

Podocyte Melanocortin 1 Receptor Mediated Signaling

**A potential new target for patients with
kidney diseases**

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A learning experience is one of those things that says,
"You know that thing you just did? Don't do that."

- *Douglas Adams*

ABSTRACT

Treatment of patients with nephrotic syndrome (NS) is currently unspecific and directed at ameliorating the symptoms rather than eliminating the cause. NS is actually a multitude of glomerular diseases characterized by poorly understood disease mechanisms and symptoms that include proteinuria, hypoalbuminemia and edema. Originally described in the 1950s, treatment of NS with adrenocorticotrophic hormone (ACTH) was rediscovered lately and its potentially beneficial effects on proteinuria and glomerular function have been studied in patients with different nephrotic diseases.

Our research group has shown that the effects of ACTH treatment are mediated through cells in the glomerulus. Thus, the melanocortin 1 receptor (MC1R) was found to be colocalizing with the podocyte marker synaptopodin. Treatment with MC1R specific agonists had beneficial effects in an experimental model of membranous nephropathy, Passive Heymann Nephritis (PHN). The aims of this thesis have therefore been to examine the intracellular signaling pathways and beneficial mechanisms following MC1R stimulation both *in vitro* and *in vivo*.

The hypothesis is that MC1R stimulation activates a number of beneficial effects in podocytes and stabilizes the actin cytoskeleton. To study these mechanisms, we performed experiments with MC1R selective agonists in the *in vivo* models of nephrotic syndrome; PHN and adriamycin nephropathy (AN). MC1R stimulation had ameliorating effects in the PHN model, but not in the AN model. In addition, we did *in vitro* experiments in order to analyze the intracellular effects induced by MC1R stimulation, and to perform a large-scale pathway analysis. MC1R stimulation induced a number of protective effects in podocytes, including increased catalase activity, decreased oxidative stress and protection against apoptosis. Furthermore, MC1R stimulation affected the actin cytoskeleton by inducing RhoA activity and increasing stress fiber formation. Subsequently, the MC1R stimulation had protective effects in both the puromycin and protamine sulfate *in vitro* models.

We conclude that MC1R stimulation has beneficial effects in different models of NS through activation of endogenous protective pathways and by stabilizing of the actin cytoskeleton. Building on these results, we believe that it is possible to create new, specific drugs with minimal side effects to treat patients with nephrotic syndromes in the future.

POPULÄRVETENSKAPLIG SAMMANFATTNING

Med globalt ökande livslängd, fetma och typ 2-diabetes ökar även antalet fall av kronisk njursjukdom dramatiskt. Patienter med grav kronisk njursvikt behöver dialys eller transplantation för sin överlevnad med ökande sjukvårdskostnader som följd. Nefrotiskt syndrom är ett kliniskt samlingsnamn för en rad njursjukdomar där patienterna drabbats av kraftigt läckage av proteiner till urinen, generell svullnad, minskad mängd protein i blodet och ökad mängd blodfetter. Vid dessa njursjukdomar ses sjukliga förändringar hos njurarnas podocyter och skador på deras form och funktion. Nefrotiska syndrom är kroniska och allvarliga sjukdomar vars orsaker är ofullständigt kända. Behandlingsalternativen är ofta ospecifika med allvarliga biverkningar.

Adrenokortikotrop hormon (ACTH) användes redan på 1950-talet men försvann när kortison kunde ges i tablettform. ACTH har under senare år fått ett uppsving som behandlingsalternativ för patienter med nefrotiskt syndrom. Vår forskningsgrupp har tidigare visat att effekten av ACTH kan förmedlas via melanocortin 1-receptorn (MC1R) i njurarnas glomeruli, närmare bestämt i podocyterna. Podocyterna är extremt specialiserade celler med viktiga funktioner för njurarnas filtration. Målet med denna avhandling har varit att studera effekter av MC1R aktivering i podocyter.

Effekterna av MC1R stimulering i olika sjukdomsmodeller i försöksdjur och i cellodling har studerats och de intracellulära effekterna i podocyter har kartlagts. MC1R stimulering leder till flera positiva effekter såsom skydd mot oxidativ stress och stärkt cellulärt skelett. MC1R stimulering skyddar både råttor och odlade podocyter i flera olika modeller för nefrotiskt syndrom via effekter på podocyterna.

Baserat på denna avhandling borde man utveckla specifika läkemedel riktade mot MC1R för att behandla patienter med nefrotiska syndrom med avsevärt lägre risk för biverkningar än med nuvarande terapi.

LIST OF PUBLICATIONS

This thesis is based on the following studies, referred to in the text by their Roman numerals.

I. Effects of Melanocortin 1 Receptor Agonists in Experimental Nephropathies

Lindskog Jonsson A, Granqvist A, Elvin J, Johansson ME, Haraldsson B, Nyström J (2014). PLoS ONE 9(1): e87816. doi:10.1371/journal.pone.0087816

II. Melanocortin 1 Receptor Agonist Protects Podocytes Through Catalase and RhoA Activation

Elvin J, Buvall L, Lassén E, Bergwall L, Lindskog Jonsson A, Granqvist A, Nyström J, Haraldsson B. *Manuscript under revision.*

III. Melanocortin 1 Receptor Activation Influences Podocyte Cytoskeletal Dynamics

Elvin J, Bergwall L, Sihlbom C, Olsson BM, Wallentin H, Haraldsson B, Nyström J, Buvall L. *In manuscript.*

TABLE OF CONTENTS

| | |
|---|-----|
| ABSTRACT | I |
| POPULÄRVETENSKAPLIG SAMMANFATTNING | II |
| LIST OF PUBLICATIONS | III |
| TABLE OF CONTENTS | IV |
| ABBREVIATIONS | VII |
| DEFINITIONS IN SHORT | IX |
| 1 INTRODUCTION | 1 |
| 1.1 The kidney | 1 |
| 1.1.1 The glomerulus..... | 2 |
| 1.2 Podocytes | 4 |
| 1.2.1 Actin cytoskeleton..... | 6 |
| 1.2.2 RhoGTPase signaling in podocytes..... | 7 |
| 1.3 Glomerular disease..... | 9 |
| 1.3.1 Nephrotic syndrome | 9 |
| 1.3.2 Minimal change disease | 10 |
| 1.3.3 Membranous nephropathy..... | 11 |
| 1.3.4 Focal segmental glomerulosclerosis..... | 11 |
| 1.3.5 Diabetic nephropathy | 12 |
| 1.3.6 Treatment strategies for nephrotic syndrome | 13 |
| 1.4 The melanocortin receptors..... | 15 |
| 1.4.1 Melanocortin 1 receptor | 16 |
| 1.4.2 Melanocortin 1 receptor agonists | 16 |
| 2 AIM..... | 19 |
| 3 METHODS | 20 |
| 3.1 Animal studies | 20 |
| 3.1.1 <i>In vivo</i> nephrotic models | 21 |
| 3.1.2 Treatment with Melanocortin 1 Receptor agonists | 22 |
| 3.1.3 Investigation of Nephrotic Syndrome | 23 |

| | | |
|-------|---|----|
| 3.2 | Cell culture..... | 25 |
| 3.2.1 | Immortalized podocytes | 25 |
| 3.2.2 | DNA constructs and molecular cloning..... | 27 |
| 3.2.3 | E92K mutant..... | 28 |
| 3.2.4 | Lentivirus..... | 28 |
| 3.2.5 | <i>In vitro</i> nephrotic models..... | 30 |
| 3.2.6 | Melanocortin 1 receptor agonists | 31 |
| 3.2.7 | <i>In vitro</i> readouts..... | 31 |
| 3.2.8 | Actin cytoskeleton..... | 34 |
| 3.3 | Gene expression and protein analysis | 36 |
| 3.3.1 | Immunocytochemistry..... | 36 |
| 3.3.2 | Real-time RT-PCR | 36 |
| 3.3.3 | SDS-PAGE Western Blot..... | 37 |
| 3.4 | Mass spectrometry | 37 |
| 3.4.1 | Ingenuity Pathway Analysis | 38 |
| 3.5 | Statistical analysis | 39 |
| 4 | RESULTS AND DISCUSSION | 40 |
| 4.1 | Effects of Melanocortin 1 Receptor agonists in Experimental Nephropathies (Paper I)..... | 40 |
| 4.1.1 | Amelioration of Passive Heymann Nephritis | 40 |
| 4.1.2 | No effect on adriamycin nephropathy | 41 |
| 4.1.3 | Expression in nephrotic models..... | 43 |
| 4.1.4 | Differences between treatments | 44 |
| 4.2 | Melanocortin 1 Receptor Agonist Protects Podocytes Through Catalase and RhoA Activation (Paper II)..... | 46 |
| 4.2.1 | Gene and protein expression in cultured podocytes | 46 |
| 4.2.2 | Podocyte-protective effects | 46 |
| 4.2.3 | Protection against puromycin..... | 48 |
| 4.2.4 | Proposed signaling pathway..... | 50 |
| 4.3 | Melanocortin 1 receptor activation influences podocyte cytoskeletal dynamics (Paper III) | 52 |

| | |
|--|----|
| 4.3.1 Cyclic AMP response..... | 52 |
| 4.3.2 Protection against protamine sulfate | 52 |
| 4.3.3 Phosphoproteomic analysis | 54 |
| 4.3.4 Actin dynamics..... | 55 |
| 4.4 General discussion | 57 |
| 5 CONCLUDING REMARKS | 61 |
| 5.1 Paper I..... | 61 |
| 5.2 Paper II..... | 61 |
| 5.3 Paper III | 62 |
| 5.4 Future implications | 63 |
| 6 ACKNOWLEDGEMENTS..... | 66 |
| 7 REFERENCES..... | 68 |

ABBREVIATIONS

| | |
|------------------|---|
| AC | Adenylate cyclase |
| ACE | Angiotensin-converting enzyme |
| ACTH | Adrenocorticotrophic hormone |
| AKI | Acute kidney injury |
| AMD | Apical membrane domain |
| AMT | 3-amino-1, 2, 4-triazole |
| AN | Adriamycin induced nephropathy |
| ARB | Angiotensin receptor blocker |
| BCA | Bicinchoninic acid |
| BMD | Basal membrane domain |
| BMS-470539 | Synthesized MC1R selective agonist, MC1R-a |
| BUN | Blood urea nitrogen |
| cAMP | Cyclic adenosine monophosphate |
| CKD | Chronic kidney disease |
| CNF | Congenital nephrotic syndrome of the Finnish type |
| CREB | cAMP response element-binding protein |
| C _T | Threshold cycle number |
| DMEM | Dulbecco's modified Eagle's medium |
| DMSO | Dimethyl sulfoxide |
| DN | Diabetic nephropathy |
| EC ₅₀ | Half maximal effective concentration |
| EGFP | Enhanced green fluorescent protein |
| ESL | Endothelial surface layer |
| ESRD | End-stage renal disease |
| FBS | Fetal bovine serum |
| FP | Foot process |
| FSGS | Focal segmental glomerulosclerosis |
| GBM | Glomerular basement membrane |
| GEF | Guanine nucleotide exchange factor |
| GFP | Green fluorescent protein |
| GFR | Glomerular filtration rate |
| GPCR | G-protein coupled receptor |
| HFRW | His-Phe-Arg-Trp peptide fragment |

| | |
|----------------|--|
| IBMX | 3-isobutyl-1-methylxanthine |
| IFN- γ | Interferon-gamma |
| IPA | Ingenuity Pathway Analysis |
| LDL | Low-density lipoprotein |
| LMW-PTP | Low-molecular-weight protein tyrosine phosphatase |
| MC1R | Melanocortin 1 receptor |
| MC1R-a | The MC1R selective agonist BMS-470539 |
| MCD | Minimal-change disease |
| MCR | Melanocortin receptor |
| MN | Membranous nephropathy |
| MPGN | Membranoproliferative glomerulonephritis |
| MSH | Melanocyte-stimulating hormone |
| MS05 | Custom made MC1R selective peptide agonist |
| NF- κ B | Nuclear factor kappa-light-chain-enhancer of activated B cells |
| PFA | Paraformaldehyde |
| PHN | Passive Heymann Nephritis |
| PKA | Protein kinase A |
| PLA2 | Phospholipase A2 |
| POMC | Proopiomelanocortin |
| PS | Protamine sulfate |
| Puromycin | Puromycin aminonucleoside |
| RT-PCR | Reverse transcriptase PCR |
| ROCK | Rho-associated protein kinase |
| ROS | Reactive oxygen species |
| SD | Slit diaphragm |
| SDS-PAGE | Sodium dodecyl sulfate polyacrylamide gel electrophoresis |
| TEM | Transmission electron microscopy |
| TMT | Tandem mass tags |
| TNF- α | Tumor necrosis factor alpha |
| UACR | Urinary albumin to creatinine ratio |
| WT | Wild-type |
| WT-1 | Wilm's tumor 1 |
| Y-27632 | ROCK inhibitor |

DEFINITIONS IN SHORT

| | |
|----------------------------------|---|
| Cytoskeletal rearrangement | A term describing the event in which the actin cytoskeleton of a cell undergoes physiological as well as pathological changes. |
| Podocyte foot-process effacement | The process in which the characteristic interdigitating structure of the podocyte foot-processes is altered and lost. |
| Nephrotic syndrome | A collection of symptoms including edema, massive proteinuria (>3.5 g/24h), hypoalbuminemia, and hyperlipidemia. Nephrotic syndrome can arise from several different kidney diseases. |
| C_T | Threshold cycle number is defined as the number of PCR cycles required to exceed background level for the signal. The C_T level is inversely proportional to the amount of target mRNA in the sample. |

1 INTRODUCTION

Worldwide socioeconomic improvements and increased life expectancy are leading to an increased proportion of elderly people in the general population. With increased age follows an increased risk of kidney disease¹. Chronic kidney disease (CKD) is a growing problem with serious implications on individual patient health and on healthcare costs for the society. The situation is aggravated by the global obesity epidemic giving rise to CKD secondary to type 2 diabetes². Thus, kidney diseases are beginning to present major problems for the healthcare systems worldwide³.

Many nephropathies are especially difficult to treat; they are idiopathic and if left untreated will likely lead to end-stage renal disease (ESRD). For the patient this often implies dialysis while waiting for a suitable kidney transplant. The annual cost of dialysis is close to US\$ 100,000 per patient, which brings quite a burden on the healthcare budget of most societies.

However, new treatment regimens of kidney disease are being developed. Earlier studies found that treatment of patients with proteinuric kidney disease with adrenocorticotrophic hormone (ACTH) had promising results⁴. ACTH is currently being used alongside other treatments in patients worldwide. Our research group has shown that the beneficial effects of ACTH are likely to be mediated by one of its natural receptors, melanocortin 1 receptor (MC1R) which is expressed on glomerular podocytes⁵.

In this thesis, different glomerular kidney diseases and the possibilities to develop new drugs targeting MC1R will be discussed. The protective effects of MC1R stimulation will be presented from studies on isolated podocytes. A mechanistic and cellular approach has been taken to unravel the intracellular signaling events of MC1R activation in podocytes, and to assess its potential as a prospective new treatment target in patients with glomerular kidney disease. The advantage of selectively targeting MC1R would be to reduce the side effects of ACTH which include elevated cortisol levels, and severe insomnia⁶.

1.1 The kidney

The human kidney is an organ with multiple responsibilities, such as filtration of blood, excretion of waste metabolites through urine, and maintenance of blood pressure and electrolyte composition throughout the body. The kidneys produce approximately 180 liters of plasma water as

glomerular filtrate daily but only excrete around 1.5 liters of urine ³. The bodily composition has to be maintained within tight limits and becomes extremely affected by changes in kidney function.

Each kidney contains about one million functional units, nephrons, and each nephron has a filtration unit – a glomerulus.

1.1.1 The glomerulus

The glomerulus consists of a small capillary network surrounded by a structure called Bowman's capsule. In each glomerulus, there is a glomerular filtration barrier consisting of different layers separating the blood from the primary urine (Figure 1). The glomerular barrier is arguably one of the most complex biological membranes. Because of its selective properties, water and small molecules can pass through at a high rate, while larger molecules and proteins are almost totally restricted from passing through ⁷.

Between the glomerular capillaries are the intraglomerular mesangial cells. The main function of the mesangial cell is to support and maintain the structure and function of the glomerulus. Mesangial cells have contractive abilities in similarity to smooth muscle cells and they produce matrix components of the glomerular basement membrane^{8, 9}. The mesangial cells have little influence on the filtration, but they secrete several growth factors important for the surrounding cells ¹⁰.

The capillary walls are covered on the luminal side by an endothelial surface layer (ESL). The ESL is important for capillary barrier function and consists of a glycocalyx and the endothelial cell coat. It is composed of membrane-associated, negatively charged glycoproteins and glycosaminoglycans as well as proteoglycans. The negative charge of the ESL contributes to the high permselectivity of the glomerular filtration barrier ⁷.

The glomerular capillaries have fenestrated endothelial cells. Capillaries found in skeletal muscle, cardiac muscle and skin consist of continuous endothelial cells. The intestine, pancreas and the salivary glands also have fenestrated capillaries. The endothelial fenestrations are large enough for proteins, such as albumin, to pass through, but in fact proteins are markedly restricted in their passage as shown for pancreas ¹¹ and glomeruli ¹²⁻¹⁴, clearly demonstrating the size and charge selective properties of the endothelial cell coat ^{7, 15-17}.

