of approximately 80%. Therefore, macrophage MR signalling is more likely to be consistently suppressed in MyMRKO mice compared to eplerenone-treated mice. Secondly, the dose of eplerenone administered in our studies (100 mg/kg/day) was derived from previously published experiments (16-17). Although commonly used, this dose may not provide complete protection against pathological MR signalling. My experimental data showed that eplerenone treated mice had normal range plasma potassium – suggesting that they could potentially tolerate higher doses, which can be examined in future studies.

5.3.2 Effect of macrophage MR deficiency on macrophage phenotype in acute GN

Deletion of the MR in macrophages suppressed the kidney expression of inflammatory markers associated with a classical macrophage M1 phenotype (e.g. TNF-α, iNOS) during anti-GBM GN. However, this reduction of M1 markers correlated with the diminished infiltrate of macrophages, indicating that the macrophage phenotype may not change substantially in the absence of MR signalling. Furthermore, when CD11b+ macrophages were isolated from kidneys at day 15 of anti-GBM disease and characterised, the analysis demonstrated that the MR-deficient macrophages had a similar phenotype to the wild type macrophages, which showed a shift towards a M2 phenotype. Therefore, the shift towards M2 should be seen as a response to the GN disease process, rather than an effect mediated by the macrophage MR. My

results are comparable to those described by Frieler *et. al.* in a mouse model of cerebral vascular disease (18), where MyMRKO mice had reduced macrophage and microglia infiltration, attenuated M1 cytokines, and no significant change to M2 cytokine profile when compared to wild type mice. Taken together, these results suggest that activation of macrophage MR does not significantly influence the kidney macrophage phenotype during the advanced stages of anti-GBM disease. These findings are in contrast to a previous *in vitro* study suggesting that the macrophage phenotype was dictated by its MR signalling (19). However, it is possible that macrophage MR signalling may play a role in determining the macrophage phenotype at earlier stages of anti-GBM GN or in other types of kidney disease, which requires further investigation.

An area that requires additional study is the identity of the ligand that occupies the macrophage MR during kidney inflammation. Both aldosterone and glucocorticoids can occupy the MR with equal affinity. However due to the 1000-fold higher concentration of glucocorticoids in the circulation, aldosterone can only occupy the MR in tissues that express 11β-HSD2 (20). Our results demonstrated that MR activation in macrophages contributes to renal injury during GN. Given that monocytes and macrophages do not express 11β-HSD2, a reasonable assumption is that the macrophage MR was occupied by glucocorticoids. In fact, there is evidence that activated macrophages express 11β-HSD1, which converts inactive cortisone to the active cortisol/corticosterone (21). However, one also needs to take into account the environment these macrophages are infiltrating. Almost all cells in the kidney

have been found to express 11β-HSD2, including: glomerular cells such as podocytes (22), mesangial cells (23); tubular cells at multiple levels, and the renal medulla (24). Therefore, it is plausible that the levels of glucocorticoids seen by kidney macrophages may be influenced by 11β-HSD2 produced locally. Currently, there is a paucity of information on the identity of the major ligand responsible for macrophage MR signalling in the kidney, nor the balance of 11β-HSD isoforms in the inflamed kidney, which may be an area of future research.

5.3.3 Macrophage MR signalling promotes renal Injury in type 1 diabetes

Previous work has shown both macrophage inhibition and systemic MR antagonism to attenuate renal injury from type 1 diabetes – suggesting perhaps a role of macrophage MR in the pathogenesis of DN. Results in Chapter 4 showed macrophage MR to be important in mediating glomerular hypertrophy and hypercellularity. Other parameters affected by macrophage MR include glomerular macrophage infiltration and tubulointerstitial apoptosis, which are similar to our findings in Chapter 3 in the anti-GBM GN model. Despite these protective findings, there were no obvious benefits of macrophage MR deletion in terms of loss of renal function, the development of albuminuria and renal fibrosis. Furthermore, macrophage MR gene deletion did not alter the increased levels of proinflammatory cytokine gene expression observed in the diabetic kidney. These findings would suggest a limited role of macrophage MR in the progression of DN due to type 1 diabetes.

The limited protective effects of macrophage MR deletion in DN need to be interpreted cautiously. Even though diabetes was induced successfully and resulted in markedly elevated HbA_{1C} levels, my model of DN displayed a relatively mild degree of nephropathy in terms of decline in renal function and histological injury. Therefore, it is possible that macrophage MR gene deletion may provide considerably more benefit in a more advanced stage of DN. Examining the effects of macrophage MR deletion in a more severe model of DN achieved by either a longer duration of diabetes or by using a model that incorporates hypertension, or greater genetic susceptibility to diabetic renal injury may help to overcome this problem. In addition, it is should be noted that the degree of renal inflammation is relatively mild in chronic DN compared to acute anti-GBM GN, which may affect the macrophage promoter (LysM) used to induce expression of Cre-recombinase. Hence, it is possible that the level of Cre mediated MR gene deletion in the macrophages may be less in diabetic kidneys compared to anti-GBM GN kidneys, making it more difficult to demonstrate the importance of macrophage MR signalling in DN. If so, future studies using the Cre-LoxP system to examine the role of macrophage MR in DN may need to utilise a different macrophage specific promoter in order to achieve more complete MR gene deletion in macrophages in diabetic kidneys.

Despite mild and early nephropathy, an obvious benefit in the reduction of glomerular hypertrophy was observed with macrophage MR KO. Glomerular hypertrophy is a secondary phenomenon after a reduction in renal filtration. It is

accepted that glomeruli undergo hypertrophy in the setting of hyperfiltration, such as following unilateral nephrectomy. With time, hyperfiltration of the remnant kidney undergoes fibrosis and loss of kidney function. Diabetic kidneys also demonstrate glomerulomegaly (16, 25). Others have shown activation of the renin-angiotensin-aldosterone axis (26) and increased oxidative stress by ROS (27) to cause glomerular hypertrophy. My results show, for the first time, that macrophage MR is involved in this process, as the MyMRKO mice had near-complete reversal of glomerular hypertrophy. This finding has implications for the prevention of progressive kidney disease and FSGS in patients post unilateral nephrectomy, as well as renal transplant recipients and warrants further research.