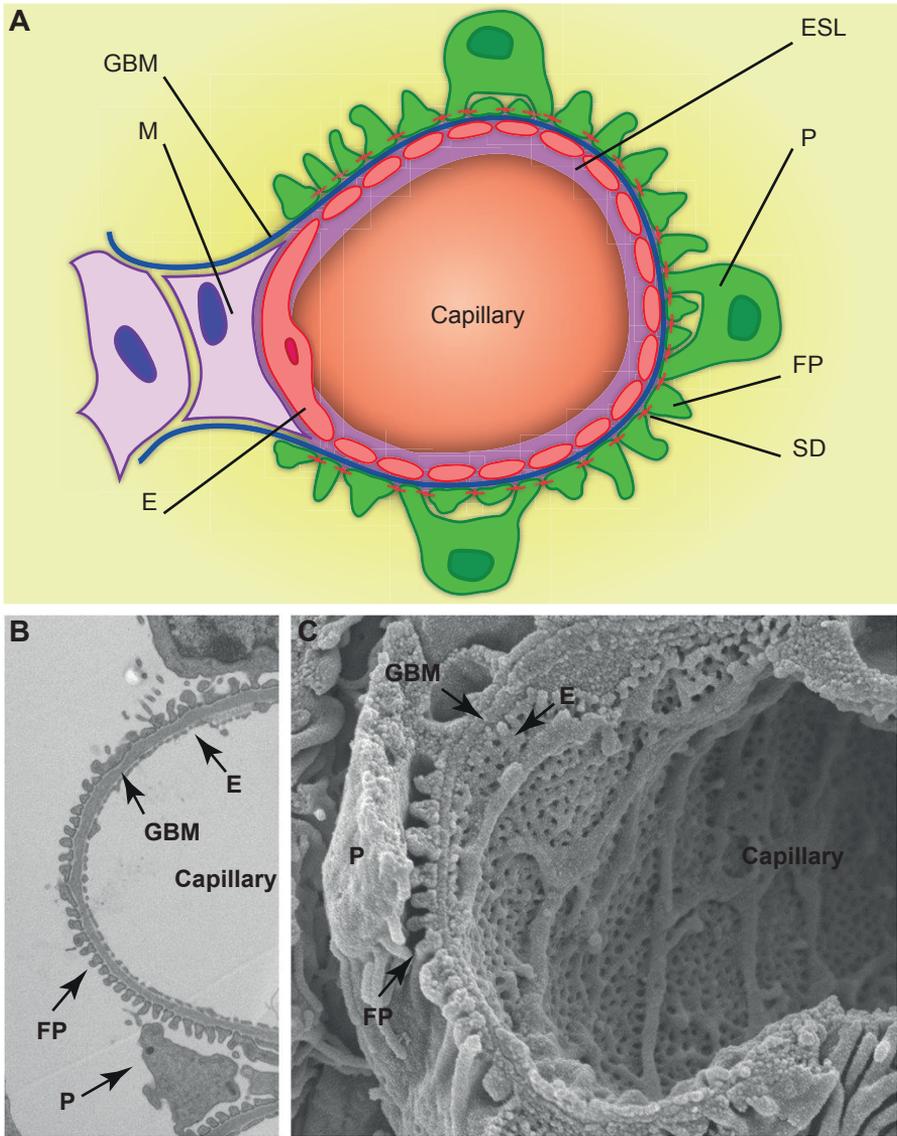


Figure 1. Glomerulus and cell types. Schematic image of glomerular capillary loop (A). Transmission electron micrograph of glomerular capillary (B), and scanning electron micrograph of a mouse glomerulus (C). ESL: Endothelial surface layer, E: Endothelial cell, GBM: Glomerular basement membrane, M: Mesangial cell, P: Podocyte, FP: Podocyte foot process, SD: Slit diaphragm. Micrographs courtesy of Dr Ebefors 2015.

Between the endothelial cells and the podocytes is a network consisting of type IV collagen, laminin and proteoglycans and glycoproteins. These proteins constitute the glomerular basement membrane (GBM). The GBM is thicker than basement membranes from other vascular beds. This is probably due to the high capillary pressure of the glomerulus – a pressure that is at least twice as high as the capillary pressure of any other vascular bed.

Residing outside of the glomerular capillaries and anchored to the GBM, are the podocytes, a specialized and highly differentiated epithelial cell type facing the Bowman's capsule.

1.2 Podocytes

The podocytes, or visceral epithelial cells, are found exclusively in the glomerulus and constitute the outermost part of the permselective glomerular barrier between the blood and primary urine¹⁸. These highly differentiated cells have a limited capability to divide. The GBM provides the primary structural support for the podocytes, which wrap around the capillaries with their characteristic morphology (Figure 2)¹⁸. The podocyte consists of a cell body with extending major processes, which branch out into actin-rich foot-processes (FP). The FPs interdigitate and form a zipper-like structure. Between adjacent FPs filtration slits are formed, which are covered by a slit diaphragm (SD). The SD is consisting of several molecules including P-cadherin, nephrin, CD2AP, ZO-1, podocin, Neph1-3 and FAT^{19, 20} of which some are critical to its integrity (Figure 3). One of the key components of the zipper-like SD, nephrin²¹⁻²³ bridges the slits between neighboring podocytes constructing a modified adherens junction²⁴ essential for the maintenance of glomerular permselectivity and structural support of the podocytes¹⁹. The podocyte actin cytoskeleton and morphology are crucial for maintenance and podocyte function. In particular, highly ordered parallel actin filament bundles are important for the structure and plasticity of the FPs. One of the most important podocyte actin-associated proteins is synaptopodin²⁵, which is highly enriched in podocyte foot processes and has found to be essential for the organization of the actin rich stress fiber bundles through RhoA²⁶⁻²⁸. Injury and modifications to the FPs disrupts the organized actin bundles which results in podocyte effacement and loss of the interdigitating FP pattern, and ultimately proteinuria²⁹.

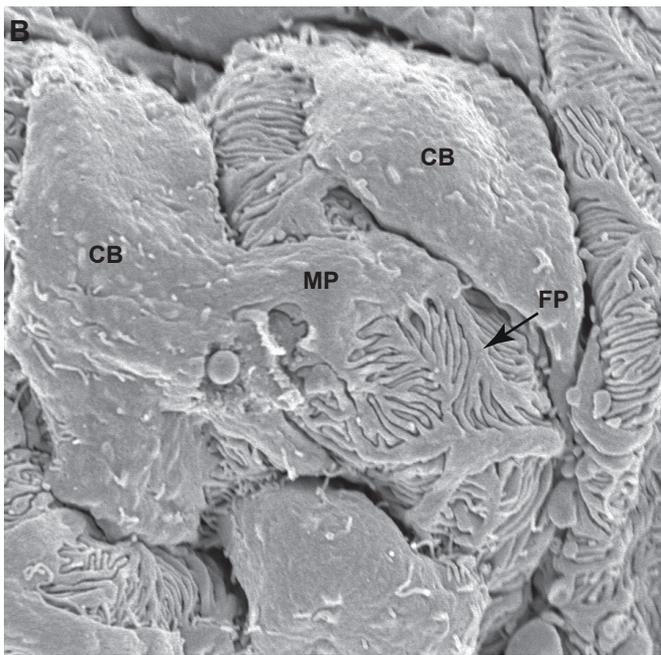
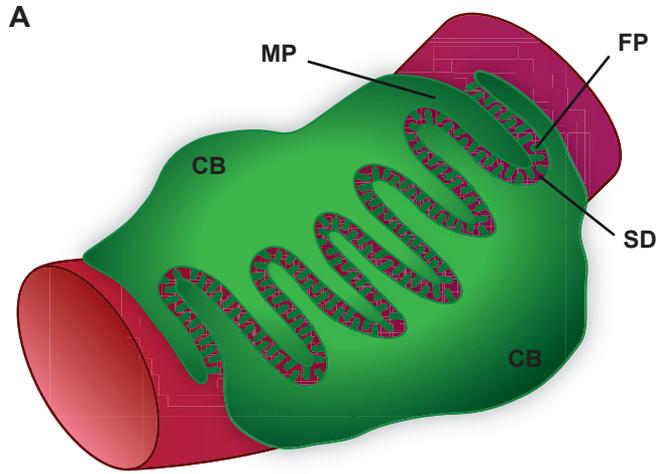


Figure 2. Podocytes wrapping around glomerular capillaries. Schematic image (A) and scanning electron micrograph (B) of podocytes wrapping around a capillary. CB: Cell body, MP: Major process, FP: Foot process, SD: Slit diaphragm. Micrograph courtesy of Dr Ebevors 2015.

Stress to the podocytes can affect the dynamics and phenotype of the actin cytoskeleton. The maintenance of a stationary podocyte phenotype is crucial for filtration and function, whereas mutations in genes encoding podocyte essential proteins or uncontrolled activation of the actin regulating proteins Cdc42 or Rac1 can lead to a more motile phenotype. This can in turn lead to “podocyte foot process effacement” which destabilizes the FPs and is a precursor for proteinuria^{20, 29, 33} (Figure 5). FP effacement requires the active reorganization of the actin filaments³⁴.

Integrins are transmembrane receptors with a heterodimeric composition, consisting of an α - and a β -subunit. The actin cytoskeleton of the FPs are physically linked to the GBM through integrins^{35, 36}. Integrin $\alpha3\beta1$ is crucial to podocyte FP maturation, and genetic deletion of the $\alpha3$ ³⁷ or $\beta1$ subunit leads to kidney failure in mice³⁶.

There are factors that can affect the activity of the integrins. Most notably is B7-1 (or CD80), which induces podocyte injury and proteinuria³⁸. B7-1 is a putative biomarker found in some patients with focal segmental glomerulosclerosis (FSGS). In a recent study B7-1 was shown to inactivate the $\beta1$ subunit, and thereby cause proteinuria, whereas treating FSGS patients with the B7-1 inhibitor abatacept was able to stabilize the $\beta1$ activity and reduce proteinuria³⁹.

1.2.2 RhoGTPase signaling in podocytes

Actin organization is directed by the family of small Rho GTPases; RhoA, Rac1, Cdc42^{28, 40}. The RhoGTPases RhoA, Rac1 and Cdc42 have different effects in cells. Activation of RhoA promotes stationary stress fiber formation⁴¹, Rac1 promote lamellipodia⁴² and Cdc42 promotes filopodia formation⁴³. Both Rac1 and Cdc42 promote cell motility at the leading edge.

Binding of GTP or GDP modulates the activity of the Rho GTPases (Figure 4). When GTP is bound, the Rho GTPases acquire an active state, whereas GDP inactivates the proteins. The switch is controlled by different guanine nucleotide exchange factors (GEFs) and GTPase-activating proteins (GAPs). The GEFs promote exchange of GDP to GTP, thereby activating the protein, whereas the GAPs inactivates the protein by GTP hydrolysis⁴⁴.

Overexpression of dominant active Rac1 leads to proteinuria in mice⁴⁵ and inhibition of the calcium channel TRPC5 protected against proteinuria by preventing activation of Rac1⁴⁶. Synaptopodin has been shown to prevent degradation of RhoA by inhibiting smurf1 mediated ubiquitination of RhoA

⁴⁷ and by mediating Nck1 activation of RhoA ²⁷. Synaptopodin also inhibits Cdc42 activity, thereby protecting against proteinuria ⁴⁸.

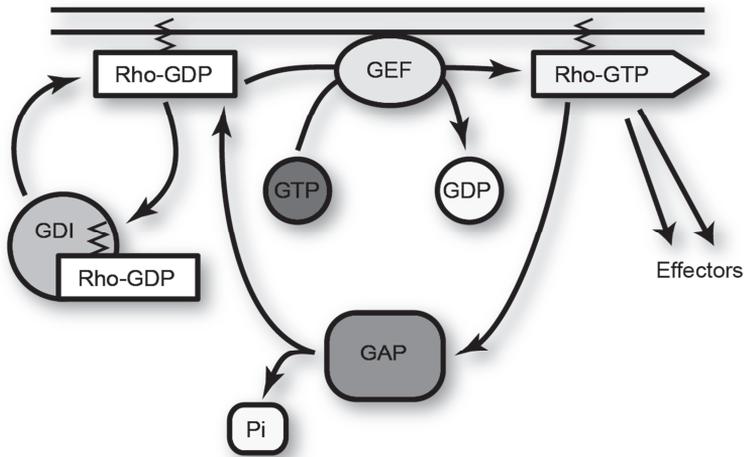


Figure 4. Schematic image of Rho GTPase activity regulation. The Rho GTPases (Rho, Rac1 and Cdc42) cycle between an active (GTP-bound) and an inactive (GDP-bound) form. This regulation is controlled by three different classes of proteins and the activity is dependent of guanosine triphosphate (GTP) exchange. Guanine exchange factors (GEFs) activate the Rho GTPases, whereas GTPase activating proteins (GAPs) deactivate them. The guanine nucleotide exchange inhibitors (GDIs) are responsible for shuttling the RhoGTPases to and from the plasma membrane. When activated, the Rho GTPases interact with a multitude of different downstream effector molecules.

Even though activation of Cdc42 and Rac1 promotes proteinuria and RhoA is beneficial, it is a delicate balance to maintain a balance of the RhoGTPases, which results in dynamic regulation of actin. Deletion of Cdc42 has been shown to induce proteinuria in mice ^{49, 50} but deletion of Rac1 was not harmful in physiologic steady-state ⁵⁰. RhoA activity was inhibited by Rac1 induced increase in levels of reactive oxygen species (ROS) through the activity of the GAP p190RhoGAP ⁵¹. In addition, the p190RhoGAP has also been shown to decrease Rac1 activity ⁵². Activation or inhibition of RhoA activity can also lead to proteinuria, by two opposing mechanisms. Enhancement of RhoA increased actin polymerization and reduced nephrin expression, which promoted podocyte apoptosis. Inhibition of RhoA caused a loss of podocyte stress fibers but did not cause apoptosis or alter glomerular nephrin expression ⁵³.

Other studies support the notion that stabilization of the kidney filter by synaptopodin is due to promotion of RhoA, which prevents the FP cytoskeleton from transition to a migratory phenotype ⁵⁴.

1.3 Glomerular disease

Glomerular disease is hard to detect since kidney function needs to be significantly reduced before any symptoms manifest in the patients³. Podocyte dysfunction and foot process effacement (Figure 5) are common denominators, and many glomerular diseases and podocytopathies have underlying genetic causes (Table 1)^{55,56}.

The initial identification of genetic mutations of nephrin, and its connection to congenital nephrotic syndrome of Finnish type (CNF), placed the podocyte at the epicenter of research into molecular and cellular causes of proteinuria. Mutations in the nephrin gene, NPHS1, were identified in 1998 as the cause of CNF²¹. Following the discovery of mutations in the NPHS1 gene, a number of mutations causing nephrotic syndrome were discovered and characterized (Table 1). Many of these are podocytopathies and the different mutations are found in essential proteins for the podocyte actin cytoskeletal organization and anchorage to the GBM such as podocin⁵⁷, α -actinin⁵⁸ and synaptopodin⁵⁹.

In addition to the podocyte-associated diseases, there are genetic renal diseases coupled to the GBM, as well as systemic genetic diseases leading to proteinuria and kidney failure⁵⁵. Not all glomerular diseases are of genetic origin and notable examples are hemolytic uremic syndrome and preeclampsia⁶⁰.

1.3.1 Nephrotic syndrome

Nephrotic syndrome (NS) is a group of symptoms associated with different renal diseases. NS is defined as edema, hypoalbuminemia, hyperlipidemia and daily protein excretion into the urine exceeding 3.5 g per 1.73 m² body-surface area⁶¹. The most common cause for NS is diabetic nephropathy⁶², but there are a number of different primary glomerular diseases which can lead to nephrotic syndrome, such as minimal-change disease (MCD), focal segmental glomerulosclerosis (FSGS) and membranous nephropathy (MN). Many nephrotic syndromes are idiopathic and treatment options are scarce⁶¹. The focus of this thesis is the podocytes; therefore a number of common podocytopathies will be discussed.

Table 1. Podocyte-associated genetic diseases.

| Protein | Gene | Associated disease | Reference |
|----------------------------------|-------------|---|-------------------------------------|
| Nephrin | NPHS1 | Congenital NS of the Finnish type (CNF) | Kestila et al. 1998 ²¹ |
| Podocin | NPHS2 | Corticosteroid-resistant NS (SRNS) | Boute et al. 2000 ⁵⁷ |
| Phospholipase Cε1 | PLCE1 | Inherited NS | Hinkes et al. 2006 ⁶³ |
| Laminin β2 | LAMB2 | Pierson's syndrome | Morello et al. 2001 ⁶⁴ |
| α -Actinin | ACTN4 | FSGS | Kaplan et al. 2000 ⁵⁸ |
| TRPC6 | TRPC6 | FSGS | Winn et al. 2005 ⁶⁵ |
| Nonmuscle Myosin Heavy Chain IIA | MYH9 | Epstein/Fetcher syndrome | Arrondel et al. 2001 ⁶⁶ |
| LMX1B | LMX1B | Nail-patella syndrome | Dreyer et al. 1998 ⁶⁷ |
| Wilm's tumor 1 | WT1 | Denys-Drash/Frasier's syndrome | Pelletier et al. 1991 ⁶⁸ |
| CD2AP | CD2AP | Sporadic FSGS | Kim et al. 2003 ⁶⁹ |
| Synaptopodin | SYNPO | Sporadic FSGS | Dai et al. 2010 ⁵⁹ |
| Myosin 1E | MYO1E | Childhood FSGS | Mele et al. 2011 ⁷⁰ |
| Apolipoprotein L-1 | APOL1 | Sporadic FSGS | Genovese et al. 2010 ⁷¹ |
| Glypican 5 | GPC5 | Acquired NS | Okamoto et al. 2011 ⁷² |

1.3.2 Minimal change disease

MCD is most common in small children, but can also occur in adults. In children, treatment of MCD with corticosteroids usually leads to remission within a few weeks. Treatment of adults poses a far bigger challenge, with steroid resistance and frequent relapses ⁷³.

Most MCD cases are idiopathic and one of the key characteristics of MCD is the fusion of podocyte FPs, or podocyte FP effacement. These changes are hard to detect in the light microscope, hence the name minimal change disease ⁷³. The pathophysiology of MCD is not fully understood, but loss of negative charge has been detected causing increased permeability of the glomerular barrier ⁷⁴. In addition, immunological abnormalities have been

described in MCD patients ⁷⁵. The B7-1 protein has also been found in the urine of MCD patients. This raised the hypothesis that B7-1 can be used as a biomarker for MCD ^{38,39}.

1.3.3 Membranous nephropathy

Almost 75 % of all MN cases are idiopathic and the rest are secondary cases, occurring from malignancies, autoimmune diseases, infections or drugs. Spontaneous remission occur in about a third of the MN patients, but another third progress to ESRD within 5-15 years ⁷⁶.