5.4 MR signalling in podocytes

5.4.1 Podocyte MR signalling does not affect the development of injury in acute GN

Previous *in vivo* studies involving aldosterone infusion or MR antagonist treatment have shown that MR activation causes podocyte injury which results in proteinuria. To examine whether MR expression in podocytes is responsible for MR-mediated albuminuria, I investigated the development of anti-GBM GN in mice with specific genetic deletion of MR in podocytes. Before establishing the GN model, successful deletion of podocyte MR in mice was verified by a lack of immunostaining of MR on glomerular podocytes and a reduction of MR gene expression in isolated glomeruli. Despite lacking podocyte MR signalling,

the PodMRKO mice exhibited podocyte injury and albuminuria comparable to WT mice with GN. Moreover, the development of renal impairment, crescent formation, markers of renal inflammation and fibrosis, were all independent of podocyte MR signalling. Our experimental findings were unexpected, as injury to podocytes has previously been shown to cause albuminuria and glomerulosclerosis (28).

Several reasons can help explain my observations. Firstly, previous evidence supporting the involvement of MR in podocyte injury and proteinuria was obtained from models of aldosterone excess, hypertension or models of type 1 diabetes. Although these animal models are dependent on inflammation for their pathogeneses, inflammation is slow to progress and only plays a limited role. This contrasts to the murine accelerated anti-GBM GN model, where acute inflammation is the predominant disease mechanism and causes widespread renal injury that often results in renal failure and death by 3 weeks. Furthermore, studies in this thesis have shown that most of the MR-dependent renal injury in the anti-GBM GN model is due to myeloid MR signalling. Therefore, the contribution of podocyte MR signalling in models of acute inflammatory kidney disease may be limited.

Secondly, podocyte injury during anti-GBM GN may arise from MR activation in other renal cell types. Anatomically, podocytes along the glomerular basement membrane are supported by the mesangial cell network. Disruption of the glomerulus by inflammation can result in the rupture of Bowman's capsule, or crescent formation that can lead to mesangial cell injury, and subsequent

podocyte loss. Mesangial cells are also capable of generating AngII, pro-inflammatory cytokines and promote local oxidative stress (29) that can affect neighbouring podocytes and promote apoptosis (30). My studies in the anti-GBM GN model only examined the direct effects of deleting MR signalling in podocytes, and thus, damage to podocytes from MR signalling in neighbouring kidney cells was not prevented. However, it is noteworthy that eplerenone treatment did not suppress albuminuria in mouse anti-GBM GN, suggesting that MR activity may not be important for podocyte damage in this antibody-dependent form of acute GN.

Even though podocyte MR deletion did not prevent progression of GN, this result is novel and has significant implications. It demonstrates that the pathological importance of MR signalling in specific cell types may vary with different renal diseases and that MR antagonism is not required in all cells. Other renal cells that warrant further investigation of selective MR blockade include mesangial cells and renal fibroblasts.

5.4.2 Podocyte MR signalling may be important for glomerular injury in other kidney diseases

Podocyte MR signalling may have more relevance in chronic diseases affecting the kidneys. Previous studies have demonstrated the importance of MR signalling in contributing to podocyte injury and proteinuria – such as in models of STZ-induced diabetic nephropathy (31), and exposure to high salt and

aldosterone (32). Both these models contribute to mild inflammation over several weeks or months, and generate enhanced oxidative stress in the kidneys. Therefore, it is possible that injury to podocytes in these models is due to the effects of oxidative stress, rather than direct destruction from infiltrating inflammatory cells. One method of evaluating this hypothesis would be to develop models of STZ-induced DN in mice with podocyte MR deletion, and examine for changes in albuminuria, podocyte injury (i.e. loss of podocytes or podocyte proteins), and glomerulosclerosis. Animal models of hypertension-associated renal injury may also be important in determining whether podocyte MR plays a major role in the pathogenesis of proteinuria. This could be achieved by administering excessive levels of mineralocorticoid-salt to PodMRKO mice, or by establishing models of renal injury in PodMRKO mice with genetic hypertension due to deletion of endothelial nitric oxide synthase.

Another subset of kidney disease that may involve podocyte MR includes the nephrotic syndromes – in particular, membranous nephropathy. MR antagonists have been shown to reduce proteinuria in patients with nephrotic syndrome who were already on ACEi (33). Experimental studies indicate that MR activation leads to increased kidney Rho-kinase signalling (34), and that Rho-kinase activation result in podocyte cytoskeletal disruption and subsequent proteinuria (35). Therefore, inhibition of podocyte MR may reduce the extent of podocyte injury during nephrotic syndrome. This could be examined in a murine model of Heymann nephritis or other models of immune-mediated podocyte injury (35), using PodMRKO mice.

5.5 Study limitations

It is important to acknowledge the potential limitations of the results arising from this thesis. In Chapter 2, the optimal dose of aldosterone used to stimulate NRK-49F cells or primary kidney fibroblasts was sometimes 10-fold higher than that observed in the circulation. Therefore, these levels may not be representative of conditions *in vivo*. However, given that multiple cells within the kidney have been shown to express aldosterone synthase, the possibility of high aldosterone concentrations in diseased kidneys cannot be excluded. Furthermore, disease conditions have also been shown to increase the expression of MR on kidney cells, indicating a greater likelihood of ligand-receptor interaction. Another issue influencing the interpretation of my *in vitro* studies is that cultures of a single cell type in plastic wells may yield results that are considerably different to those that occur in a mixed cell cultures, or in an *in vivo* environment. These issues may be partially addressed by future studies examining co-cultures of fibroblasts and other renal cell types, such as tubular cells or podocytes, in matrix-coated wells.

In Chapters 3 and 4, gene KO mice were given anti-GBM GN and DN to assess the relative importance of MR signalling in selective cell types. Cell selectivity was achieved by the Cre-LoxP technology, which is a widely used and validated method. However the degree of MR gene deletion achieved in the selected cells during disease was difficult to quantify. For example, the deletion of MR in

myeloid cells was dependent on the promoter LysM, which is up-regulated during active infection or inflammation. The extent of Cre-mediated deletion of MR is therefore dependent on the degree of LysM up-regulation. Hence, it is possible that not all myeloid cells will have adequate MR gene deletion during mild inflammatory states, resulting in incomplete MR blockade. This effect may not be obvious in the model of anti-GBM GN; however in the model of DN, incomplete MR blockade may lead to reduced renal protection. Furthermore, the exact contribution of MR from macrophages, neutrophils or sub-populations of dendritic cell leading to renal injury during GN is difficult to determine. This is due to the shared expression of LysM in these cells. Our assumption that macrophage MR was predominantly responsible for the pathological effects targeted in MyMRKO mice in anti-GBM disease was based on our understanding of the pathogenesis of this disease model, which has been well characterised and reviewed extensively in the literature. Despite this, further experiments are required – perhaps through different techniques of selective macrophage MR inhibition as described earlier – to specifically demonstrate the role of macrophage MR during GN.

Animal models utilised in this thesis were chosen for their reproducibility, as well as laboratory expertise. In Chapter 4, the resultant DN was mild, and may have contributed to the limited benefits achieved with myeloid MR deletion. Future studies examining the cell type specific roles of MR in diabetic kidney disease will require optimisation of DN models to identify the best disease conditions

and time points, in order to assess the impact of selected gene deletions on particular features of disease.

In this thesis, I used eplerenone for systemic blockade of MR, which is widely used in clinical practice. However, the use of eplerenone has some limitations. Even though eplerenone is a more specific inhibitor of the MR than spironolactone, it is of lesser potency in terms of competing for the receptor (36). Findings obtained in the anti-GBM GN studies (Chapter 3) demonstrated that the degree of renal protection obtained with eplerenone was sometimes less than that obtained with myeloid MR deletion, suggesting that that systemic MR blockade was incomplete. Therefore, it would be a reasonable assumption that higher doses of eplerenone may provide greater renal protection – especially when our results showed no evidence of hyperkalaemia. Furthermore, from a pharmacokinetic point of view, eplerenone may have provided more efficacy if it was allowed to reach steady state prior to the induction of GN. This could have been achieved by giving an initial calculated loading dose, or allowing five half lives of eplerenone to reach steady state.

5.6 Human translation

The Achilles heel of all laboratory studies is the applicability of novel results in the treatment of kidney disease in patients. Positive findings in Chapter 2, where aldosterone stimulated fibroblast proliferation in rodents, may not apply directly to fibroblasts of a human. In order to translate our cell culture results,

similar experiments must be carried out using fibroblasts sourced from people. This can be examined using primary human dermal fibroblasts, which are available commercially, or could be harvested from patients with specific medical conditions such as diabetes or existing kidney disease. Another potential source could be fibroblasts obtained from rejected transplanted kidneys, although this would be technically challenging. Whatever the source, primary human fibroblasts would require characterisation of their phenotype: proliferative potential, generation of ECM, and also to confirm the expression of MR; before being able to test the effect of aldosterone. Subsequent receptor activation and intracellular signalling pathways may also differ between rodents and humans – although these generally are highly conserved between species.

The ability to translate my animal model findings to patients is somewhat limited by the lack of effect of myeloid MR deletion on the development of albuminuria in models of anti-GBM GN and STZ-induced type 1 DN. In contrast, most clinical studies examining the effects of MR antagonist treatment in kidney disease have identified a reduction in albuminuria as the primary outcome (37). This discrepancy may be explained by differences in the nature of the kidney diseases examined in animal and clinical studies. In my mouse model of accelerated anti-GBM GN, albuminuria developed acutely at day 1 and maintained a similar level throughout the disease. Neither eplerenone nor deficiency of MR in podocytes or myeloid cells provided any substantial protection to the development of albuminuria, suggesting that MR signalling was not a critical contributor to the acute podocyte injury seen in this model. In

comparison, eplerenone blockade has been shown to be protective against models of GN and DN that progress more slowly (16, 38) and chronic kidney disease in patients (37). In my DN model, albuminuria was not progressive and was not affected by myeloid MR deficiency, which suggests that renal injury in this model was probably too mild to see any benefit of cell-specific or systemic MR blockade; however, eplerenone treatment was not investigated in this model. These findings emphasize the importance of using disease models that have a similar pathological progression and measurable endpoints to those seen in clinical trials.

Involvement of the MR-mediated pathology during GN is demonstrated in Chapter 3, where both macrophage-selective and systemic MR antagonism reduced overall renal injury by inhibiting inflammation and fibrosis. These results support a role for the use of MR antagonists as an adjunct therapy for patients with GN, particularly when conventional therapies (ACEi or ARB) are insufficient to prevent disease progression. Given the current availability of systemic MR antagonists, randomised controlled clinical trials could be set up to examine the effects of MR antagonists alone, or in combination with ACEi, during GN. The obvious concern is hyperkalaemia; however our results suggest – at least in mice – that the doses of MR inhibitor required for renal protection are smaller than that is required to induce hyperkalaemia. Furthermore, newer classes of MR antagonists with reduced affinity for renal tubular cell MR, and thus have reduced theoretical risk of hyperkalaemia, are already being trialled in experimental studies (39). Results from this thesis also suggest that specific

targeting of myeloid MR can be effective in reducing the extent of kidney injury during GN without affecting tubular function. This may lead to the future development of therapeutic drugs or drug delivery systems that allow cell selective MR antagonism.

5.7 Future perspectives

Based on the results of this thesis, further investigations are required to assess the effectiveness of single cell MR inhibition in other kidney diseases, as well as to explore methods of delivering cell selective MR blockade.

As described earlier, cell selective MR blockade in fibroblasts and podocytes should also be examined in models of renal fibrosis and DN respectively. Another experiment of interest is the assessment of MyMRKO mice during ischaemic reperfusion injury (IRI). MR antagonists have been shown to abolish the extent of injury during IRI, whether it was given pre-injury, or post (40-41). Given that macrophages also play a role in the acute inflammatory response post IRI (42), as well as contributing towards renal fibrosis late in the disease process (43), an experiment using mice with selective macrophage MR inhibition may provide insight into the role of macrophage MR in IRI.