Central to the pathogenesis of MN is the formation of subepithelial immune complexes, which is the basis of diagnosis. Three different hypotheses have been postulated for the formation of these immune complexes ⁷⁶. The first states that circulating immune complexes found in lupus nephritis are passively trapped in the glomerular subepithelial space. The second hypothesis implies the incorporation of circulating antigens into the subepithelial space, which then forms *in situ* complexes with antibodies. This is supported by findings from patients with hepatitis B virus, where clusters of viral-like particles were found in immune deposits in the kidneys ⁷⁷. The third hypothesis is based on the idea that autoantibodies bind to antigens in the podocytes. A major breakthrough was the finding of antibodies against the podocyte receptor phospholipase A2 (PLA2) in a majority of cases, confirming the third hypothesis ⁷⁸. The anti-PLA2R antibodies are promising biomarkers to discriminate between primary and secondary MN. Despite these findings, the mechanisms behind MN are not yet fully understood.

1.3.4 Focal segmental glomerulosclerosis

FSGS is not a specific glomerular disease, but refers to a morphologic injury pattern that can be detected in kidney biopsies ⁷⁹. Sclerotic and fibrotic lesions in glomeruli characterize the injury. The pathological changes are focal and segmental, which indicates that not all glomeruli are affected and only discrete areas of each glomerulus are affected. In familial FSGS many different genetic mutations have been found in genes encoding proteins critical for the podocyte structure, of which some are presented in Table 1.

Patients with primary FSGS often suffer from acute nephrotic syndrome whereas patients with secondary FSGS have a more chronic disease. There is no known cause for the primary form of FSGS, but the secondary forms have a number of underlying causes. FSGS might be recurrent and can even recur after successful kidney transplantation ⁸⁰. There are different theories

explaining primary FSGS, and one of them is that a circulating factor (soluble urokinase receptor) causes the disease⁸¹.

Foot process effacement followed by apoptosis and reduction of podocyte number can lead to podocyte detachment and subsequent uncovering of the GBM. This produces areas of the GBM without structural support that can expand uncontrollably^{79, 82}.

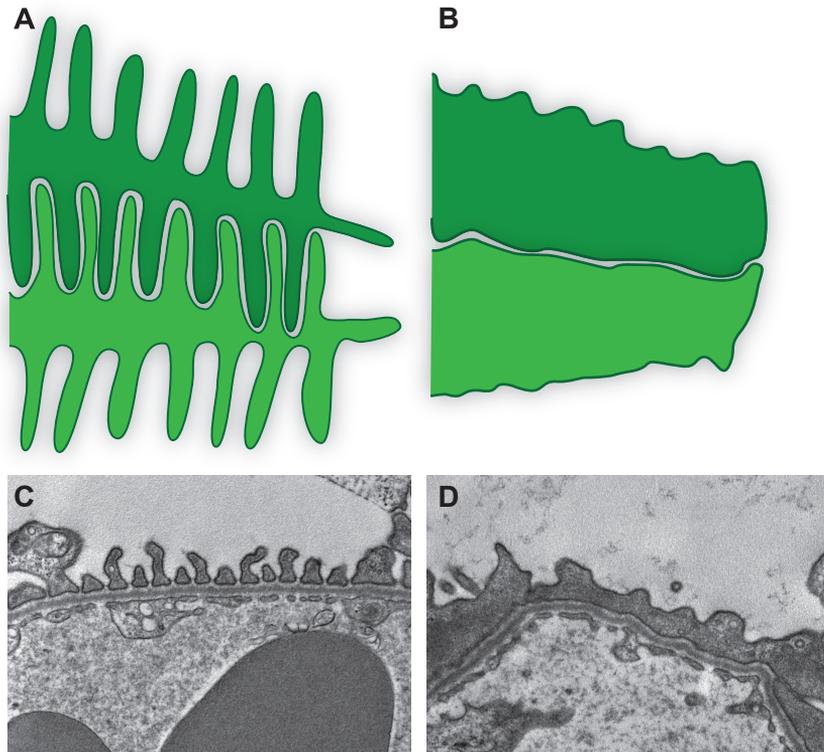


Figure 5. Podocyte foot process effacement. Healthy podocyte foot processes form an interdigitating zipper-like structure (A, C). Foot process effacement following disease or injury leads to the disappearance of the zipper like structure (B, D). Transmission electron micrographs of foot process effacement, healthy mouse glomerulus with pronounced foot processes (C), effaced podocytes (D). Image courtesy of Dr Lindskog Jonsson 2014.

1.3.5 Diabetic nephropathy

Diabetic nephropathy (DN) is a severe complication of diabetes mellitus and represents one of the largest problems facing the healthcare systems

worldwide ². DN has emerged to be the most prevalent chronic kidney disease and the primary cause of ESRD. Interestingly, a considerable percentage of diabetic patients with impaired renal function do not have elevated proteinuria and the progression of DN is slow ^{2, 62}.

The primary underlying cause is diabetes, which is usually coupled to overweight, hypertension, hypercholesterolaemia, hyperglycemia and elevated insulin tolerance. DN can cause NS, and can also lead to diffuse glomerular scarring ⁸³ and nephrinuria has been suggested as a potential mechanism involved in DN ^{84, 85}.

1.3.6 Treatment strategies for nephrotic syndrome

Many of the underlying mechanisms of nephrotic syndromes remain unclear and treatment is therefore not curative, but focused on amelioration of the symptoms. In the most severe cases of NS there is a large risk of uncontrolled progression into ESRD, dialysis and need for a kidney graft. Examples of treatment strategies for nephrotic syndromes will be presented here.

The first priority in nephrotic syndromes is to treat the underlying cause, but if the NS is idiopathic, this is not always possible. It is common to use a combination of different treatments in order to stabilize and treat the different nephrotic diseases. Many renal patients have increased risk of cardiovascular events ⁸⁶. Most patients are given angiotensin-converting enzyme (ACE) inhibitor ⁸⁷ or an angiotensin receptor blocker (ARB) ⁸⁸. This is known to have positive effects on proteinuria and halt disease progression in various nephrotic syndromes ⁸⁹. These compounds probably exert their effect by lowering the glomerular capillary pressure since the locally produced angiotensin II exerts tonic vasoconstriction on the efferent arteriole. Most patients are also treated with loop diuretics ⁹⁰.

About a third of the MN cases remit spontaneously, but in many patients treatment is required to ameliorate prolonged proteinuria or other complications due to the nephrotic syndrome. In MN, the primary evidence-based therapies include the usage of broadly immunosuppressive agents such as alkylating agents or calcineurin inhibitors in combination with corticosteroids ⁹¹. Other agents have been tested such as rituximab and ACTH ⁹² with variable results. All therapies have adverse side effects.

The most common treatments for FSGS includes initial treatment with ACE inhibitors or ARB ⁸⁰. This is followed by glucocorticoids, but if the FSGS is

steroid resistant, therapy includes a calcineurin inhibitor such as cyclosporine or tacrolimus⁹³. When patients are unresponsive to either of these treatments, another agent is usually tested⁹⁴. As an example, Rituximab has been tested in different studies⁹⁵, as well as ACTH⁹⁶. In addition, abatacept has emerged as a new and promising treatment option in patients with B7-1 positive FSGS³⁹.

Patients with MCD normally respond to treatment with glucocorticoids. However, relapses are common, and additional treatments are often required. Second line treatments include cyclosporine or calcineurin inhibitors⁷³. Studies has also been performed in which ACTH⁹⁷ and rituximab has been tested⁹⁸ with moderate success.

There is no established specific therapy for DN. ACE inhibitors or ARBs are used to lower hypertension, which has been shown to substantially decelerate the progression of DN. ACE inhibitors and ARB also have direct renoprotective effects as described above^{99, 100}. For mild DN, reduction of dietary protein intake is encouraged as well as improved glycaemic control^{83, 100}.

ACTH treatment

The use of ACTH as treatment of nephrotic patients was started in the 1950s^{101, 102}, but was replaced as oral steroids became available. In 1999 however, ACTH treatment had a revival when Berg et al found that a synthetic ACTH peptide analog had beneficial effects on the glomerular function of patients with membranous nephropathy⁴. The treatment was designed to lower the serum lipoproteins and the renoprotective effects were discovered by chance.

Since then, a number of studies have been performed on nephrotic patients with different glomerulopathies¹⁰³. In the United States, a purified ACTH-based gel formulation had FDA approval for treatment of nephrotic syndrome and a retrospective trial was constructed to establish its effects¹⁰⁴. A number of clinical studies have since been performed in which ACTH gel is being tested on patients with a variety of nephrotic syndromes⁹⁷. However, few randomized controlled studies have been performed and the role of ACTH in the treatment of nephrotic syndrome is yet to be defined.

ACTH treatment stimulates cortisol release, which is coupled to some side effects described in the different studies. Among the side effects reported was disturbed night sleep, fluid retention⁴, refractory edema, severe insomnia⁶ and tanning of the skin¹⁰⁵. Elevated cortisol levels has also been correlated to complications in type-2 diabetics¹⁰⁶.

Adding to the different ACTH studies were the results published by our research group in 2010. In that study, the melanocortin 1 receptor (MC1R) was found in human kidney biopsies co-localizing with the podocyte marker synaptopodin⁵. Treatment of rats with Passive Heymann Nephritis (PHN) using MC1R specific peptide agonists was successful in reducing proteinuria and improving glomerular morphology. On the basis of these findings, the ACTH induced stimulation of MC1R was hypothesized as the main mechanism behind the ameliorating effects of ACTH⁵.

1.4 The melanocortin receptors

To date, five melanocortin receptors (MCRs) have been detected and characterized, MCR 1-5. Their genes are intronless and they belong to the family of G-coupled receptors (GPCRs)¹⁰⁷. When stimulated with ligands, the MCRs activates adenylate cyclase (AC), which in turn forms cyclic adenosine monophosphate (cAMP)¹⁰⁸.

There is a number of naturally occurring agonists to the MCRs, which are called melanocyte-stimulating hormones (α - β - and γ -MSH) as well as ACTH. These ligands are all derived from posttranslational modifications of the proopiomelanocortin (POMC) gene transcript¹⁰⁹.

MC1R is highly expressed in melanocytes and is coupled to pigmentation. A famous set of MC1R polymorphisms are the recessive loss of function mutations coupled to red hair and fair skin¹¹⁰ with increased risk of melanoma¹¹¹.

MC2R is primarily expressed in the adrenal cortex and only responds to stimulation by ACTH¹¹² It is an important mediator of the cortisol release following ACTH stimulation^{113,114}.

MC3R has been detected in brain, placenta, heart and gut tissue. In addition to activating the cAMP pathway, MC3R also activates the inositol phospholipid signal transduction pathway¹¹⁵. MC3R is proposed to be connected to energy homeostasis, and a mutation in the MC3R associated with severe obesity has been identified¹¹⁶.

MC4R is present in mouse and human genitalia, hypothalamus, brain stem and pelvic ganglion. It is believed to be associated with energy homeostasis as well as sexual function and behavior¹¹⁷.

MC5R is widely expressed in both central and peripheral exocrine glands and tissues. It is involved in thermoregulation and functions of the exocrine glands ¹¹⁸.

1.4.1 Melanocortin 1 receptor

The melanocortin 1 receptor (MC1R) has a role in skin pigmentation and is highly expressed in melanocytes. MC1R has been extensively studied in melanocytes where it is responsible for the switch in pigmentation synthesis, from yellow or red pheomelanin to brown or black eumelanin ^{108, 119} (Figure 6). MC1R is an integral GPCR membrane protein consisting of 7 transmembrane fragments with an extracellular N-terminus and an intracellular C-terminus ¹²⁰.

Human MC1R consists of 317 amino compared to 315 in the mouse gene. There is only 76% sequence homology between the two species ¹²¹. Human MC1R is involved in tanning, it is much more sensitive to stimulation, and has a lower expression in skin, than mouse MC1R which is important for fur pigmentation ¹²².

Stimulation of the MC1R has been reported to induce a number of signaling events (Figure 6). Stimulation with the ligand α -MSH increases cAMP levels and also increases catalase activity, resulting in decreased ROS levels and a reduction of oxidative stress ¹²³⁻¹²⁵. Protection and repair of DNA damage in a protein kinase A (PKA)-dependent pathway has also been demonstrated ^{124, 126}. Secondary signaling includes increased levels of cAMP response element-binding protein (CREB) ¹²⁷, as well as activation of extracellular-signal-regulated kinases (ERK1/2) ¹²⁸. The MC1R also undergoes desensitization and internalization following stimulation ¹²⁹.

MC1R has been reported to have anti-inflammatory effects ^{130 131}, and is present in immune cells, ^{130, 132, 133} B-lymphocytes, natural killer cells and cytotoxic T cells ¹³⁴. Stimulation of MC1R with α -MSH suppresses activation of the inflammatory transcription factor nuclear factor kappa-light-chain-enhancer of activated B (NF- κ B) ¹³⁵, further adding evidence to the anti-inflammatory effects of MC1R.

1.4.2 Melanocortin 1 receptor agonists

Several different agonists are capable of activating MC1R ^{121, 136}. There is a strongly conserved amino acid sequence found in ACTH and all MSHs, which is His-Phe-Arg-Trp (HFRW) ¹³⁷. This sequence is essential for receptor activation. Different synthetic peptides have also been described and

one of the most interesting peptides is MS05, which is highly selective for MC1R¹³⁸. In addition, the NDP-MSH peptide is notable, since it is extremely potent in activation of all different MCRs¹³⁹, except for MC2R¹²¹.

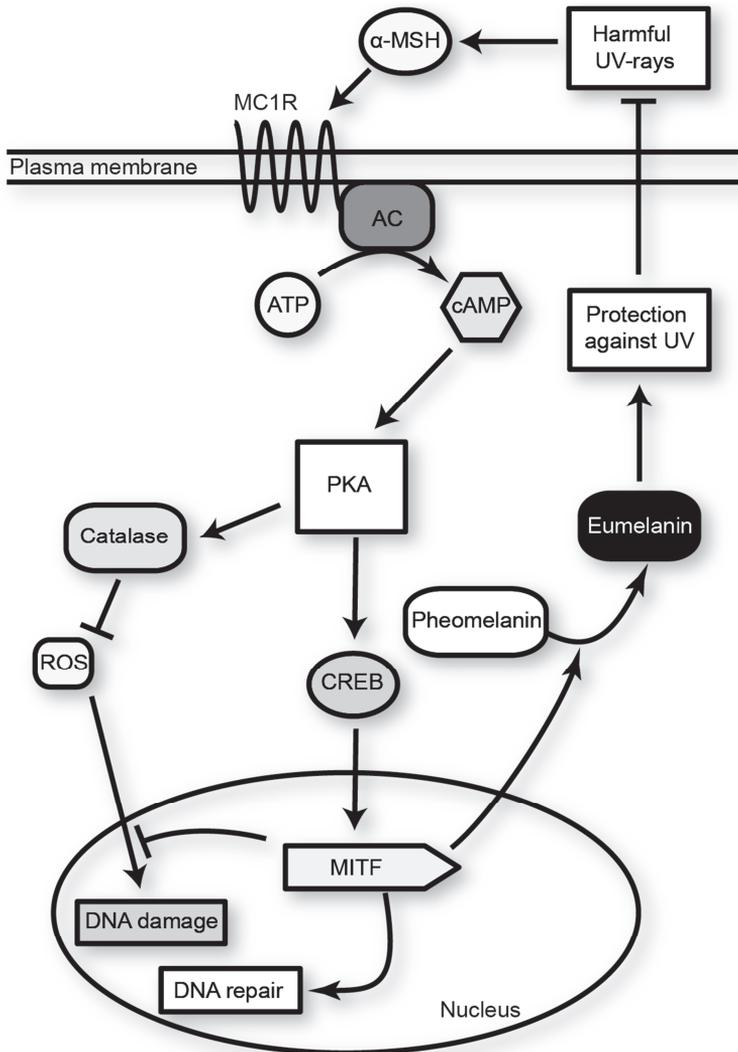


Figure 6. Schematic image of Melanocortin 1 receptor (MC1R) signaling pathway in melanocytes. Stimulation of MC1R with the natural agonist α -MSH induces a cascade of signals to synthesize pigment (eumelanin) and protect from UV-induced damage. MC1R stimulation also decrease levels of reactive oxygen species (ROS) associated with DNA damage. AC: Adenylate cyclase, ATP: adenosine triphosphate, cAMP: cyclic adenosine monophosphate, CREB: cAMP response element, MITF: Microphthalmia-associated transcription factor, PKA: protein kinase A.

In 2003, Herpin et al synthesized a non-peptide small molecule MC1R agonist with excellent selectivity. The compound was designed to mimic the HFRW core of the MSHs and was named BMS-470539. In all subsequent references to BMS-470539 in this thesis, it will be referred to as *MC1R-a*. The agonist MC1R-a was found to be avidly selective in activation of MC1R, although not as potent as NDP-MSH. MC1R-a also possesses anti-inflammatory properties, protects against LPS-induced cytokine accumulation, decreases tumor necrosis factor alpha (TNF- α) levels and prevents leukocyte infiltration in mice^{130, 132, 133}. The facts that MC1R-a is a small molecule with extreme selectivity towards MC1R and has reasonable potency are qualities that make it an interesting drug prospect.

2 AIM

The aims of this thesis have been to identify the intracellular signaling following MC1R activation and its downstream effects in different experimental nephropathies.

The specific aims of the papers included in this thesis:

I. Effects of Melanocortin 1 Receptor Agonists in Experimental Nephropathies

The aims of the first paper were to test MC1R agonists on *in vivo* nephrotic models. The models used were *Passive Heymann Nephritis* (PHN) in rats, and adriamycin nephropathy (AN) in mice.

II. Melanocortin 1 Receptor Agonist Protects Podocytes Through Catalase and RhoA Activation

The aims of the second paper were to determine MC1R signaling pathways in cultured podocytes using a MC1R overexpression model. Further aims included determining downstream and protective effects of MC1R activation against the puromycin aminonucleoside (puromycin) model.

III. Melanocortin 1 Receptor Activation Influences Podocyte Cytoskeletal Dynamics

The aims of the third paper were to build on the findings from paper II and examine the connection of MC1R and the podocyte cytoskeleton by using a phosphoproteomic approach. In addition, the protective effects of MC1R stimulation and the constitutively active MC1R mutant E92K were examined in the protamine sulfate (PS) model of cytoskeletal rearrangement.