My thesis findings are unique in that, for the first time, I have been able to discern the roles of MR activation in selective kidney cells during kidney

disease. Similar techniques have led to exploration of cell specific MR deletions in cardiac and cerebrovascular disease. These findings will help to provide a foundation for cell selective MR blockade, which allows avoidance of salt disturbance, and will benefit patients, not only with renal disease, but also cardiovascular diseases in general. To achieve this goal, newer therapeutics require a drug design that will promote cell selectivity.

There are multiple potential strategies for selectively delivering MR antagonists to macrophages. One example is the use of liposome delivery systems (44), which capitalises on the innate ability of the macrophage to phagocytose liposomes – which can be loaded with drugs such as MR antagonists. Another method is to taking advantage of scavenger receptors that are unique to macrophages. Therapeutics can be attached to molecules that are designed to specifically engage activated macrophage expressing scavenger receptors (45-46). This approach has been shown to accurately target macrophages responsible for the pathogenesis of atherosclerosis (46). Both these techniques have the potential to achieve macrophage selective MR antagonism during disease, without affecting tubular MR activity or the function of resident tissue macrophages.

Developments in novel non-steroidal MR antagonists have also seen improvements in reducing endocrine side effects as well as reducing effects on tubular MR (39). This could facilitate a broader use of combined therapies which

utilise both MR antagonists and ACEi, and potentially overcome the effects of aldosterone breakthrough.

An alternative method of reducing the deleterious effects of MR activation is by targeting downstream signalling pathways. It is likely that some of the MR-activated cell signalling pathways involved in the pathological effects mediated by macrophages, podocytes and fibroblasts will not be required for MR regulation of normal tubular function. Once identified, these pathways could potentially be blocked by specific inhibitors and provide an alternate therapeutic strategy for preventing the pathological effects of MR in kidney diseases.

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Publications

Original Paper

nephron Experimental Nephrology

Nephron Exp Nephrol 2012;120:e114–e121
DOI: 10.1159/000339500

Received: January 19, 2012
Accepted: May 11, 2012
Published online: July 18, 2012

Aldosterone Induces Kidney Fibroblast Proliferation via Activation of Growth Factor Receptors and PI3K/MAPK Signalling

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Key Words
Kidney · Fibroblast · Proliferation · Aldosterone · Growth factors · Signal transduction

Abstract
Background/Aims: The mineralocorticoid hormone, aldosterone, has pro-fibrotic properties which can cause kidney damage. The severity of kidney interstitial fibrosis is dependent on the accumulation of fibroblasts, which result largely from local proliferation; however, it is unknown whether aldosterone stimulates kidney fibroblast proliferation. Therefore, we examined the effects of aldosterone on the proliferation of cultured kidney fibroblasts. ***Methods:*** Uptake of ^{3}H-thymidine and cell number quantitation were used to determine the proliferative effects of aldosterone on a rat kidney fibroblast cell line (NRK49F cells) and interstitial fibroblasts extracted from mouse kidneys after unilateral ureter obstruction. The role of different mitogenic signalling pathways in aldosterone-induced proliferation was assessed using specific inhibitors of receptors and kinases. ***Results:*** Physiological levels of aldosterone induced a doubling of proliferation of kidney fibroblasts ($p < 0.0001$), which was inhibited by pre-treatment with the mineralocorticoid receptor antagonist, eplerenone. Aldosterone-induced fibroblast proliferation was dependent upon the kinase activity of growth factor receptors [platelet-derived growth factor receptor (PDGFR) and epidermal growth factor receptor]. Notably, PDGF ligands were not involved in aldosterone-induced PDGFR activation, indicating receptor transactivation. Aldosterone-induced fibroblast proliferation also required signalling via PI3K, JNK and ERK pathways, but not via the transforming growth factor-β_1 receptor. ***Conclusion:*** Aldosterone ligation of the mineralocorticoid receptor in kidney fibroblasts results in rapid activation of growth factor receptors and induction of PI3K/MAPK signalling, which stimulates proliferation. This suggests that increased levels of aldosterone during disease may promote the severity of kidney fibrosis by inducing fibroblast proliferation.

Introduction

Increased levels of aldosterone promote fibrosis in the heart and kidneys [1]; however, it is unclear whether these pro-fibrotic effects are simply an indirect consequence of aldosterone-induced hypertension or a direct effect of aldosterone on fibroblasts via the mineralocorticoid receptor (MR).

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Studies in hypertensive rats demonstrate that MR blockade with eplerenone, at doses that do not reduce blood pressure, can inhibit renal fibrosis in the presence or absence of diabetes [2, 3]. Eplerenone can also reduce renal fibrosis in diabetic mice which do not develop hypertension [4]. These findings suggest that aldosterone can induce renal fibrosis in the absence of an effect on hypertension.

In vitro studies indicate that aldosterone can directly promote fibrotic responses. In kidney fibroblasts, physiological levels of aldosterone (10^{-9} to 10^{-8} M) can enhance the production of transforming growth factor (TGF)-β_1, plasminogen activator inhibitor (PAI)-1 and connective tissue growth factor [5, 6] and increase mRNA levels of collagen and ^{3}H-proline incorporation [7]. These findings suggest that aldosterone may regulate matrix production by fibroblasts.

Aldosterone may also promote fibrosis by increasing fibroblast accumulation. In diseased kidneys, interstitial fibroblast number is a strong predictor of progression to end-stage renal failure [8]. Aldosterone can induce epithelial to mesenchymal transition of cultured kidney tubular cells resulting in their transformation into myofibroblasts [9]. However, regardless of the origin of kidney interstitial fibroblasts, it is the local proliferation of these fibroblasts that is a major factor driving their accumulation in the diseased kidney [10]. Studies have reported that aldosterone can induce proliferation of cardiac myofibroblasts [11, 12] and that a high level of aldosterone (10^{-7} M) can induce the proliferation of a kidney fibroblast cell line [13]. However, it is unknown whether physiological levels of aldosterone stimulate the proliferation of kidney fibroblasts, especially primary cells isolated from fibrotic kidneys. Therefore, this study was devised to examine whether kidney-derived fibroblasts proliferate in response to aldosterone and to determine the cell signalling mechanisms involved.