3 METHODS

This section describes the different methods and experimental strategies used in the studies. The purpose is to highlight some of the strengths and weaknesses of the various methods and motivate why they were chosen. Detailed descriptions of experimental protocols are given in the respective papers.

3.1 Animal studies

Mice and rats as model organisms

There are a number of reasons to use animal models instead of research on humans, such as safety aspects, ethical considerations, and the need for highly controlled and standardized conditions. Animal studies can also be more time and cost effective and are very useful for performing systematic research. Clinical research on humans is usually performed by cohort studies, but more directed studies are often limited to costly, tightly regulated medicinal trials. Even though there are differences between humans and rodents in terms of physiology and metabolism, there are also a lot of similarities. The genetical, biological and behavioral characteristics of mice and rats closely resemble those of humans and there are many well-established experimental models resembling human diseases available for study¹⁴⁰.

Ethical statement

Approval was obtained from the Gothenburg Ethical Board for Animal Experiments at the Swedish Board of Agriculture prior to all animal experiments. For paper I, the ethical permits are numbered 237-2009 and 124-2012.

Housing and surgery

The animals were housed in rooms with 12-hour dark-light cycle and had unrestricted access to standard food and water. Prior to and during surgery, the animals were anaesthetized by inhalation of an isoflurane-air mixture. After operative procedures, a pain reliever was administered to reduce effects of stress on the study parameters.

3.1.1 *In vivo* nephrotic models

There are many different models to simulate human nephrotic syndrome in laboratory animals *in vivo*¹⁴⁰. In this thesis, two commonly studied models of nephrotic syndrome have been utilized to explore the mechanisms and beneficial effects coupled to MC1R stimulation. A rat model of Passive Heymann Nephritis (PHN), which is a model resembling human MN and adriamycin-induced nephropathy (AN) in mice, which is a model of human FSGS were utilized and are described in detail in paper I.

Experimental Membranous Nephropathy

PHN was first described in 1959 and is an experimental nephrotic rat model¹⁴¹. The model has since been thoroughly characterized and is widely used to model human MN¹⁴². Injection of anti-Fx1A antibody intravenously into the rat induces the disease symptoms, which primarily are characterized by subepithelial IgG-deposits in the glomeruli. The symptoms include lowered glomerular filtration rate (GFR), proteinuria, decreased serum levels of albumin, increased serum levels of creatinine, increased oxidative stress, podocyte foot process effacement and “spike” formation in GBM¹⁴³. The antigen responsible for the IgG deposit formation is megalin¹⁴⁴, a low-density lipoprotein (LDL)-receptor glycoprotein expressed in the podocyte foot-processes¹⁴⁵.

PHN disease progression is characterized by the fast appearance of subepithelial IgG-deposits 3-5 days after anti-Fx1A injection, whereas proteinuria peaks 2 weeks post anti-Fx1A-injection. Proteinuria persists in the animals if not treated, which enables a sustained and stable disease model. A downside associated with PHN is the slow disease progression; it takes a couple of weeks for the animals to exhibit detectable proteinuria.

The PHN model was assessed prior to the studies in paper I by a dose-response study, in which two subsequent injections of Anti-Fx1A IgG were found to induce a nephrotic syndrome with stable proteinuria⁵. In paper I, PHN experiments were performed on male Sprague Dawley rats. Anti-Fx1A IgG antibody was injected into the tail vein at day 0 followed by a booster dose at day 7 to induce PHN, whereas controls received sterile saline.

Experimental Focal Segmental Glomerulosclerosis

Adriamycin induced nephropathy (AN) is an experimental model of human FSGS, which was at first developed and tested on rats¹⁴⁶. The AN model has since also been described in mice¹⁴⁷. Notably, AN does not cause renal failure in all mice strains, some are resistant but not BALB/c mice^{148, 149}. The

symptoms include proteinuria, decreased serum levels of albumin and increased serum levels of creatinine, oxidative stress and progressive renal injury followed by glomerulosclerosis.

The AN model is quick and the disease symptoms, most notably proteinuria, are visible already after a couple of days. The mode of action in the AN model, however, has not been fully understood, but an increase in ROS levels has been detected¹⁵⁰ and the β -Catenin scaffolding protein has been shown to mediate the injury to the podocytes¹⁵¹. A drawback with the model is that the mice normally are heavily affected; they lose appetite and quickly lose weight. However, in a previous study from our research group¹⁴⁸ we showed that such changes could be avoided by giving intraperitoneal injections twice daily of glucose-electrolyte solutions. Indeed, with such procedures body weight can be maintained and the health status of the animals can be restored¹⁴⁸. The severity of FSGS can be hard to quantify since the lesions are focal and segmental, i.e. certain sections of glomeruli are affected while others are not.

Male BALB/c mice were used in the AN model. Prior to the experiments, the optimal dosage of adriamycin required to induce a stable nephropathy was determined by a dose-response study. AN was induced at day 0 by a tail vein injection of adriamycin and an equal amount of saline was given to controls.

3.1.2 Treatment with Melanocortin 1 Receptor agonists

The aim of paper I, was to determine the treatment effects of MC1R stimulation in the two different models of nephrotic syndrome. Because of differences in the model systems, the treatment regimens had to be tailored for the animals and therefore differed.

Experimental Membranous Nephropathy

Two weeks after induction of PHN in rats, treatment with MS05 was started via subcutaneous injection or an osmotic pump placed subcutaneously in the neck. The osmotic pumps are a great way to administer treatment for a long time without repeated injections and stress. A drawback associated with use of osmotic pumps is that not all substances are compatible with the pumps. We treated the animals for four weeks and the osmotic pumps were replaced two weeks into the treatment. Weight was recorded and spot urine samples were collected twice a week, analyses were based on weekly observations of the animals. The studies were continued for two additional weeks after the

end of treatment and the rats were terminated and kidneys were harvested for further analysis.

Experimental Focal Segmental Glomerulosclerosis

Treatment with either BMS-470539 (MC1R-a) or α -MSH was started one day prior to adriamycin injection, administered either via osmotic pumps subcutaneously placed in the neck or via daily intraperitoneal injections. Intraperitoneal injection is an easy, direct way to administer drugs. It does, however, entail repeated stress to the animals, but is easier and faster than repeated intravenous injections. Controls were given vehicle only or adriamycin and vehicle. Mice were given daily intraperitoneal injections of isotonic glucose-electrolyte solution from day 1 to 11 to prevent weight loss. Body weight measurements were recorded and spot urine samples collected daily. The mice were terminated during days 7-12 and kidneys were harvested for further analyses.

3.1.3 Investigation of Nephrotic Syndrome

The two experimental models induces nephrotic syndrome in mice and rats respectively. To be able to follow the severity and effects of treatment of the nephrotic syndrome, we chose a number of parameters for analysis. The objective of paper I was to determine the effects of MC1R stimulation on parameters such as proteinuria, blood urea nitrogen (BUN) and to describe the kidneys morphologically.

Measurement of proteinuria

Spot urine was collected and formed the basis for the examination of kidney function in mice and rats. By following proteinuria over time, we could analyze the severity of the nephrotic model as well as the effects of treatment.

Albumin levels in rat urine were analyzed using a Rat Albumin Elisa Quantitation Kit and urine creatinine concentrations were corrected for with the Jaffé reaction using standards. Mice urine albumin was analyzed using the Albuwell M ELISA Kit and creatinine concentration was corrected for by using the Creatinine Companion Kit. Concentrations were determined by measuring optical density on a Spectra max plus reader.

The collection of urine and subsequent analysis of proteinuria is a non-invasive method, which is good for examining changes in kidney function over time. It does not require any special equipment or handling of the animals. The only drawbacks are that it can be difficult to get the animals to urinate during stress.

Blood Urea Nitrogen

Blood urea nitrogen (BUN) is a metabolite of protein breakdown and measurable in serum. Increased BUN serum levels are likely to be correlated to impaired kidney function and decrease in GFR. BUN serum measurement is a well-documented and useful analysis to determine kidney function. However, it is only possible to measure by analyzing blood samples, therefore we could only obtain a value at the end-point of the study.

Plasma from mice was analyzed by taking blood from the caval vein prior to termination, 8 or 10 days after induction of AN. Blood samples were centrifuged and plasma was frozen and stored at -20°C. BUN was measured in the plasma with the QuantiChrom™ Urea Assay Kit.

Gene expression

Gene expression analysis can be used in *in vivo* models to analyze changes in the expression of a certain gene, either following treatment or as a result of damaging stimuli. The analysis of gene expression patterns are standardized, easily performed analyses, but expression analysis of a gene in the glomerulus requires access to the entire kidney since the material is so sparse, which was only made available upon termination of the animals.

Mouse glomeruli were isolated from the mouse kidneys through a sieving process. The glomerular fraction was then prepared for analysis by Real-time reverse transcriptase PCR (RT-PCR) and changes in expression of the MC1R gene was examined in the mouse model of AN to determine the effects of adriamycin and treatment with MC1R agonists.

Morphological analysis

Changes of glomerular morphology are indicators of disease progression and severity. Podocyte foot process depletion or fusion are typical features of AN and are readily analyzed by transmission electron microscopy (TEM) micrographs¹⁴⁷. TEM analysis of the morphology is a costly method, but the micrographs obtained provide reliable information about the morphology of the investigated tissue.

Following termination of the animals, the collected kidneys were prepared for morphological analysis. The renal vein and artery were clamped and the kidney was fixed by injection of Karnovsky's fixative. The kidneys were then sliced into mm-slices and processed according to standard procedure¹⁵². Micrographs of the slides were taken with a Leo 912AB Omega electron microscope and were examined by a pathologist in a blinded fashion. 15

glomeruli from each group were used; podocyte foot processes were counted along 10 μm of the GBM in 2-5 places of the glomeruli.

3.2 Cell culture

In vitro cell culture is a useful tool that allows for more detailed and convenient in-depth analyses of intracellular mechanisms. It is easy to modify, purify and examine proteins of choice. Cellular localization and co-localization of different proteins are possible to examine and there are many methods for overexpression of interesting proteins. However, *in vitro* studies have their drawbacks and cells in culture may react differently to stimulation since the presence of and interplay with other cell types and tissues are not easily mimicked in cell culture. It might be challenging to extrapolate findings and relate them to the model organism.

In papers II and III, culturing of immortalized mouse podocytes was used to study intracellular signaling pathways, downstream effects and coupling of MC1R activation to the cytoskeleton. In these experiments MC1R was overexpressed for amplification of the response to MC1R activation and for easier determination of the downstream effects. In addition, it allowed for analysis of MC1R-mediated protection against harmful stimuli and connection between the cytoskeleton and nephrotoxic models.

The main part of this thesis work (papers II and III) was performed *in vitro* with an immortalized mouse podocyte cell line, a kind gift from Professor Peter Mundel at Massachusetts General Hospital, Boston, MA, USA.

3.2.1 Immortalized podocytes

Isolated primary human podocytes can only be obtained from patient biopsies and are so highly differentiated that they cannot be cultured without previous modification. Primary cultures are usually limited in the number of passages they can grow. Passaging also changes them over time; primary cells in culture become less and less similar to the cells they originate from with every new passage. Even after immortalization these cells are challenging to maintain in culture with increasing risks of de-differentiation and loss of specific podocyte markers such as nephrin, podocin, Wilm's tumor 1 (WT-1) and synaptopodin¹⁵³.

In 1997, an immortalized mouse podocyte cell line was established by Mundel and the technique has since been widely used for *in vitro* podocyte experiments¹⁵⁴. The immortalized podocyte cell line is derived from isolated

glomeruli of the H-2K^b-tsA58 transgenic mouse, which express the temperature sensitive SV40 large tumor antigen TAg¹⁵⁵. Interferon-gamma (IFN- γ) and temperature is used to control proliferation and cells derived from the H-2K^b-tsA58 transgenic mouse subjected to IFN- γ do proliferate under permissive conditions at 33°C. When thermo-shifted into 37°C without IFN- γ , the cells proliferation stops and they differentiate into mature cells.

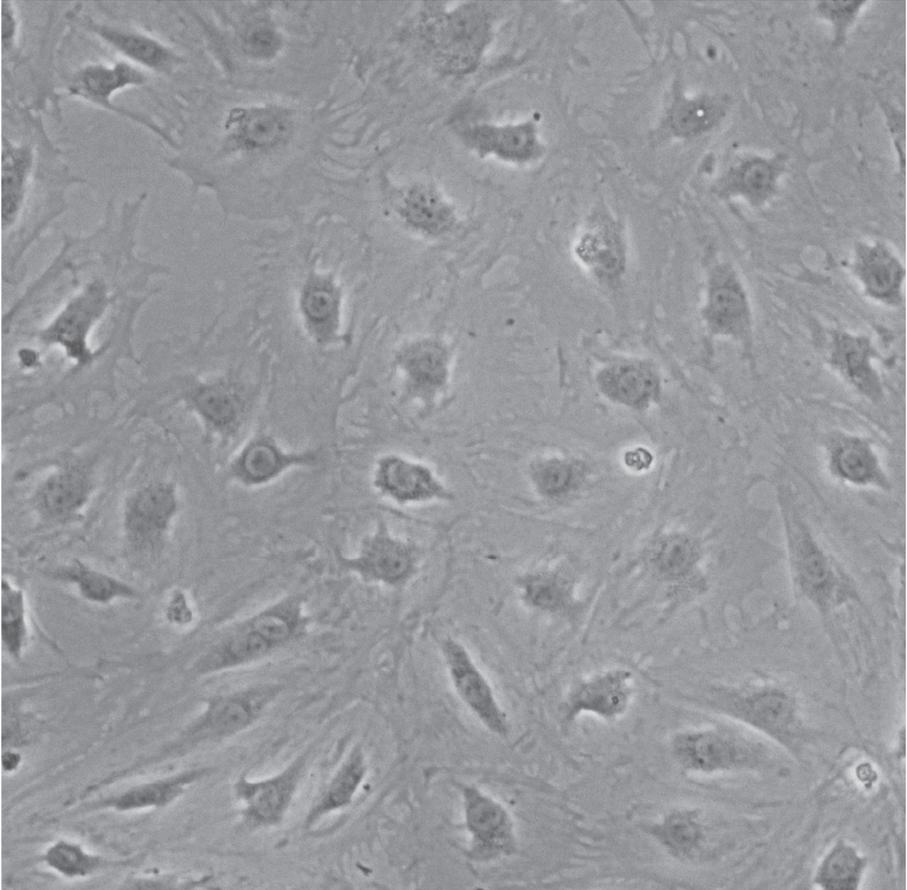


Figure 7. Immortalized, differentiated mouse podocytes in cell culture growing on a collagen coated surface.

The podocytes are cultured in two steps. Primarily, they are cultivated during permissive conditions, in RPMI 1640 medium supplemented with 10 % fetal bovine serum (FBS) and antibiotics at 33°C with addition of IFN- γ . This allows the podocytes to proliferate and grow in a non-differentiated phenotype. After sub-cultivation by trypsinization and seeding into different cell culture vessels, podocytes are thermo-shifted into 37°C and cultured in

non-permissive conditions without IFN- γ . After thermo-shift they are growth-arrested and differentiate into mature podocytes over the course of 6-10 days (Figure 7). Fully differentiated podocytes in cell culture exhibit podocyte markers such as nephrin, synaptopodin, and WT-1¹⁵⁶. The immortalized podocytes also present a slit-diaphragm like structure²⁴ as well as actin stress fibers¹⁵⁴. They do however not exhibit foot processes. In addition to the mouse podocytes, there is a human immortalized cell line established by Saleem et al by retroviral transfection of isolated primary podocytes with the SV40 large tumor antigen. These human podocytes express nephrin and podocin¹⁵⁷.

There are some pitfalls associated with culturing of the immortalized mouse podocytes. Most notably they are confluence-sensitive and de-differentiate if left to grow to more than 90 % confluency before thermo-shift.

3.2.2 DNA constructs and molecular cloning

In papers II and III, recombinant DNA vectors was created by subcloning cDNAs encoding different proteins into vectors used for overexpression of the proteins of interest. All of the cloned recombinant proteins are fused together with a fluorescent protein; either enhanced green fluorescent protein (EGFP) or mCherry. This enables easy detection of the protein by fluorescence microscopy.

In paper II, DNA encoding human MC1R was subcloned from a pEGFP-N1 vector (kindly provided by Professor Richard Sturm at the University of Queensland, Brisbane, Australia) into a VVPW overexpression vector in conjunction with a sequence encoding the EGFP protein, resulting in a vector encoding for a hMC1R-EGFP fusion protein.

In paper III, mouse MC1R and the mouse MC1R-E92K mutant (both provided in pcDNA3.1+ vectors by Professor Mette Rosenkilde at the University of Copenhagen, 7TM Pharma, Denmark) were subcloned into different VVPW vectors encoding either EGFP or mCherry.

The LifeAct peptide described in paper III was obtained from the Mundel lab in the VVPW vector. The VVPW-LifeAct-GFP vector enabled the visualization of F-actin in living cultured podocytes by overexpression of the LifeAct-GFP fusion protein.

Table 2. Vectors used for lentiviral overexpression of recombinant proteins in cultured podocytes

| Vector | Paper | Applications |
|-------------------------|--------------|---|
| VVPW-EGFP | II, III | Virus control |
| VVPW-hMC1R-EGFP | II | cAMP, AlamarBlue, Catalase activity, ROS levels, RhoA activity, Actin stress fibers, WB |
| VVPW-mMC1R-EGFP | III | cAMP, MS |
| VVPW-mMC1R-E92K-EGFP | III | cAMP |
| VVPW-mCherry | III | PS, LifeAct |
| VVPW-mMC1R-mCherry | III | PS, LifeAct |
| VVPW-mMC1R-E92K-mCherry | III | PS, LifeAct |
| VVPW-LifeAct-GFP | III | PS, LifeAct |

3.2.3 E92K mutant

Mutations of the E92 residue in mouse MC1R (E94 in human) produces constitutive activity of the receptor¹⁵⁸. In 2011 Benned-Jensen et al. further described the E to K mutation at residue 92 in murine MC1R and residue 94 in human¹⁵⁹. They found that the E92K mutation induces an active conformational state coupled to constitutive activity, which was distinct from the activity and conformational changes induced by α -MSH.