Methods

Rat Kidney Fibroblast Cells

A fibroblast cell line derived from normal rat kidney (NRK49F) was obtained from the American Tissue Culture Collection (Rockville, Md., USA).

Isolation of Fibroblasts from Fibrotic Mouse Kidneys

Interstitial fibroblasts were obtained from fibrotic mouse kidneys after 7 days of unilateral ureter obstruction (UUO) [10], in accordance with institutional ethics guidelines. These fibroblasts have stronger proliferative activity than those obtained from normal kidneys and more closely represent fibroblasts in diseased kidneys [14]. Dissected kidneys were incubated in collagen-coated wells in 10% FCS/DMEM/penicillin/streptomycin. The primary outgrowth cells were trypsinized and subcultured for experiments. Primary cells were cultured with colony-stimulating factor receptor antibody (20 μg/ml AFS98) to deplete contaminating macrophages [15]. After 2 weeks, the remaining kidney cells were found to be fibroblasts based on flow cytometry analysis (see Results).

Cell Proliferation Assays

Kidney fibroblasts were added to 96-well plates (2.5×10^3 cells/well) in 5% FCS/DMEM and, after overnight culture, the FCS was reduced to 0.5% for 24 h to render cells quiescent. After fasting, cells were incubated with and without stimuli for 24 h in DMEM containing 0.1% BSA and 1% insulin/transferrin/selenium supplement (Invitrogen, Grand Island, N.Y., USA). The stimuli used were aldosterone (Sigma) or recombinant human platelet-derived growth factor (rhPDGF)-AB (10 ng/ml; Peprotech, Rocky Hill, N.J., USA). During the last 18 h of culture, ^{3}H-thymidine (0.5 μCi/well; Amersham, UK) was added to the medium. After incubation, cells were washed and lysed, and the lysate analysed with a β-counter. In some experiments, the cell number was determined by adding a tetrazolium compound (MTS) to the media and measuring its conversion to formazan (Celltiter, Promega, Madison, Wisc., USA).

Inhibition of Cell Signalling Pathways

To identify signalling mechanisms involved in aldosterone-induced proliferation, cells were pre-incubated with various inhibitors for 30 min before stimulation. Preliminary studies identified the minimal effective dose of these inhibitors and showed that these doses were not cytotoxic by measuring the cellular release of lactate dehydrogenase (Cytotoxicity Detection Kit, Roche Diagnostics, Mannheim, Germany). The inhibitors used were: MR antagonist – eplerenone (Sigma); PDGF receptor (PDGFR) kinase inhibitor – STI-571 (Novartis, Sydney, Australia); recombinant mouse PDGFRα/Fc chimera and recombinant mouse PDGFRβ/Fc chimera (R&D Systems, Minneapolis, Minn., USA); epidermal growth factor receptor (EGFR) kinase inhibitor – AG1478 (Calbiochem, La Jolla, Calif., USA); PI3K inhibitor – LY294002 (Calbiochem); JNK inhibitor – SP600125 (Calbiochem); ERK inhibitor – UO126 (Calbiochem); and ALK5/TGF-β_1 receptor (TGF-β_1R) kinase inhibitor – SB431542 (Calbiochem).

Immunoprecipitation and Western Blots

NRK49F cells were grown in 6-well plates to near confluency and then starved in 0.5% FCS/DMEM for 24 h. Cells were then incubated in DMEM with 10^{-4} M vanadate in the presence of either aldosterone (10^{-9} or 10^{-8} M; Sigma) or rhPDGF-AB (10 ng/ml; Peprotech) or no stimuli for 15, 30 or 60 min. After stimulation, cells were washed and placed in lysis buffer (1% Triton X-100, 50 mM Tris/HCl, pH 7.5, 150 mM NaCl, 100 mM NaF, 2 mM EGTA and phosphatase/protease inhibitor cocktail from Sigma).

For immunoprecipitation studies, cell lysates were incubated with rabbit anti-PDGFRβ antibody (sc-432; Santa Cruz Biotechnology, Santa Cruz, Calif., USA). The lysate-antibody solution was then incubated with protein G-Sepharose, and bound proteins were released in SDS-PAGE sample buffer. Proteins were separated by SDS-PAGE and electroblotted onto nitrocellulose membranes. After blocking with Odyssey buffer (LI-COR, Lin-