Effects on the MC1R in terms of cAMP accumulation and activation of the transcription factor CREB were detected as well as recruitment of β -arrestin. However, increase in phosphorylation of ERK1/2 was not detected nor did the constitutive activity increase internalization of the receptor, which is coupled to MC1R activation with ligands such as α -MSH¹⁵⁹. This indicates that the constitutive signaling resulting from the E92K mutation does not activate all pathways associated with MC1R signaling.

3.2.4 Lentivirus

Lentiviral gene transfer methods are used in cell culture because of the lentiviral ability of reverse transcription. Infection of non-replicating cells with lentiviruses can be used to introduce recombinant DNA into the genome of the host cells. The transduction is stable and is used to either overexpress proteins or to knock down protein expression by using shRNA.

The lentivirus used in papers II and III is not capable of self-replication, the virus cannot replicate after infection of target cells. The production of infectious virus requires three different vectors and a packaging host.

Production of lentivirus

A packaging cell line was required to produce large amounts of lentivirus for protein overexpression. The HEK293T cell line is commonly used since it is easily transfected and capable of producing large amounts of lentiviral particles.

HEK293T cells were cultivated in high glucose Dulbecco's modified Eagle's medium (DMEM), supplemented with 10 % FBS and antibiotics. Cells at 80 % confluence were transfected with the vector encoding the protein of interest as well as the packaging plasmid pCMV dR8.91 and the envelope plasmid pCMV-VSV-G in a 3:2:1 ratio^{160, 161}. The transfection was carried out in antibiotic free DMEM with the addition of FuGene® transfection reagent. Medium containing virus particles was collected and stored at -80°C.

Lentiviral overexpression

Both mouse and human MC1R were overexpressed in podocytes. In paper III, the LifeAct-GFP plasmid was overexpressed in addition to mMC1R-mCherry. The different vectors used for overexpression in papers II and III are summarized in Table 2.

The method of lentiviral overexpression is particularly useful in podocytes, because the differentiated podocytes do not proliferate. The lentiviral transduction induces stable protein expression in the podocytes. The virus itself might have some negative effects on the cells, since it requires entry through the membrane for incorporation of DNA into the genome, but titrating the virus concentration and using a virus control can be used to avoid this.

Medium containing lentiviral particles was added to the podocytes. The virus medium was titrated by determining overexpression efficiency using real-time RT-PCR analysis of the MC1R gene expression. The lentiviral mediated overexpression of the different constructs were successful and produced stable levels of the protein constructs that were confirmed with Western blot, real-time RT-PCR and confocal microscopy (paper II).

The overexpression efficiency of the LifeAct-GFP peptide was determined by fluorescence microscopy. A virus titer, which induced a stable expression

resulting in clearly visible stress fibers in the confocal fluorescence microscope without signs of virus toxicity, was used.

Table 3. MC1R gene expression levels in WT and transduced podocytes. Values presented as mean $C_T \pm SEM$.

| Vector | Mouse MC1R C_T | Human MC1R C_T |
|-------------------------|------------------|------------------|
| WT (no vector) | 32.0 \pm 0.3 | - |
| VVPW-EGFP | 32.4 \pm 0.4 | - |
| VVPW-hMC1R-EGFP | 31.8 \pm 0.3 | 24.2 \pm 0.5 |
| VVPW-mMC1R-EGFP | 21.0 \pm 0.1 | - |
| VVPW-mMC1R-E92K-EGFP | 21.9 \pm 0.1 | - |
| VVPW-mMC1R-mCherry | 20.7 \pm 0.1 | - |
| VVPW-mMC1R-E92K-mCherry | 20.8 \pm 0.1 | - |

3.2.5 *In vitro* nephrotic models

There are many different *in vitro* models that can be used to mimic disease conditions and to simulate nephrotic type injury to podocytes. One of the most important cellular changes observed in glomerular diseases is the flattening of foot-processes in podocytes. This is termed podocyte foot process effacement, and is a result of cytoskeletal rearrangement (or disruption). In cell culture, this is characterized by transition from actin stress-fibers to a more cortical actin cytoskeleton. Different nephrotoxic agents such as puromycin aminonucleoside (puromycin), adriamycin, protamine sulfate (PS) and lipopolysaccharides derived from bacteria (LPS) are *in vitro* models widely used to induce cytoskeletal rearrangement^{38, 162}. A problem when using nephrotoxic agents *in vitro* is that the effects do not fully mimic the *in vivo* situation. Some effects are observable, but not all.

Puromycin aminonucleoside

Puromycin aminonucleoside (puromycin) is a nephrotoxic agent used to induce a condition similar to MCD in rats¹⁶³. Puromycin does also induce cytoskeletal disruption, increased oxidative stress and decrease in viability of cultured podocytes¹⁶⁴. The rearrangements induced by *in vitro* administration of puromycin are dose-dependent and the effects usually appear after at least 24 h¹⁶⁵. Puromycin has been extensively studied both *in vivo* and *in vitro*, which makes it suitable for cell culture experiments.

Puromycin was administered to cultured podocytes in paper II according to different treatment regimens. To induce loss of stress fibers and cytoskeletal rearrangement or decrease viability, puromycin was added in different doses to the podocyte medium for 72 h.

Protamine sulfate

Protamine sulfate (PS) is a cationic peptide, described both *in vivo* and *in vitro*, which induces a nephrotic syndrome similar to puromycin in rats ¹⁶⁶, foot process effacement in mice ⁴⁶ and cytoskeletal rearrangement in podocytes *in vitro* ¹⁶⁵. PS causes fast *in vitro* loss of stress fibers in a TRPC5 dependent model ⁴⁶. PS has been extensively studied and one of the drawbacks of the model *in vitro* is that it is irreversible when administered in high doses.

PS was administered at a final concentration of 600 µg/ml for up to 60 m in cultured mouse podocytes overexpressing LifeAct, during which micrographs were obtained every 30 s using confocal fluorescence microscopy.

3.2.6 Melanocortin 1 receptor agonists

A number of different agonists were used to activate MC1R in podocytes. In paper II, MC1R-a and MS05 were used, and in paper III, only MC1R-a was used. MC1R-a was dissolved in dimethyl sulfoxide (DMSO), MS05 was dissolved in PBS and all agents were sterile-filtered prior to usage. MC1R-a was administered with 0.1 % DMSO. The agonists were chosen for their high specificity and selectivity towards the MC1R receptor ^{133, 138}. The different MC1R agonists were administered in a range of doses between 0.1 nM to 1 µM, and for different lengths of time depending on experiments.

Using several agonists for MC1R stimulation is important to be able to minimize spontaneous effects of a compound compared to the effects of MC1R stimulation. Some of the problems by using multiple agonists are differences in solubility and concentrations required to induce the same response effects.

3.2.7 *In vitro* readouts

To determine the effects of MC1R stimulation in podocytes *in vitro*, a number of different readouts were chosen for analysis in papers II and III.

Cyclic adenosine monophosphate

Upon activation of G-protein coupled receptors (GPCRs) including MC1R, increased intracellular levels of cyclic adenosine monophosphate (cAMP) is a

response to activation¹⁰⁸. The increased cAMP levels have downstream effects, such as stimulation of PKA dependent cascades¹⁶⁷. We analyzed changes in cAMP following MC1R activation in paper II and III. The effects of the constitutively active MC1R mutant E92K on cAMP concentrations were studied in paper III. Analysis of changes in cAMP level is interesting since it provides direct functional information. The elevation of cAMP is usually closely connected to activation of a GPCR. However, it does not provide information on which GPCR has been activated, and the analysis requires lysis of the cells.

Differentiated podocytes were subjected to different concentrations of MC1R-a in starvation medium with 0.1 % FBS in the presence of 3-isobutyl-1-methylxanthine (IBMX) for 30 min. The experiment was terminated by addition of lysis buffer and the intracellular cAMP content of the cells were analyzed with a cAMP-Screen® cAMP Immunoassay System. The agent forskolin was used as a positive control.

Catalase assay

Catalase is an antioxidative enzyme, which catalyzes the reaction of hydrogen peroxide into water and oxygen, leading to decreased levels of intracellular ROS¹²⁴. In paper II, the catalytic activity of catalase following MC1R-stimulation was examined. The effects of catalase inhibition were also studied with the use of the irreversible catalase inhibitor 3-amino-1, 2, 4-triazole (AMT)¹⁶⁸. Following the activity of an enzyme provides more reliable data than examination of the expression. The usage of the catalase activity kit was a fast and reliable method to determine changes in activity. The only problem with the method was that the buffer and lysis compositions were found to affect the results, and were therefore examined thoroughly before experiments.

Protein concentrations were determined from podocyte protein lysates subjected to 100 nM MC1R-a for up to 120 h. Equal amounts of proteins were then analyzed with the Amplex® Red Catalase Assay Kit.

Reactive oxygen species

Reactive oxygen species (ROS) is a collective label for chemically reactive oxygen-containing compounds. ROS convey important signaling in cells and is formed as a result of oxygen metabolism. However, during stress or disease conditions ROS levels can increase dramatically, resulting in significant damage to cells and cell structures. In paper II, the effects of MC1R stimulation on ROS levels were examined. Using a plate-reader based assay for ROS analysis has the advantages of being fast and allowing for

multiple measurements at the same time. The technique does, however, require suitable plates; the bottom of the plate has to be of a material suitable for fluorescence measurements with low background interference.

Podocytes were treated with 1 and 10 nM MC1R-a and the ROS levels were determined using a plate reader assay. CellROX® Deep Red reagent was added to the podocytes, in which the nuclei were simultaneously counterstained with NucBlue® Live ReadyProbes®. After 90 min incubation the relative CellROX fluorescence was examined with a SpectraMax i3 reader and normalized to the NucBlue fluorescence intensity.

RhoA

RhoA is a member of the family of small Rho-GTPases, and is coupled to the actin cytoskeleton where it promotes the formation of stress fibers⁴¹. The p190Rho-GAP is a GTPase-activating protein, which inhibits the activity of RhoA. The p190Rho-GAP activity is regulated upon pTyr-phosphorylation of p190Rho-GAP⁵¹. Co-immunoprecipitation and subsequent western blot was performed in paper II for p190Rho-GAP, analyzing the change in active (pTyr-phosphorylated) protein. The analysis of p190Rho-GAP activity is robust because it relies on analysis of the phosphorylated form of p190Rho-GAP on western blot. However, co-immunoprecipitation relies on a good primary antibody.

Podocytes subjected to 1 nM MC1R-a for 72 h were lysed and p190Rho-GAP was immunoprecipitated using an anti-p190Rho-GAP antibody. Protein G dynabeads were added to the lysate and incubated. The elute was analyzed on western blot using antibodies to detect both the total amount and the tyrosine-phosphorylated form of the p190Rho-GAP protein.

Alamar Blue measured viability

AlamarBlue® assay is a non-toxic, repeatable method to measure viability of cells in culture. Resazurin is a cell permeable compound that is converted into the fluorescent compound resorufin upon entry into cells. The amount of reduced resorufin fluorescence can then be compared to control, and the effects of toxic compounds assessed. The advantages of using AlamarBlue are that it is a non-toxic and non-invasive method. But the readout does only give information about the general metabolic status of the cells. In paper II, AlamarBlue assays were used to determine the protective effect of MC1R stimulation on podocytes subjected to puromycin.

Differentiated podocytes were pre-incubated for 72 h with 10 nM MC1R-a or MS05, and then 10 µg/ml puromycin was added for 72 h. The cells were then

allowed to recover for 120 h and viability measurements were conducted at day 0, 3 and 8.

3.2.8 Actin cytoskeleton

The actin cytoskeleton is an essential part of every eukaryotic cell, and changes in the cytoskeletal dynamics are responsible for cellular mechanics such as phenotype, morphology, migration and resilience to mechanic stress¹⁶⁹. In the podocytes the actin cytoskeleton creates the framework for foot processes and an intact filtration barrier.

The actin cytoskeleton was visualized in cultured podocytes by fixation of podocytes with paraformaldehyde (PFA) and subsequent staining with rhodamine-phalloidin.

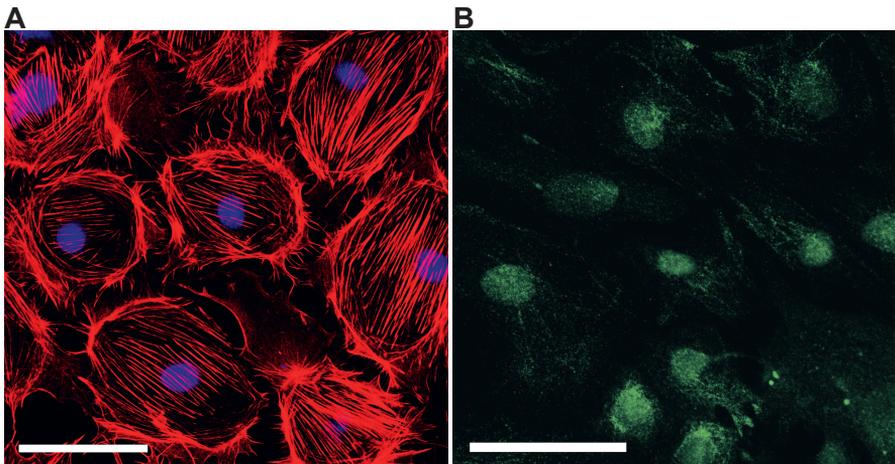


Figure 8. Confocal micrographs of podocytes in culture. (A): Podocytes stained with rhodamine-phalloidin to visualize actin stress fibers (red) and nuclear stain DAPI (blue). (B): Podocytes stained with an anti-MC1R antibody and visualized by a secondary Alexa 488 antibody. The melanocortin 1 receptor is localized in the plasma membrane, cytoplasm and nucleus of podocytes. Scale bars represent 50 μm .

Stress fiber quantification

In podocytes fixated and stained with rhodamine-phalloidin the stress-fibers of the actin cytoskeleton are clearly visible (Figure 8). Podocytes subjected to a harmful agent, such as puromycin or PS display signs of cytoskeletal rearrangement and loss of stress fibers. Healthy, non-affected podocytes, however, contain thick parallel stress fibers spanning over the entire cytosol.

In paper II, podocytes were pre-treated with MC1R-a, subjected to puromycin and allowed to recover prior to fixation with PFA. In addition to puromycin and MC1R-a, podocytes were also subjected to the catalase inhibitor AMT, the Rho-associated protein kinase (ROCK) inhibitor Y-27632 and also treated with α -MSH. For a list of treatment regimens, see Table 4. Fixated, rhodamine-phalloidin-stained podocytes were photographed in a blinded fashion with a Zeiss axioplan-2 fluorescence microscope. The micrographs were subsequently analyzed and podocytes were scored according to stress-fiber content. A podocyte with thick stress fibers spanning across the entire cytosol was scored as a healthy, unaffected podocyte, whereas a podocyte with broken, bent or diminished stress fibers was considered affected.

Staining of podocytes with rhodamine-phalloidin is a robust and well-tested method, but quantification of stress-fibers is a somewhat biased method. The researcher must be relied on to score podocytes uniformly according to appearance and occurrence of stress fibers. Anonymization of the fixated cells as well as the acquired photos is essential to guarantee reliable analyses.

*Table 4. Treatment regimens of podocytes in culture prior to fixation. The experiments were designed to determine the effects of puromycin on actin stress-fiber composition. * AMT was added before and after addition of puromycin. ** Y-27632 was added 90 min prior to fixation of the cells.*

| Pre-treatment 72h | Treatment 72h | Recovery 120h | Additional |
|------------------------------|--------------------------|--------------------------|-------------------|
| - | Puromycin | Medium | - |
| MC1R-a | MC1R-a + puromycin | Medium | - |
| MC1R-a | MC1R-a + puromycin | Medium | AMT 2 x 1 h * |
| MC1R-a | MC1R-a + puromycin | Medium | Y-27632 90 m ** |

LifeAct

LifeAct® is a peptide consisting of 17 amino acids which binds selectively to F-actin¹⁷⁰. When LifeAct fused to GFP is overexpressed in cells, it binds to the actin cytoskeleton and stress fibers, thereby allowing real-time analysis of the cytoskeleton in living cells. This enables analysis of changes in cytoskeletal modulation, stress fiber regulation and cell area⁴⁶. The opportunity to follow real-time events in living cells makes the method powerful, but it also has some limitations. The overexpression is usually not prominent in all cells, which results in that not all cells have detectable F-actin.

LifeAct-GFP was overexpressed in podocytes in paper III, which were treated with MC1R-a and subjected to PS. Micrographs of the podocytes were taken in 30 s intervals on a Zeiss Confocal LSM700 microscope. Fluorescence of LifeAct-GFP was quantified using Visiopharm® software and used to determine of stress-fiber retraction by PS and prevention of cytoskeletal rearrangement by MC1R stimulation.

3.3 Gene expression and protein analysis

Prior to examination of proteins or gene expression, samples need to be purified and quantified. The following methods are considered to be standard techniques and are described in greater detail in the corresponding papers (I, II and III).

3.3.1 Immunocytochemistry

Immunocytochemistry is used to determine protein expression and localization in fixated cells, *in vitro*. The method relies on antibodies against proteins of interest, which are used to stain fixated cells. A secondary antibody with affinity for the primary is then applied, often fused to a fluorescent small molecule or peptide. Immunocytochemistry was used to determine MC1R expression in podocytes.

Differentiated podocytes were fixated, permeabilized and incubated with antibodies against MC1R. Micrographs of the podocytes were obtained using a Zeiss Confocal LSM700 microscope. The expression and intracellular localization of endogenous MC1R was determined by analyzing the micrographs. In addition, overexpression of MC1R was determined both by analyzing the fluorescence signal of the fused EGFP protein as well as an antibody against MC1R.

3.3.2 Real-time RT-PCR

Gene expression analysis is used to examine gene expression or changes in mRNA levels corresponding to a gene of interest. It is a simple method, which gives reliable results. The mRNA levels do not prove anything about the amount of functional protein but provides information of changes in regulation. It was used in paper I to determine the mRNA levels of MC1R in the different nephrotic models. In paper II the gene expression of MC1R following puromycin stimulation of podocytes was examined. In paper II and III it was also used to determine virus titers and protein overexpression.

RNA was harvested and homogenized in RLT buffer and purified by the RNeasy Mini Protocol with DNase digestion. RNA quality was confirmed using a Standard Sensitivity Kit on Experion™, and RNA concentrations were determined using a NanoDrop spectrophotometer. Reverse transcription was performed on equal amounts of RNA with the High Capacity RNA-to-cDNA kit. The mRNA gene expression levels were quantified by analyzing 50 ng of the cDNA by TaqMan® real-time RT-PCR on the QuantStudio 7 Flex real-time RT-PCR system.

3.3.3 SDS-PAGE Western Blot

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) with subsequent analysis on western blot is a well-established standard laboratory method. In paper I, western blot was used to determine MC1R expression in mouse glomeruli and podocytes. In paper II overexpression of MC1R-EGFP was confirmed with western blot as well as p190Rho-GAP phosphorylation.

Prior to SDS-PAGE, the protein concentrations were measured by Bicinchoninic acid (BCA). Equal amounts of proteins were loaded into the wells of a polyacrylamide gel. The samples were run using SDS-PAGE and subsequently transferred to a membrane according to standard protocol. To analyze the samples by western blot, they were incubated with different antibodies for protein analysis. The protein bands were visualized using a secondary horseradish-peroxidase-conjugated antibody and Immuno-Star™ WesternC™ chemiluminescent kit in a Bio-Rad ChemiDoc™ XRS+ Imaging system.

3.4 Mass spectrometry

Mass spectrometry (MS) using an Orbitrap Fusion Tribrid mass spectrometer in combination with mass tandem tags (TMT) was performed in paper III to determine effects on the phosphoproteome of podocytes following MC1R stimulation. By using TMT labeling, the mass spectrometer provides the possibility to determine relative quantification of proteins in multiple samples simultaneously. The MS method with TMT labeling and database quantification used in paper III is a complex method that requires expertise to return data of good quality. It is however very sensitive and can return large amounts of data from only a small sample set.

In paper III, cell lysates from podocytes subjected to 10 nM MC1R-a for 15, 30 and 60 min and time-matched controls were collected and prepared for

LC-MS/MS analysis. Additional details regarding the protocol are described in paper III.

Sample preparation and phosphopeptide enrichment

Podocytes were homogenized in lysis buffer supplemented with PhosStop and total protein concentration was determined with a BCA assay. Up to 500 µg protein was used for the phosphopeptide analysis and 30 µg of protein was used for total protein quantification. The proteins were digested into peptides using trypsin and a previously described sample preparation method¹⁷¹.

The phosphopeptides were enriched using a TiO₂ Phosphopeptide Enrichment and Clean-Up Kit, where samples were applied to columns containing TiO₂. The TiO₂ retains the phosphopeptides during washing and the bound phosphopeptides can then be eluted and purified.

The eluted samples were then evaporated in a vacuum centrifuge prior to addition of isobaric mass tagging reagent TMT®. Each sample in a set was labeled with a unique TMT tag from an isobaric mass tag labeling kit. The same TMT labeling was performed for analysis of the total, unmodified peptides. Labeled samples in a set were pooled, concentrated and desalted.

LC-MS/MS analysis

The dried, desalted TMT-labeled samples were reconstituted in 0.1 % formic acid, 3 % acetonitrile and analyzed on an Orbitrap Fusion Tribrid mass spectrometer connected to an Easy-nLC1000. 2 µl of the samples were injected and separated using an analytical column. Following separation, ions were injected into the mass spectrometer and detected in the Orbitrap.

Tandem mass tags database quantification

Data files from the MS for each TMT set were merged to allow relative quantification. The peptides were identified using Proteome Discoverer v 1.4 and database searches were performed with the Mascot search engine using the *Mus musculus* Swissprot Database v April 2015. Quantification of the peptides was normalized using protein median of all peptides in each comparison, only the peptides unique to a given protein were analyzed in the relative quantification. This excluded peptides found in proteins of the same family or in other isoforms.

3.4.1 Ingenuity Pathway Analysis

The Ingenuity Pathway Analysis (IPA) software obtained from Qiagen is a tool used to analyze large amounts of data and to classify post-translational

modifications into cellular processes and pathways. The IPA tools are capable of analyzing changes in the phosphoproteome and determining the most significantly affected pathways. The IPA tools are very useful in examining changes in large data sets. However, the analysis does not say anything about the specific activities of different phosphopeptides and the results needs to be analyzed with this in mind. In paper III, the data obtained through mass spectrometry was analyzed through the IPA software.

The phosphopeptide data obtained from the MS was analyzed with the IPA tools. Cutoff was set to 15 %, depending on the margin of error of the MS system, indicating that differences of 15 % or more was considered as an increased phosphorylation or dephosphorylation of a peptide. The data was then run through a network analysis in the IPA software and the most significantly affected intracellular pathways were determined from changes in the phosphoproteome. The IPA software was also used to group proteins according to cellular functions and into pathways. Interaction maps of the significantly affected pathways were then produced, and interesting proteins and their respective phosphorylation sites were identified.

3.5 Statistical analysis

All results are presented as mean \pm SEM and all statistical analyses were performed using Prism 6 software if not otherwise stated.

The data processed in the IPA software was analyzed with a Fisher's exact test, $P < 0.001$ was considered statistically significant. Differences between groups were compared with a Student's T-test. When comparing more than 2 groups, one- or two-way ANOVA was used with Bonferroni's or Dunnett's post-hoc test. Differences in phosphorylation of p190Rho-GAP were determined with a ratio paired Student's T-test. Differences in albuminuria between groups in paper I were examined with the exact Mann-Whitney non-parametric test. $P < 0.05$ was considered statistically significant.

4 RESULTS AND DISCUSSION

This thesis is based on three papers. In paper I, the effects of Melanocortin 1 receptor (MC1R) stimulation is examined in *in vivo* models of nephrotic syndrome. In paper II, some of the intracellular mechanisms and pathways activated by MC1R stimulation are studied in podocytes. In paper III, further studies of the MC1R pathways and connection to the cytoskeleton are carried out in podocytes by a phosphoproteomic approach. In both papers II and III, the effects of MC1R stimulation are examined in different *in vitro* models of cytoskeletal rearrangement using nephrotoxic agents.

In this section, the results from papers I, II and III are summarized, presented and discussed in separate sections. A general discussion can be found in the end of the section.

4.1 Effects of Melanocortin 1 Receptor agonists in Experimental Nephropathies (Paper I)

In paper I, two animal models were used to induce nephrotic syndrome, namely Passive Heymann Nephritis (PHN) and adriamycin nephropathy (AN). The former is a model of human MN and the latter, resembles human FSGS. Proteinuria and morphological changes in the kidneys were examined during and after treatment.

4.1.1 Amelioration of Passive Heymann Nephritis

The PHN model caused proteinuria, which was apparent 2 weeks after induction of the disease model. The rat weights in the different treatment groups were not affected by induction of PHN and did not significantly differ at any time during the study.

Proteinuria in PHN rats was defined as urinary albumin to urinary creatinine ratio (UACR) in mg/g. The UACR increased less following 4 weeks of treatment with 100 µg/day of the MC1R agonist MS05 than it did in rats with untreated PHN. The improvement continued even after treatment withdrawal, and 5 weeks after treatment start the proteinuria was further reduced. This confirms earlier findings from our research group, which showed that treatment with a low dose of MS05 (10 µg/day) also decreased proteinuria in PHN rats⁵. Treatment of PHN in rats with the MC1R agonist MS05 was

earlier shown to improve podocyte morphology,⁵ but this was not examined in Paper I.

Discussion

The PHN model induces a peak proteinuric level 1-2 weeks after disease induction that persists if left untreated¹⁴². The PHN rats in this study showed decreasing proteinuria over time following the 2-week time point. This was in contrast to a previous study, where proteinuric levels varied over time⁵. Even though the proteinuric levels in the rats decreased overall, proteinuria in the PHN rats treated with the MC1R agonist MS05 was significantly lower compared to animals with untreated PHN. It would be of great interest to continue following PHN rats for an even longer period of time. This would allow examination of whether MC1R treatment can fully resolve the proteinuria and completely restore podocyte morphology. It would also be of interest to test the synthetic MC1R agonist MC1R-a in PHN rats to determine if it is superior or equivalent to the peptide MS05 for treating PHN in rats.

4.1.2 No effect on adriamycin nephropathy

The AN model rapidly caused proteinuria in BALB/c mice. The effect of adriamycin on proteinuria was measured after 7 days and found to be dose-dependent. Because of the broad range of proteinuria induced by AN, the high doses of 8 and 10 µg/kg were chosen since they resulted in a pronounced disease model and was consistent with earlier studies^{147, 172}. The proteinuria in the AN mice was also estimated as urinary albumin to urinary creatinine ratio (UACR) in mg/g.

In this model, treatment of the experimental nephrosis with the MC1R agonists MC1R-a and α -MSH did not significantly decrease UACR. If anything, MC1R-a treatment increased UACR levels in the 10 mg/kg AN group. In addition, all of the mice subjected to AN showed decreased appetite leading to weight loss over time.

BUN levels were measured in plasma samples taken from mice during the end of the study, at 8-10 days after AN induction. Mice in both groups (8 and 10 mg/kg adriamycin) had increased BUN serum values compared to normal BALB/c clinical values¹⁷³. There were no differences in BUN levels in either of the 8 or 10 mg/kg AN mice treated with MC1R-a or α -MSH compared to untreated.

The glomerular morphology and podocyte foot-processes from harvested kidneys were examined by TEM and quantified. The AN mice displayed pathological changes in the glomeruli, with apparent lesions, foot-process

effacement and flattening, whereas controls had healthy glomeruli with intact foot-processes. There was no significant improvement in podocyte morphology (in terms of number of foot-processes / 10 μm GBM) following the MC1R-a treatment (Figure 9). Because of the nature of the FSGS disease model, the mice showed both disrupted glomerular parts, with podocyte foot process effacement, as well as entirely unaffected parts.

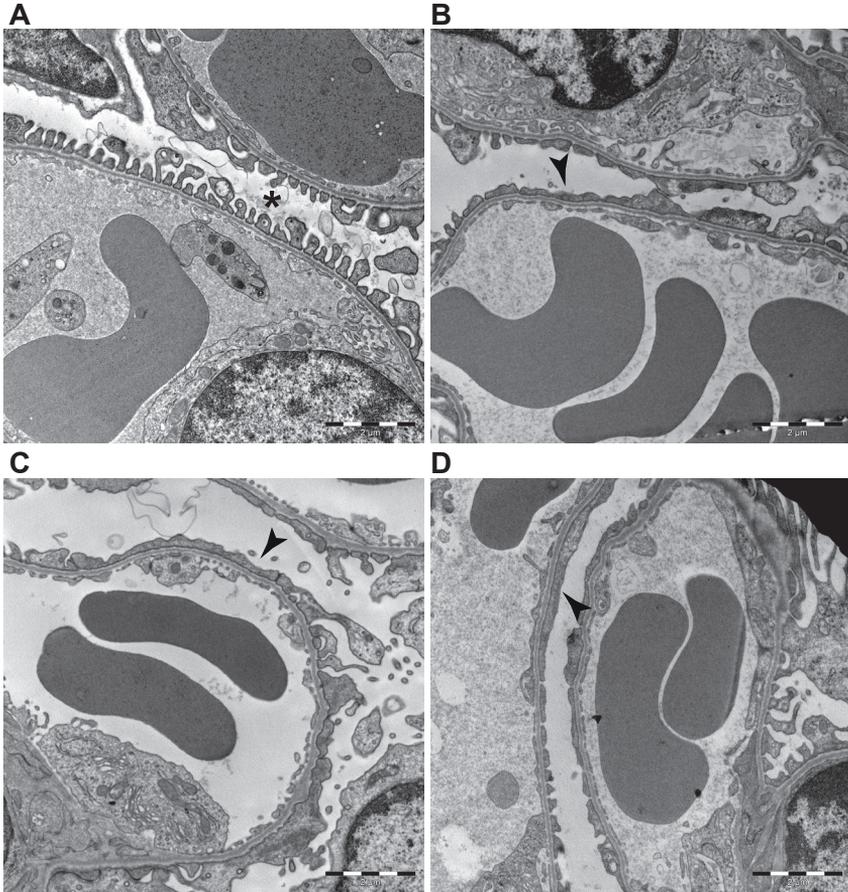


Figure 9. Transmission electron microscopy of glomerular morphology in mice. Glomerulus from control (A) and subjected to adriamycin (B), adriamycin and treatment with the melanocortin 1 receptor (MC1R) agonist MC1R-a (C), adriamycin treatment with the unspecific agonist α -MSH (D). Asterisk indicates normal podocyte foot process morphology, arrows denote regions where podocyte foot process effacement is detectable. MC1R stimulation was not able to restore pathological changes in morphology caused by adriamycin. Scale bars represent 2 μm .

Discussion

One possible explanation for the lack of beneficial effects of treatment is the fact that AN in mice is a rapid process, but FSGS in patients develops over a long time. It is noteworthy that treatment of FSGS in patients with Acthar® gel (containing ACTH) is only moderately successful, suggesting that FSGS may be resistant to MC1R-based treatment^{96, 103}. Previous studies have also shown that the complex signaling between podocytes and endothelial cells is disturbed in FSGS, and that endothelial cell injury precedes effects on podocytes^{174, 175}. Indeed, injury to the endothelial cells in FSGS might explain the lack of effect of MC1R treatment in our study. A second possibility is that we did not observe the effect of treatment long enough. The dose of ADR has earlier been shown to produce a stable model^{147, 172}, but more pronounced weight loss. Thus, provided that the mice could be substituted even more intensely to stabilize their weight, it would be interesting to examine the effects of MC1R stimulation for a longer time.

4.1.3 Expression in nephrotic models

In mice, the MC1R protein was detected using Western blot and the expression was compared to actin protein expression. This supports our previous study in which MC1R was detected in human tissue, co-localizing with the podocyte marker synaptopodin⁵.

The MC1R mRNA expression levels were examined in both mice and rats and were unchanged at termination, 8 weeks after PHN induction in the rats and day 7-12 for the mice.

Discussion

We hypothesized that the MC1R gene expression levels in podocytes could increase in the different disease models, owing to experiments in which puromycin (paper II) and adriamycin (Figure 10) stimulation increased MC1R gene expression levels in podocytes, *in vitro*. The observed increases in gene expression levels of active MC1R seem to act as a negative feedback loop, strengthening the cellular defenses against proteinuria.

Gene expression levels are subject to changes, some of which are fast and transient. If increased MC1R gene expression levels result from the induction of PHN or AN, the upregulation might have occurred earlier than examined in paper I. An interesting experiment would be to harvest the kidneys for gene expression analysis at different time points, either directly after induction of the disease model or at peak proteinuria.

4.1.4 Differences between treatments

As showed in paper I, there were differences in the effects of treatment between the two different models. The treatment of PHN in rats with different MC1R agonists had beneficial effects, such as decreased proteinuria even after treatment withdrawal. The mice with AN, on the other hand, were unaffected by MC1R agonist treatment, and proteinuria even increased in one of the AN groups.

There are some differences between the two models besides the obvious in terms of species (mice vs. rats). In the PHN model it takes longer time for the animals to develop proteinuria and in contrast to the mice, the rats are not affected in terms of appetite and weight loss. The PHN model is characterized by subepithelial immune-deposits leading to proteinuria and podocyte foot-process effacement⁵, whereas AN affects the endothelial cells prior to the podocytes¹⁷⁵. In addition, inflammatory cells have not been detected in glomeruli of PHN rats¹⁷⁶, but is a sequential effect following adriamycin administration in mice¹⁴⁷. PHN is induced by injection of the Anti-Fx1A IgG antibody and the model mechanisms seem to fit well with the disease. Adriamycin, however, is originally an anti-cancer drug that was identified to cause proteinuria and the model is crude. The therapeutic window is narrow, strain-specific and different batches of adriamycin can cause different amounts of proteinuria¹⁷⁷. These pathological differences could partly explain differences in treatment effects.

The treatment time is an interesting factor. The mice were only treated for about 1 week after induction of AN. In the PHN rats, the effects of the MC1R treatment were followed for 4 weeks, and proteinuria was examined even 1 week after treatment withdrawal. This was not possible in the AN mice, because of a rapid decline in body weight. The ethical permit did not allow more than a 10 % reduction in body weight and because of this, the AN mice were terminated after only 7-12 days following AN induction.

Naturally, the results might illustrate differences in these podocytopathies in terms of underlying mechanisms and disease progression. The PHN model induces a podocyte injury coupled primarily to actin reorganization and nephrin dissociation from podocin¹⁴³, whereas loss of podocytes is a more prominent feature of AN¹⁴⁷.

Rationale for using the adriamycin nephropathy model

Adriamycin increases mRNA MC1R expression in podocytes, which we showed by adding increasing doses of adriamycin to podocytes and

measuring the MC1R gene expression after 24, 48 and 72 h (Figure 10). We hypothesized that the increase in MC1R gene expression was a response of the podocytes to increase protection against a harmful stimulus. This led us to test whether MC1R stimulation was beneficial in the AN model. However, there might be differences in the response of cells in culture and *in vivo* animal models.

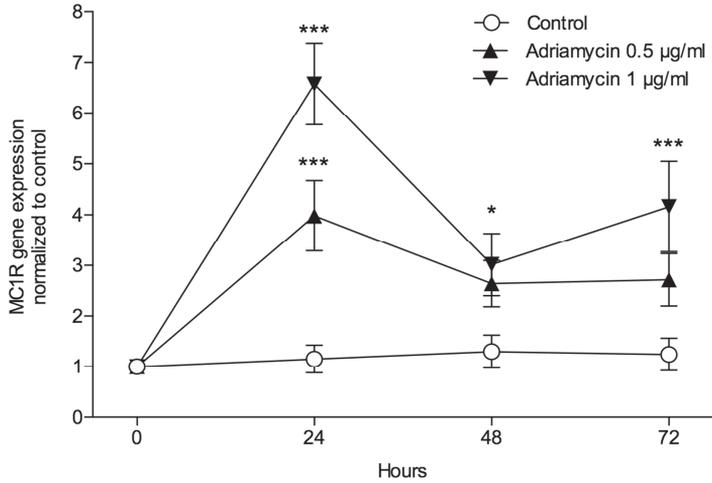


Figure 10. Melanocortin 1 receptor (MC1R) gene expression in cultured podocytes following adriamycin stimulation. Addition of adriamycin to podocytes upregulates MC1R gene expression, which might be a response to induce protection. Gene expression normalized to control and shown as fold change, \pm SEM, $n = 6$, Two way ANOVA, * $P < 0.05$, *** $P < 0.001$.