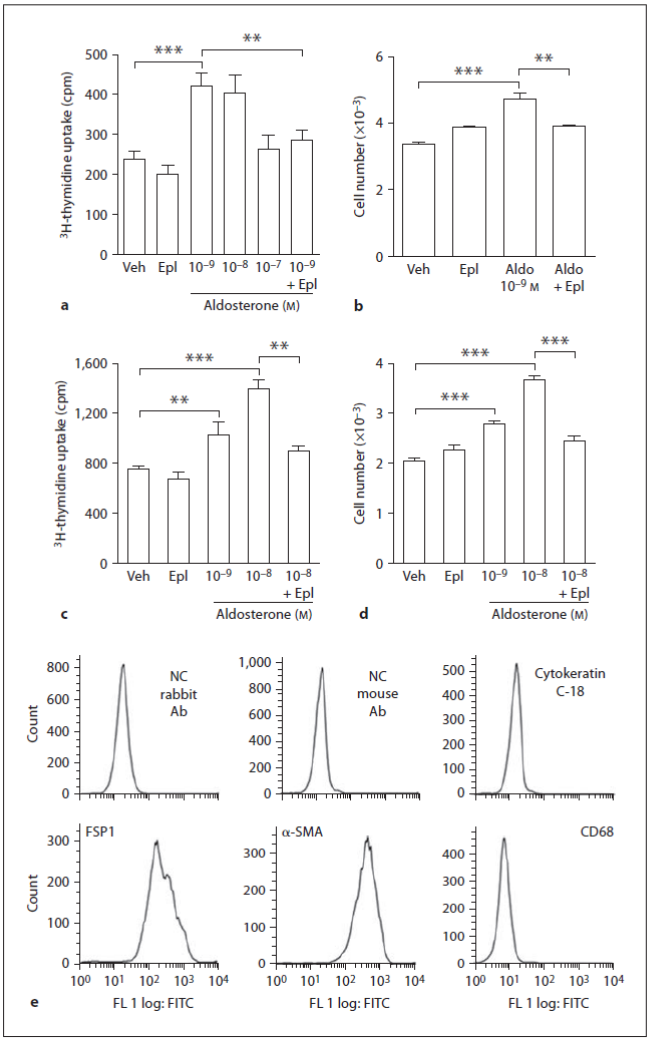

Fig. 1. Aldosterone induces renal fibroblast proliferation via the MR. Data are the mean ± SEM; n = 12 (**a**, **c**); n = 6 (**b**, **d**). Veh = Vehicle; Epl = eplerenone; Aldo = aldosterone. ** $p < 0.01$; *** $p < 0.001$. **a** Dose-response effect of aldosterone on ^{3}H-thymidine uptake in NRK49F cells at 24 h and impact of eplerenone blockade. **b** Effect of aldosterone and eplerenone blockade on the number of NRK49F cells. **c** Analysis of aldosterone-induced ^{3}H-thymidine uptake and eplerenone blockade in primary fibroblasts extracted from mouse UUO kidneys. **d** Effect of aldosterone and eplerenone blockade on the number of mouse UUO kidney fibroblasts. **e** Flow cytometry analysis of fibroblasts isolated from mouse UUO kidneys. Compared to labelling with negative control (NC) antibodies (Ab), fibroblasts from UUO kidneys had strong expression of fibroblast-specific protein 1 (FSP1) and α-smooth muscle actin (α-SMA), but did not express cytokeratin C-18 or CD68.

coln, Nebr., USA), membranes were incubated with mouse anti-phospho-tyrosine antibody (PY20; Upstate Biotechnology, Lake Placid, N.Y., USA) followed by donkey anti-mouse IRDye 800 (Rockland, Gilbertsville, Pa., USA). Phosphorylated PDGFR was detected using the Odyssey Infrared Image Detection System (LI-COR). Blots were reprobed to determine total PDGFR levels by incubation with rabbit anti-PDGFRβ (sc-432; Santa Cruz) followed by goat anti-rabbit Alexa Fluor 680 (Invitrogen, Carlsbad, Calif., USA) and LI-COR analysis.

For analysis of cell signalling pathway activation, proteins in cell lysates were separated by SDS-PAGE and electroblotted onto nitrocellulose membranes. After blocking in Odyssey buffer,

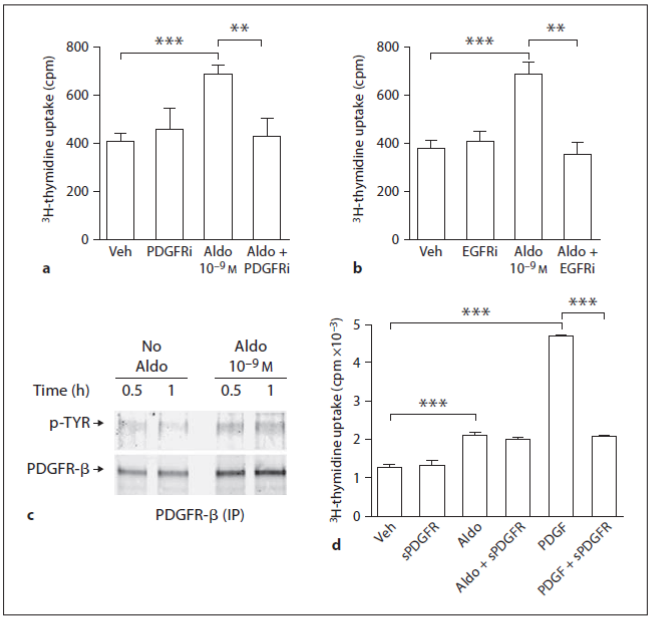

Fig. 2. Aldosterone-induced kidney fibroblast proliferation requires activation of PDGFR and EGFR. Data are the mean ± SEM; n = 9 (**a**, **b**), n = 12 (**d**). Veh = Vehicle; Aldo = aldosterone; p-TYR = phospho-tyrosine. ** $p < 0.01$; *** $p < 0.001$. Pretreatment with inhibitors of PDGFR (PDGFRi, STI571) (**a**) and EGFR (EGFRi, AG1478) (**b**) prevented aldosterone-induced proliferation of NRK49F fibroblasts. **c** A Western blot of PDGFR-β immunoprecipitate (IP) from NRK49F cell lysate showed that aldosterone increases tyrosine phosphorylation of the PDGFR-β in these cells. **d** Pretreatment with soluble PDGFR (sPDGFR) was unable to prevent aldosterone-induced proliferation of NRK49F fibroblasts.

membranes were incubated with rabbit primary antibodies [anti-phospho-ERK1/2 (Thr202/204), anti-phospho-JNK1/2 (Thr183/Thy185) or anti-phospho-Akt (Ser473); all from Cell Signaling Technology, Beverly, Mass., USA] followed by goat anti-rabbit Alexa Fluor 680 (Invitrogen). Protein bands were detected using the Odyssey Infrared Detection System (LI-COR). Protein loading was determined using mouse anti-α-tubulin (Sigma) followed by donkey anti-mouse IRDye 800 (Rockland).

Statistical Analysis

Statistical differences were analysed by Student's t test or one-way ANOVA. Data were recorded as the mean ± SEM, and $p < 0.05$ was considered significant. All analyses were performed using GraphPad Prism 5.0 (GraphPad software, San Diego, Calif., USA). Each experiment was performed 3 times.