4.2 Melanocortin 1 Receptor Agonist Protects Podocytes Through Catalase and RhoA Activation (Paper II)

In paper II, stimulation of the MC1R was performed on podocytes exposed to the drug puromycin in culture and downstream signaling events were examined. The different vectors used to create lentivirus for subsequent transduction and protein overexpression in podocytes are summarized in Table 2. In paper II, human MC1R (hMC1R) fused to EGFP was overexpressed in cultured mouse podocytes and wild-type (WT) podocytes with low endogenous MC1R expression were used as controls. Podocytes transduced with the EGFP virus vector were used to determine virus titration and toxicity.

4.2.1 Gene and protein expression in cultured podocytes

Endogenous as well as overexpressed MC1R was analyzed on western blot, real-time RT-PCR and by immunocytochemistry. Podocytes were subjected to different concentrations of the nephrosis-inducing agent puromycin over time, and changes in MC1R mRNA gene expression were examined.

As shown in paper I, MC1R was detected in WT cultured podocytes using western blot. The protein expression was also confirmed using both western blot and immunocytochemistry in paper II and shown in Figure 8. In addition, mouse podocytes had stable gene expression of murine MC1R with a C_T value of 32.0 (Table 3). Addition of puromycin was found to increase the MC1R gene and protein expression in podocytes.

Discussion

We hypothesized that the increase of the gene expression of MC1R was beneficial to the podocytes in terms of increased protection against a harmful stimulus. Puromycin have been shown to cause cytoskeletal rearrangement and increase oxidative stress in podocytes^{164, 178}, and MC1R stimulation has anti-oxidative properties as shown in melanocytes¹²⁴.

4.2.2 Podocyte-protective effects

Intracellular cAMP levels of podocytes overexpressing hMC1R were significantly increased in a dose-dependent fashion by stimulation with

MC1R-a for 30 min. There was no significant cAMP increase in the WT podocytes, even when subjected to high doses of MC1R-a.

We examined changes in podocyte catalase activity both in WT and hMC1R overexpressing podocytes. An increase in catalase activity was detected over time following stimulation with 100 nM MC1R-a in hMC1R overexpressing podocytes. We observed a trend in the WT podocytes, but the increase was not statistically significant. We also tested the irreversible catalase inhibitor AMT, and it substantially inhibited catalase activity in podocytes.

The amount of intracellular ROS was measured in WT podocytes and podocytes overexpressing hMC1R. The ROS levels were significantly decreased in both WT and hMC1R overexpressing podocytes after treatment with 1 and 10 nM MC1R-a for 72h. The decrease was higher in the cells overexpressing hMC1R, in which a dose-dependent effect also was observed.

Stimulation of MC1R decreased p190Rho-GAP tyrosine phosphorylation, which corresponds to decreased p190Rho-GAP activity⁵¹. This in turn leads to increased RhoA activity and promotion of stress fiber formation⁵¹. The effect was observed in podocytes overexpressing hMC1R. In WT podocytes we saw a trend of decreasing p190Rho-GAP dephosphorylation, but it was not statistically significant.

Discussion

Increased intracellular cAMP levels are observed following GPCR activation, and in this case the cAMP measurements served as a control experiment to determine the overexpression of functional and active hMC1R. In addition, measurement of cAMP levels was used as a dose-response trial of the hMC1R agonist MC1R-a. The half maximal effective concentration (EC_{50}) of MC1R-a, which was calculated to 3.232 nM in paper II, was in the same range as an EC_{50} value calculated in a previous study (11.8 nM for mouse MC1R and 16.8 nM for human)¹³³. The lower EC_{50} found in our study is likely due to higher MC1R expression in the podocytes following lentiviral overexpression.

The absence of cAMP increase in WT podocytes was probably due to low amounts of active MC1R resulting in signals close to baseline. The MC1R might be internalized in the WT podocytes, which has been described in other cell types^{159, 179}. There might also be a correlation between stress and MC1R expression as showed in the mRNA gene expression analyses, where puromycin and adriamycin time- and dose-dependently causes significant increases in the MC1R gene expression, whereas WT podocytes might

contain low levels of active MC1R. Significant changes in catalase activity could only be observed in podocytes overexpressing hMC1R, whereas decreased ROS levels was observable in both WT and podocytes overexpressing hMC1R. All the trends observed in WT podocytes also suggest low MC1R levels. Changes in catalase activity have been reported as a consequence of MC1R stimulation in melanoma cells ¹²³.

The activity of p190Rho-GAP is regulated by changes in ROS levels ⁵¹. Increased ROS levels leads to an activation of p190Rho-GAP, which in turn dephosphorylates and deactivates RhoA. Decreased ROS levels do in turn inactivate p190Rho-GAP, which promotes stress fibers and a stationary podocyte phenotype ^{20, 41}.

4.2.3 Protection against puromycin

W2 also examined the effects of MC1R stimulation in an *in vitro* model of nephrotoxic stress. In paper II, we used the nephrotoxin puromycin to induce pathological rearrangement of the actin cytoskeleton as well as decreased viability which has been described in a number of *in vitro* studies ^{164, 180}.

Puromycin induced a number of effects on the podocytes, such as a decrease in viability and stress fiber rearrangement. The viability was assessed with the AlamarBlue assay at different time-points, and it was shown that puromycin significantly decreased viability. Treatment of hMC1R overexpressing podocytes with 10 nM MC1R-a or MS05 partially ameliorated these effects and the viability was increased compared to podocytes only subjected to puromycin. This was also observed in WT podocytes, but to a lesser extent.

The effect of puromycin on the podocyte actin cytoskeleton involves disruption of stress fibers. By visualizing stress fibers in podocytes using rhodamine-phalloidin the stress fiber content could be analyzed and quantified (Figure 11). The amount of broken, bent stress fibers increased following administration of puromycin to podocytes. These pathological changes were prevented, and the stress fibers were regenerated when podocytes overexpressing hMC1R were treated with 1 nM MC1R-a (Table 4). When testing different treatment regimens, it became clear that administration of a lower dose of MC1R-a (0.1 nM), for an extended time-period had similar beneficial effects in both hMC1R overexpressing and WT podocytes.

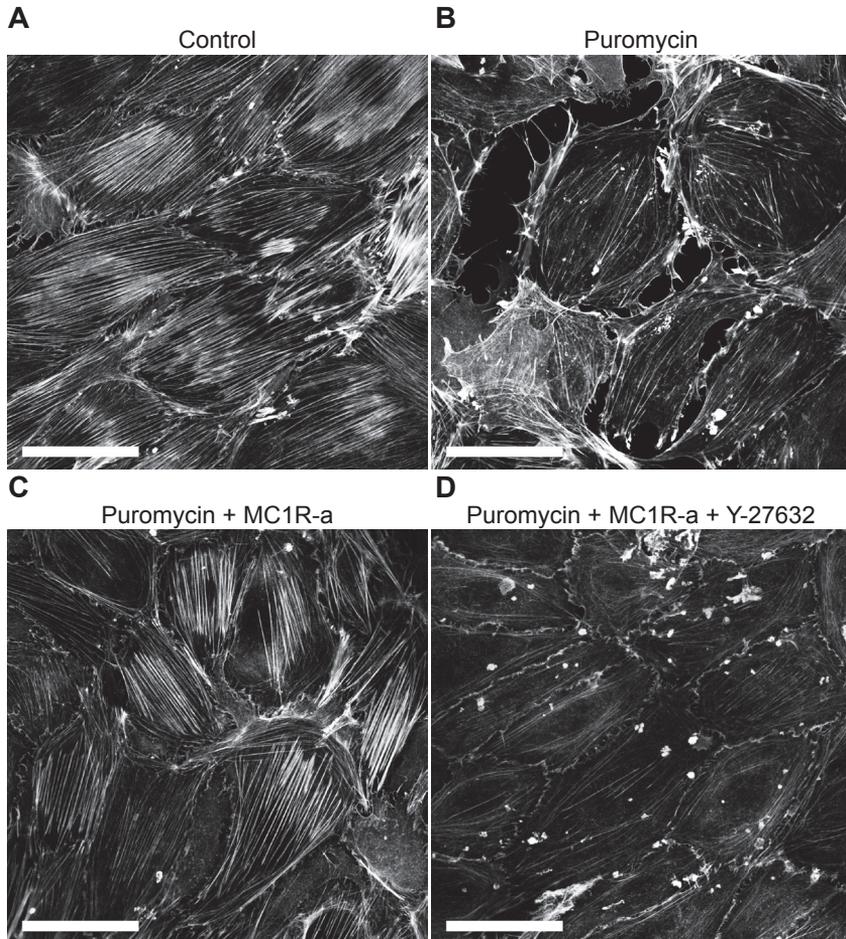


Figure 11. Confocal micrographs of rhodamine-phalloidin staining of stress fibers in cultured podocytes. Podocytes overexpressing melanocortin 1 receptor controls (A), subjected to puromycin (B), puromycin and treatment with MC1R-a (C), addition of the ROCK inhibitor Y-27632 (D). Puromycin reorganizes the stress fiber content of podocytes and treatment with MC1R-a is able to ameliorate the effects of puromycin. Addition of Y-27632 inhibits ROCK downstream of RhoA and completely disrupts stress fibers, indicating that MC1R stimulation protects by activation of the RhoA pathway to stabilize stress fibers. Scale bars represent 50 μ m.

To determine whether the protective effects on the stress fiber regeneration was a consequence of catalase activity, we tested the irreversible catalase inhibitor AMT. Addition of AMT to podocytes overexpressing hMC1R subjected to puromycin and treated with MC1R-a prevented the regeneration

of stress fibers. The AMT in itself had no toxic effect on the podocytes or stress fibers.

To ensure that the effects were a result of increased RhoA activity, we used the selective ROCK inhibitor Y-27632¹⁸¹. The inhibitor effectively disrupted almost 100 % of the stress fibers in podocytes subjected to puromycin and treated with MC1R-a (Figure 11). As expected, this effect was much more pronounced than puromycin, providing evidence to the hypothesis that MC1R stimulation activates RhoA, which is indeed involved in the promotion of stress-fibers in podocytes.

Discussion

The hypothesis in paper II relied on the findings that catalase activity was increased following several days of MC1R stimulation. In addition, we detected decreased ROS levels and p190Rho-GAP phosphorylation after 72 h of MC1R stimulation. The pre-treatment regimen developed relied on the idea that these effects would infer added protection against nephrotoxic injury. Indeed, the treatment regime of pre-incubation with MC1R-a prevented puromycin-induced viability loss as well as stress fiber disruption in both wt and hMC1R overexpressing podocytes.

RhoA was showed to be an integral part of the stress-fiber promotion in the podocytes, since the inhibitor Y-27632 efficiently disrupted almost 100 % of the stress fibers in podocytes¹⁸¹. If RhoA did not promote the stress fiber formation, the inhibitor would not have been likely to disrupt the stress fibers. The catalase inhibitor AMT added evidence to the hypothesis that the increase in catalase activity was an important event in the signaling cascade, since addition of AMT prevented the beneficial effect of MC1R-a stimulation on stress fiber formation.

4.2.4 Proposed signaling pathway

An increase in cAMP is one of the first signs of GPCR activation. Increase of cAMP usually leads to activation of PKA, which is a signal transducer into other intracellular pathways¹⁶⁷. The increase in intracellular cAMP following MC1R stimulation of hMC1R overexpressing podocytes was characterized as the first step in the MC1R activated cascade in podocytes.

There is also a connection between cAMP, increased catalase activity and decreased ROS levels following MC1R stimulation^{123, 124}. In addition to being a sign of oxidative stress in podocytes, ROS levels are signal transducers and have been confirmed to affect p190Rho-GAP activity through inhibition of the low-molecular-weight protein tyrosine phosphatase

(LMW-PTP)⁵¹. These events do in turn lead to inhibition of the p190Rho-GAP activity, which leads to an increased RhoA activity^{51, 182}.

The increased RhoA activity indicates a connection between MC1R and the actin cytoskeleton. RhoA activity is actively promoting stress fiber formation in the podocytes¹⁸³. Following the results from the different experiments a putative signaling pathway was proposed (Paper II, Figure 6).

Discussion

The parameters examined; cAMP, catalase activity, ROS and p190Rho-GAP phosphorylation are connected to the maintenance of the podocyte phenotype. Catalase activity is coupled to changes in ROS, since catalase catalyses the reaction of H₂O₂ into oxygen and water, which leads to a decrease in the ROS levels. Decreased ROS levels leads to inactivation of p190Rho-GAP, which in turn allows for the activation of RhoA and promotion of stress fiber formation. The different steps in the proposed pathway should be further confirmed by doing additional experiments and the phosphoproteomic approach in paper III was a step to clarify the effects of the MC1R stimulation of podocytes.

4.3 Melanocortin 1 receptor activation influences podocyte cytoskeletal dynamics (Paper III)

The putative connection between MC1R stimulation and the actin cytoskeleton described in paper II was further examined in paper III. The analysis was performed using a large-scale MS-based phosphoproteomic approach and pathway analysis using IPA software. In paper III, we overexpressed mouse MC1R (mMC1R) in podocytes, in addition to the constitutively active E92K mouse MC1R mutant. LifeAct-GFP was used in certain experiments to study cytoskeletal rearrangement in real-time (Table 2).

4.3.1 Cyclic AMP response

Similarly to paper II, stimulation of mMC1R with MC1R-a produced significant increases in the intracellular cAMP levels. In addition, the constitutively E92K mMC1R mutant presented constitutive activity and podocytes overexpressing E92K mMC1R had significantly higher basal levels of cAMP, without agonist-induced activation of the receptor. The E92K mutant was less sensitive to stimulation with MC1R-a.

Discussion

The constitutive activity of E92K has been shown earlier in terms of cAMP accumulation in transfected HEK cells ¹⁵⁹, but it was important to determine the effect in our podocyte model.

4.3.2 Protection against protamine sulfate

The PS model was utilized to produce fast rearrangement of the actin cytoskeleton. PS causes rapid and irreversible effects on the cytoskeleton ⁴⁶. The actin stress fibers were visualized by overexpressing the LifeAct-GFP peptide, which is known to bind to F-actin ¹⁷⁰. This allowed detection and subsequent quantification stress fiber rearrangement and changes in the podocyte area (Figure 12). The disruption and collapse of the cytoskeleton was measured by decreased podocyte area over time.

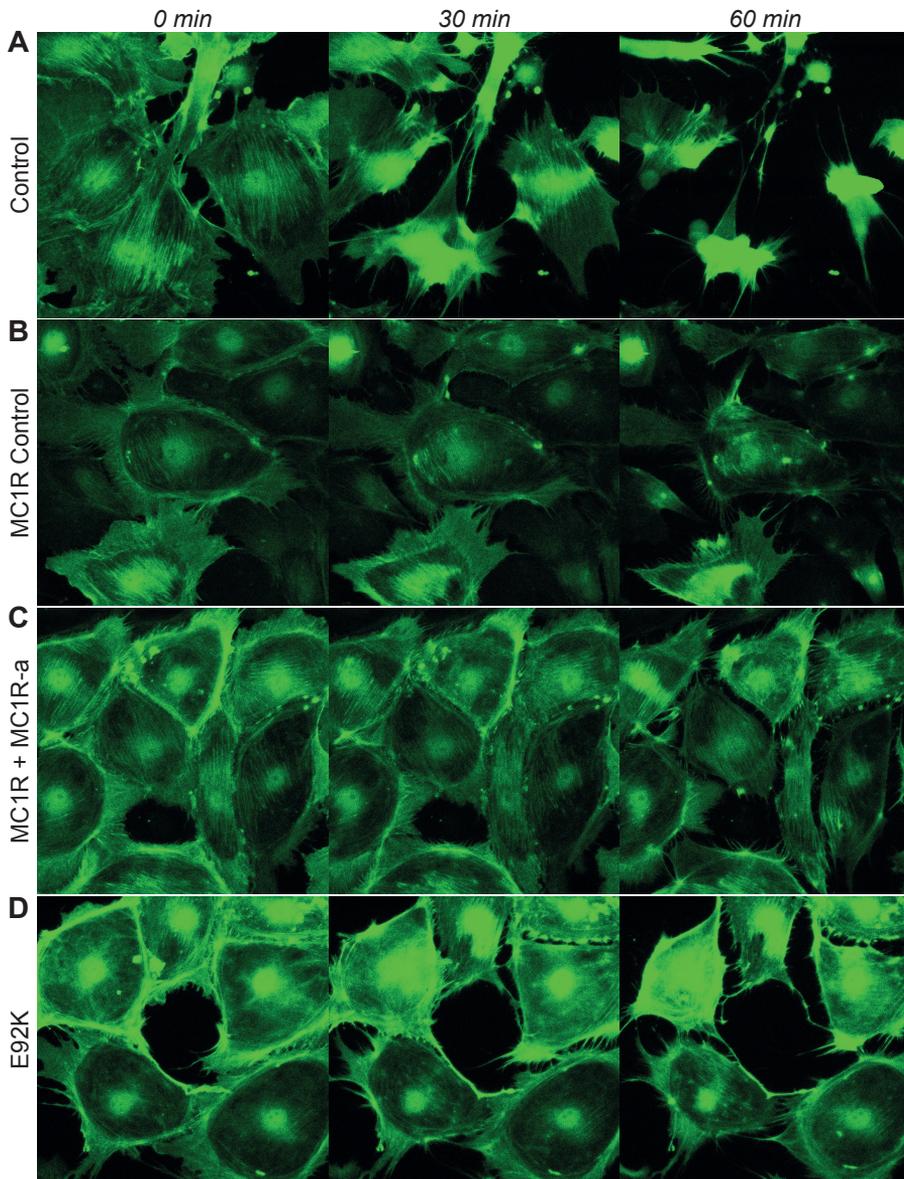


Figure 12. Podocytes overexpressing LifeAct-GFP subjected to protamine sulfate (PS). PS leads to fast cytoskeletal reorganization and the process was followed in living cells by confocal microscopy. Control podocytes (A), podocytes overexpressing melanocortin 1 receptor (MC1R) (B), overexpressing MC1R and stimulated with MC1R-a (C) and overexpressing the constitutively active MC1R E92K mutant. Stimulation of podocytes overexpressing MC1R with MC1R-a protects against cytoskeletal rearrangement following addition of PS. The activity of the E92K mutant does also partly protect the podocytes.