Results

Aldosterone Induces Renal Fibroblast Proliferation by MR Signalling

Aldosterone at 10^{-9} and 10^{-8} M increased ^{3}H-thymidine uptake in rat kidney fibroblasts (NRK49F cells) by approximately 2-fold; however, no mitogenic effect was seen at 10^{-7} M (fig. 1a). Aldosterone also increased the number of NRK49F cells (fig. 1b). Similarly, fibroblasts extracted from obstructed mouse kidneys showed a 2-fold proliferative response to aldosterone at 10^{-8} M (fig. 1c, d). Pre-treatment of kidney fibroblasts with 10^{-6} M eplerenone prevented the proliferative effect of aldosterone without altering basal proliferation (fig. 1a–d). Flow cytometry analysis of the fibroblasts isolated from obstructed mouse kidneys shows that these cells expressed fibroblast-specific protein 1 and α-smooth muscle actin (a myofibroblast marker), but not cytokeratin C-18 (an epithelial marker) or CD68 (a macrophage marker) (fig. 1e).

Aldosterone-Induced Fibroblast Proliferation Requires Activation of Growth Factor Receptors

Pre-treatment of NRK49F cells with an inhibitor of PDGFR kinase (0.5 μM STI-571) or EGFR kinase (0.25 μM AG1478) prevented the effect of 10^{-9} M aldosterone on fibroblast proliferation (fig. 2a, b), but did not influence basal proliferation. Immunoprecipitation of PDGFRβ from cell lysates of NRK49F cells and subsequent West-

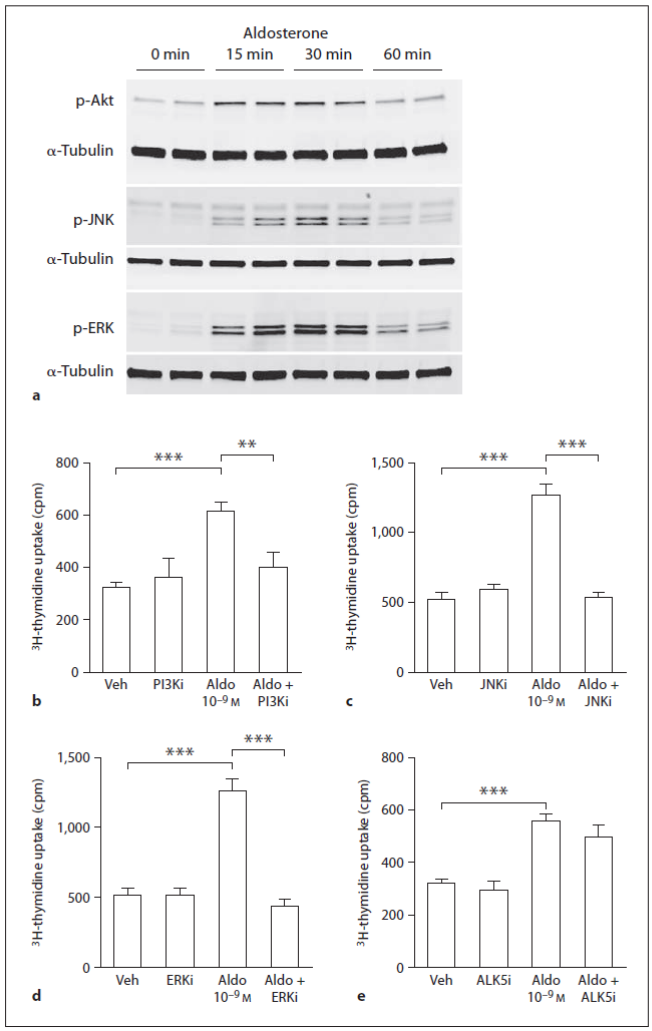

Fig. 3. Aldosterone-induced fibroblast proliferation involves activation of PI3K/Akt, JNK and ERK signalling pathways. Western blotting of NRK49F cell lysates demonstrates aldosterone-induced phosphorylation of Akt, JNK1/2 and ERK1/2 (**a**). α-Tubulin levels are shown as loading controls. Pre-treatment with inhibitors of PI3K (PI3Ki, LY294002) (**b**), JNK (JNKi, SP600125) (**c**) and ERK (ERKi, UO126) (**d**) prevented aldosterone-induced proliferation of NRK49F fibroblasts. In comparison, an inhibitor of TGF-β_1R/ALK5 (ALK5i, SB431542) (**e**) did not suppress aldosterone-induced fibroblast proliferation. Data are the mean ± SEM; n = 12 (**b**); n = 9 (**c**–**e**). Veh = Vehicle; Aldo = aldosterone. ** $p < 0.01$; *** $p < 0.001$.

ern blotting of phosphorylated tyrosine showed that stimulation with 10^{-9} M aldosterone for 30 min increased phosphorylation of PDGFRβ (fig. 2c), suggesting that the receptor was rapidly activated by aldosterone. The stimulatory effect of 10^{-9} M aldosterone on NRK49F cell proliferation was unaffected by pre-treating cells with soluble PDGFR, which comprised PDGFRα/Fc and PDGFRβ/Fc fusion proteins (2 μM each). In contrast, the same pre-treatment suppressed the proliferation induced by 10 ng/ml rhPDGF-AB (fig. 2d), showing that the soluble

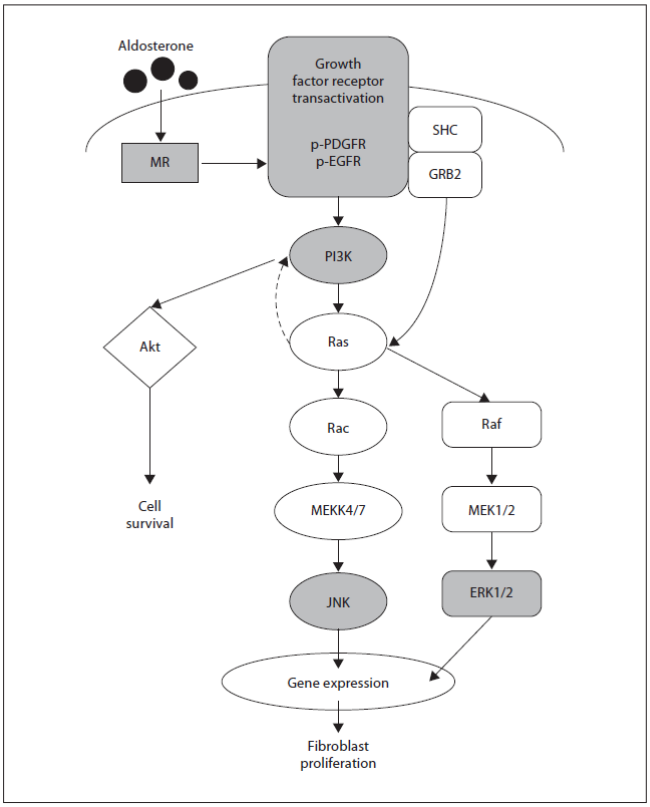

Fig. 4. Proposed mechanism of aldosterone-induced kidney fibroblast proliferation. Aldosterone binds with cytoplasmic MRs resulting in the transactivation of growth factor receptors, which facilitate activation of PI3K/Akt and Ras signalling. Activation of Ras induces JNK signalling via Rac and MEKK4/7 and ERK1/2 signalling via Raf and MEK1/2, which stimulates expression of genes that promote fibroblast proliferation.

PDGFR blocks ligand-mediated proliferation through the PDGFR.

Aldosterone-Induced Fibroblast Proliferation Involves Activation of PI3K and MAPKs

The post-receptor signalling mechanisms involved in aldosterone-induced fibroblast proliferation were examined in NRK49F cells. Stimulation of NRK49F cells with 10^{-8} M aldosterone resulted in activation of Akt, JNK and ERK signalling (fig. 3a). We then examined whether specific inhibitors of these kinases could inhibit aldosterone-induced kidney fibroblast proliferation. The proliferative effect of 10^{-9} M aldosterone on NRK-49F cells was prevented by pre-treatment with inhibitors of PI3K (3 μM LY294032), JNK (1 μM SP600125) and ERK (1 μM UO126), and the doses of inhibitors used had no effect on basal proliferation (fig. 3b–d). Since aldosterone also induces TGF-β_1 production by NRK-49F cells, we examined whether signalling via the TGF-β_1R receptor is involved in this proliferative response. Treatment with an inhibitor of TGF-β_1R/ALK5 (5 μM SB431542) did not prevent aldosterone-induced fibroblast proliferation (fig. 3a), although this inhibitor suppressed a TGF-β_1-induced increase in PAI-1 mRNA level at 8 h in NRK-49F cells (PAI-1/18S: no treatment 1.0 ± 0.1, TGF-β_1 14.2 ± 2.6, TGF-β_1 + ALK5 1.1 ± 0.1; for supplementary materials, see www.karger.com/doi/10.1159/000339500).

Discussion

Our study has shown that physiological levels of aldosterone induce proliferation of a kidney fibroblast cell line and fibroblasts extracted from fibrotic mouse kidneys, demonstrating a capacity for increased levels of aldosterone to promote fibroblast accumulation in diseased kidneys. This finding is directly relevant to kidney disease since previous research has reported elevated aldosterone levels in the plasma and urine of patients with chronic kidney disease [1] and increased MR levels in diabetic kidneys [4, 16].

Aldosterone-induced proliferation of kidney fibroblasts was found to be dependent on the activation of specific receptors. Firstly, eplerenone prevented the proliferative response to aldosterone, indicating that this effect was mediated by the established cytoplasmic MR. Secondly, aldosterone induced proliferation and required activation of two growth factor receptors PDGFR and EGFR. Interestingly, the PDGFR-mediated response was ligand independent, indicating that activated cytoplasmic MR was inducing transactivation of the PDGFR. It is also likely that the induction of EGFR signalling in kidney fibroblasts is dependent on transactivation, since aldosterone binding of MR facilitates transactivation of EGFR in other kidney cell types [17, 18]. Furthermore, it is possible that transactivation of PDGFR and EGFR may be linked by heterodimerization of these receptors, which occurs in vascular smooth muscle cells resulting in cross-talk [19]. Indeed, studies with vascular smooth muscle cells have identified that aldosterone can stimulate transactivation of both the PDGFR and EGFR in the presence of low levels of angiotensin II [20].

This study has revealed critical post-receptor signalling mechanisms which facilitate aldosterone-induced proliferation of kidney fibroblasts. Aldosterone induced rapid phosphorylation of Akt, JNK and ERK, and blockade of PI3K, JNK or ERK could prevent the aldosterone-induced proliferative response. Our finding is supported by studies of Müller glial cells in which ATP stimulates transactivation of the PDGFR and EGFR and subsequent activation of PI3K and ERK1/2, resulting in proliferation [21]. Aldosterone can also induce proliferation of cardiac myofibroblasts via activation of the Ras/Raf/MEK1/2/ERK1/2 pathway [11] and proliferation of mesangial cells by a pathway dependent on activation of EGFR, PI3K and ERK1/2 [18]. Our discovery that JNK signalling is also required for aldosterone-induced renal fibroblast proliferation is supported by studies showing that PDGF-BB induced proliferation of mesangial cells and dermal fibroblasts are JNK dependent [22, 23].

Previous work shows that aldosterone increases TGF-β_1 mRNA levels in NRK49F cells [5] and TGF-β_1 can stimulate proliferation of NRK49F cells [24] and fibroblasts isolated from renal biopsies [25]. However, our results indicate that the proliferative effects of aldosterone on kidney fibroblasts are rapid and probably precede TGF-β_1 production. Furthermore, TGF-β_1R blockade did not alter aldosterone-induced proliferation in NRK-49F cells. Therefore, aldosterone does not appear to induce fibroblast proliferation by producing TGF-β_1.

Aldosterone promotes fibrosis in animal models of hypertensive kidney disease, glomerulonephritis and diabetic nephropathy [3, 4, 26], suggesting that aldosterone-induced fibroblast proliferation may be an important pathomechanism in chronic kidney diseases. Our study demonstrates that aldosterone directly induces the proliferation of cultured kidney fibroblasts via MR-mediated activation of growth factor receptors and downstream signalling through PI3K/Akt, ERK and JNK pathways (fig. 4).

Acknowledgements

This study was supported by the Jacquot Foundation and the National Health and Medical Research Council of Australia.

Disclosure Statement

The authors have no conflicts of interest.

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