PS caused a massive disruption of the actin stress fibers in virus control cells overexpressing mCherry as well as podocytes overexpressing mMC1R-mCherry. Pre-treatment of mMC1R-mCherry overexpressing podocytes with 10 nM MC1R-a for 1 h prevented decrease of podocyte area and stress fiber collapse following addition of PS. The mMC1R mutant E92K also inferred a protective effect against the cytoskeletal rearrangement induced by PS, even without prior stimulation with MC1R-a. The protective effect of E92K, however, was not as pronounced as stimulation of mMC1R with MC1R-a.

Discussion

In paper III we wanted to test if the protective effects induced by MC1R stimulation could occur faster than what was examined in paper II. Instead of a 72 h pre-incubation with MC1R-a, the podocytes overexpressing mMC1R were pre-incubated for only 1 h. The PS experiments confirmed the hypothesis and added evidence to the theory that MC1R activation can protect podocytes through signaling to the actin cytoskeleton.

The lesser effect of E92K upon PS could be a result of the constitutive activity. The constant signaling might impair the downstream response, or the mutation might infer biased signaling. The E92K mutation increases intracellular cAMP levels, but as has been described earlier, it is not certain that the mutation affects all of the signaling pathways downstream of MC1R₁₅₉.

4.3.3 Phosphoproteomic analysis

Podocytes overexpressing mMC1R were subjected to MC1R-a for 15, 30 and 60 min. The samples were prepared for MS by enriching and labeling phosphopeptides, which were detected and quantified. The MS analysis detected 717 unique phosphopeptides belonging to 486 different proteins. Of these a large portion (between 30-60 %) displayed changes in their phosphorylation > 15 % compared to time-matched controls.

The phosphopeptide data from the MS experiment was analyzed through the IPA analysis. A cutoff of 15 % was set and all changes in phosphorylation > 15 % was considered as increased phosphorylation or dephosphorylation. The IPA software and database were able to identify a number of different pathways, which were significantly affected from the MC1R stimulation.

The top three pathways significantly changed in the 15 min sample were the actin cytoskeletal, the integrin and the paxillin pathways. The changes in these three pathways clearly implicate connections between MC1R and the actin cytoskeleton. A notable finding was the difference in phosphorylation

pattern over time. The order of significance for the different pathways was found to be time-dependent. At 15 min, the actin cytoskeletal pathway is the topmost significantly changed. After 60 min however, the tight junction pathway is the most significantly affected, indicating a sequential change in the phosphoproteome from actin to tight junctions.

Discussion

The 15 min sample was chosen for further analysis to determine which changes in phosphorylation that were induced directly following MC1R activation. It is of interest to investigate the 30 and 60 min time-points further, and crucial to repeat the experiment before extending the conclusions and building on the results.

Even though the IPA analysis found the actin cytoskeletal, integrin and paxillin pathways to be the most significantly affected, it did not provide actual information about the changes in activity of the analyzed phosphopeptides. To determine the effects on activity of the proteins the phosphorylation sites were determined by database searches, and the changes in phosphorylation were coupled to activity.

4.3.4 Actin dynamics

The IPA studies allowed analysis of the changes in the podocyte phosphoproteome following mMC1R stimulation. For a deeper analysis of the effects on the intracellular pathways we examined the actin cytoskeletal pathway from the 15 min sample. A list of the phosphoproteins coupled to the actin cytoskeletal pathway was compiled, and the different phosphorylated residues were identified.

From the list of phosphoproteins affected in the actin cytoskeletal pathway the phosphoprotein β PIX had undergone interesting changes in phosphorylation. β PIX is a protein regulating the activity of the small GTPases Rac1 and Cdc42 by acting as a GEF. β PIX has been described in different studies where it mediates cytoskeletal reorganization^{184, 185}. In addition, we found a number of proteins related to the organization of the cytoskeleton, such as cofilin, α -actinin, paxilin, vinculin, filamin-A and talin-1, but the significance of changes in phosphorylation of these proteins were hard to elucidate.

The three detected phosphorylation sites of β PIX (Ser-340, Ser-516 and Ser-525) found in the data set has also been described in earlier studies and are involved in regulation of β PIX GEF activity. The two sites Ser-340 and Ser-525 were dephosphorylated in podocytes overexpressing mMC1R after

stimulation with MC1R-a for 15 min. Phosphorylation of the site Ser-340 occurs downstream of the epidermal growth factor receptor (EGFR)¹⁸⁶ and leads to an increase of the Rac1 activation¹⁸⁷. The Ser-525 site is phosphorylated by PAK2, increases Rac1 activation¹⁸⁸ and is important for modulation of the intracellular β PIX localization¹⁸⁹. The Ser-516 residue had increased phosphorylation ratio and has earlier been described to be phosphorylated by PKA after addition of a cAMP analog¹⁹⁰. The phosphorylation of Ser-516 translocates β PIX to the focal adhesions and activates Cdc42¹⁹⁰.

The changes in phosphorylation of the different β PIX residues were interpreted as an inhibition of the β PIX ability to activate Rac1. This was considered as a connection between MC1R stimulation and promotion of stress fibers by deactivation of β PIX GEF activity on Rac1.

Discussion

We chose to examine the changes in phosphorylation of the protein β PIX because of its interesting connections to the family of small Rho GTPases; RhoA, Rac1, Cdc42. However, since we found and examined three different phosphorylation sites, the net effect of the β PIX phosphorylation is hard to determine but it points in the direction of Rac1 deactivation. In addition to these experiments, the intracellular localization of β PIX should be examined.

MC1R has shown to be important in regulating and affecting the actin cytoskeleton as well as regulation of the focal contacts. This implies that MC1R could protect the glomerular filtration barrier by activation of pathways protective for the cytoskeleton.

4.4 General discussion

This project started after the reports of beneficial effects of ACTH treatment in patients with several different kidney diseases ⁴. Based upon these findings we presented a hypothesis in which the protective effect of ACTH was due to the presence of one, or several, of the melanocortin receptors on glomerular cells. In 2010, a study from our research group was published, in which MC1R was found in human kidney tissue, co-localizing with the podocyte marker synaptopodin ⁵. In the same study, different MC1R agonists were tested in a nephrotic model of PHN in rats. The agonists tested were ACTH, α -MSH and MS05 ¹³⁸. ACTH binds to all of the 5 MCRs, and specifically MC2R, whereas α -MSH binds to all MCRs except MC2R. MS05 is highly specific to MC1R. The results from the study suggested that MC1R is the receptor responsible for the ACTH mediated effect in kidneys. It also showed that all agonists had a varying but beneficial reducing effect on proteinuria.

Following the earlier results, the three papers in this thesis were aimed at determining the intracellular signaling events activated by MC1R in podocytes. In addition to studying protective effects of MC1R signaling *in vivo* and *in vitro*, we investigated the putative connection between MC1R and the actin cytoskeleton and a proposed a signaling pathway of MC1R stimulation in podocytes.

In the *in vivo* PHN study, the selective MC1R agonist MS05 was used based upon earlier findings. In all of the *in vitro* experiments we used the synthetic and selective MC1R agonist BMS-470539 (MC1R-a), because of its selectivity and its suitability as a therapeutic agent, as well as the MS05, to confirm the findings in some of the experiments. In the *in vivo* AN study, we also tested MC1R-a, and wanted to examine the non-selective MCR agonist α -MSH. If only α -MSH would have had beneficial effects, then the explanation could have been that more than one MCR is involved.

Melanocortin 1 receptor expression

A problem during the work on this thesis has been expression of MC1R in podocytes. We detected MC1R in the cultured podocytes, though at lower levels than observed in human renal biopsies. We could also show that the nephrotoxin puromycin increased basal MC1R protein levels in podocytes. In almost all of the experiments with WT podocytes we saw effects coupled to MC1R stimulation, but the signals were weak. We addressed the problem by overexpressing MC1R, which allowed amplification of the response to MC1R stimulation and made the protective effects easier to study.

Another thing of note is the fact that in paper II, human MC1R was overexpressed, whereas in paper III, mouse MC1R was used as well. Initially, we considered the human MC1R to be most relevant and the target for the synthetic MC1R agonist. Ultimately we are interested in the human disease and to understand the mechanisms behind ACTH treatment. In paper III, we used the mouse MC1R for overexpression in the podocytes to be able to compare the results with those of the constitutively active MC1R mutant of mouse origin. In a paper described by Benned-Jenssen et al.¹⁵⁹ the mouse E92K and the human E94K MC1R mutants are described. The authors generously provided both mutants to us. We performed pilot studies with both mutants and found that the mouse E92K mutant showed higher activity in the podocytes, and hence it was selected for further studies.

Overexpression of the mouse MC1R protein in podocytes (paper III) produced a similar cAMP response as human MC1R (paper II), and the effects of stimulation were protective using mouse MC1R in the PS model. Following these results we decided to continue using the mouse construct.

Protection in nephrotic models

Both the puromycin and the PS models are commonly used *in vitro* to test effects of new pharmacological compounds and to examine protective effects on the actin cytoskeleton. MC1R stimulation had beneficial effects both in podocytes overexpressing MC1R against puromycin-induced stress, as well as against PS. An important finding is that in a majority of the experiments in paper II, a trend of MC1R-mediated protection could be observed in the WT podocytes, although not as marked as in the podocytes overexpressing hMC1R.

Signaling in podocytes

A signaling pathway following MC1R stimulation was proposed in paper II, where a connection between the MC1R and the actin cytoskeleton was presented. This connection was further examined in paper III.

The hypothesis of how MC1R induced protection would function in podocytes was further developed during the transition from paper II to III. In paper II, the hypothesis relied on the findings that the catalase activity as well as RhoA activity was significantly increased after 72 h whereas ROS levels were decreased, and therefore protection against the ROS increasing puromycin model would require stimulation of MC1R for at least 72 h. In paper III, the changes in the phosphoproteome following MC1R stimulation were found to occur very fast, and a second hypothesis was conceived. According to the latter, the protective effect of MC1R is proposed to work by

quickly stabilizing the actin cytoskeleton. Indeed, the PS model is an extremely fast way of inducing marked alterations of the cytoskeleton. Both signaling pathways are likely to be activated *in vivo*.

Two pathway effects

The two different hypotheses presented in papers II and III could represent two different sequential effects of the same signaling cascade. On the basis of the findings in paper II and III we could propose a putative MC1R pathway in podocytes (Figure 13).

The pathway described in paper II represents a more sustained and chronic MC1R signaling through increased catalase activity and subsequent protection by decreased ROS levels. The decreased ROS levels in turn connect MC1R signaling to the actin cytoskeleton through RhoA activation and promotion of stress fiber formation. The induced effects might be beneficial to the podocytes in the long term by increasing the general protection against harmful levels of oxidative stress and adding protection against damaging cytoskeletal rearrangement.

These long-term effects could be beneficial for patients with different nephrotic syndromes, since they seem to elevate the podocyte defense against oxidative stress and protect the cytoskeleton.

The pathway and effects described in paper III are more acute, and the latter seem to be exerted by quick stabilization of the actin cytoskeleton against fast reorganization. Stimulation of MC1R induces rapid signaling to the actin cytoskeleton by dephosphorylation of β PIX that would likely deactivate Rac1 and Cdc42 activity, thereby cause protection by promoting stationary stress fibers. These effects, however, are not fully confirmed, and more work is needed to determine the exact effects on the phosphoproteome and coupling between MC1R and the β PIX activity.

The fast effects connected to stress fiber promotion are difficult to translate to a clinical setting. Nephrotic syndromes generally progresses over months or years. However, there is evidence that ischemic injury to kidneys does cause loss of nephrin interaction with ZO-1 in the podocyte slit diaphragm¹⁹¹. Additionally, both the PS and the puromycin models induce fast relocalization of the nephrin binding partner ZO-1¹⁶⁶. Nephrin is a protein found bridging the SDs between the podocytes with connections to the actin cytoskeleton^{23, 192} and a mutation in nephrin causes congenital nephrotic syndrome of the Finnish type (CNF)²¹. It would be very interesting to test the effects of MC1R stimulation in an acute model such as renal ischemia to

determine whether the rapid connection to the actin cytoskeleton is of clinical importance. Also, following the analysis of the phosphoproteome, more effort should be put into examination of the phosphorylation profiles of nephrin and ZO-1 following MC1R stimulation, since tyrosine phosphorylation of nephrin has been postulated to increase nephrin binding to neph1 and this promotes stress fibers¹⁹³.

In addition, it would be of interest to test MC1R-based drugs on patients with recurring steroid resistant NS after kidney transplantation; these patients have an increased risk of acute kidney injury (AKI)¹⁹⁴. Could the combination of the rapid cytoskeletal organization in connection with the more long-term protective effects induced by MC1R be beneficial in preventing graft rejection and AKI in these patients?

5 CONCLUDING REMARKS

The main work of this thesis was the effort put into examination of the downstream signaling effects of MC1R activation in podocytes. MC1R was detected in podocytes and the receptor levels were examined. The selective agonist MC1R-a was chosen and synthesized to determine the specific effects of MC1R stimulation in podocytes, as well as the downstream signaling pathways.

5.1 Paper I

The aim of paper I was to determine the effects of MC1R stimulation with the help of different MC1R agonists against two different and well-characterized nephrotic animal models. We chose the AN model, since it is a model of the human FSGS disease¹⁴⁷. We also elected to examine readouts similar to a previous study, such as proteinuria, BUN and podocyte morphology⁵.

Treatment of the two animal models with MC1R activating agonists had different effects. The nephrotic syndrome in PHN rats was ameliorated by the MC1R agonist MS05, but the AN mice was not beneficially affected by the compounds MC1R-a or α -MSH. Podocyte morphology was not improved and proteinuria was not decreased in contrast to rats with PHN. We were however able to reconfirm previous findings where treatment with MC1R agonists could ameliorate the nephrotic syndrome in rats with PHN, and the effects of treatment were sustained even after treatment withdrawal. We believe that the reasons for this can be found in the differences in the animal disease models that can reflect differences between the two diseases, and also different responses to the MC1R mediated effects.

5.2 Paper II

The aim of paper II was to examine the intracellular signaling pathways activated in cultured murine podocytes and the possible protective effects induced. It was soon discovered that the murine podocytes do express MC1R, but in low levels and they do not respond to stimulus by MC1R-a in terms of increased intracellular cAMP levels, which can be attributed to low receptor levels. If the amount of active MC1R is low enough, or the receptor is internalized or desensitized^{129, 159, 179} it may be impossible to detect changes in cAMP levels. The overexpression of MC1R was a necessary tool to be

able to examine the downstream effects of MC1R stimulation. It was also very useful in terms of amplification of the stimulus effects.

Stimulation of podocytes overexpressing hMC1R with the synthetic and MC1R selective agonist MC1R-a protects against puromycin-induced viability loss and regenerates stress fibers. Intracellular cAMP levels were increased, ROS levels and tyrosine-phosphorylation of p190Rho-GAP were decreased, and henceforth the activity of p190Rho-GAP. All of these effects or at least trends could be detected in WT podocytes, though not very pronounced which presented a problem. Following these findings we could propose a simple pathway (Paper II, Figure 6) from MC1R stimulation to beneficial effects in terms of decrease in oxidative stress levels, connections to RhoA and stress fiber regeneration.

5.3 Paper III

In paper III we wanted to extend the hypothesis from paper II to a more acute model of *in vitro* nephrotic syndrome in addition to examine large-scale changes in the phosphoproteome. We also wanted to test the constitutive active mouse MC1R mutant E92K and its possible beneficial effects on stabilization of the actin cytoskeleton.

We found that stimulation of overexpressed mMC1R in cultured podocytes with MC1R-a protected against the rapid model of PS induced cytoskeletal rearrangement and decreased podocyte area by using a LifeAct model. Overexpression of the E92K mutant also provided partial protection through MC1R signaling. Lastly we examined the effects of MC1R stimulation on intracellular pathways following large-scale changes in the phosphoproteome of podocytes overexpressing mMC1R. Through MS, subsequent phosphopeptide labeling and database searches we could analyze the data through an intrinsic pathway analysis. We found that the actin cytoskeletal pathway was the most significantly changed pathway following 15 min stimulation with MC1R-a. We detected a number of phosphoproteins with changes in their phosphorylation patterns and focused on the protein β PIX because of its connections to the actin cytoskeleton and the family of small RhoGTPases^{184, 185}. We concluded that the Rac1 activating activity of β PIX was inhibited following MC1R stimulation for 15 min. However, this is still work in progress and we need to examine our findings more closely.

5.4 Future implications

Patients with different types of CKD have limited treatment options. Corticosteroids (prednisone), antibodies (rituximab), hormones (ACTH) and ACE inhibitors are examples of therapeutic options but they are unspecific and all have adverse side effects.

The CKD are often idiopathic diseases, which leads to the targeting of the symptoms instead of the underlying causes. There is also a large patient group emerging from the type-2 diabetics and DN is the most serious complication following diabetes. Often, the diseases progress into ESRD, where dialysis and kidney transplant are the only viable options. Dialysis is a very expensive treatment and it reduces the quality of life significantly. Kidney transplants are usually successful, but there is often a long waiting list associated with transplantation. Some diseases can also recur in the kidney graft. As an example CNF has been shown to re-emerge in patients with transplanted kidneys due to autoantibodies against the podocyte specific SD protein nephrin^{195, 196}, and the more extreme IgA nephropathy, with a recurrence of almost 50 % in transplanted kidneys¹⁹⁷.

ACTH therapy has emerged as a new and promising therapy, but there are few randomized controlled studies. One problem with ACTH is the side effects, which may prevent the use of larger, more optimal, doses. Treatment with ACTH has been reported to have beneficial effects on patients with MN, MCD, DN, and membranoproliferative glomerulonephritis (MPGN)^{4, 103}. In some patients, ACTH treatment may have been beneficial on FSGS⁹⁶.

Treatment with ACTH based drugs leads to elevated cortisol release, which in turn can cause side effects such as Cushing's syndrome, severe insomnia⁶, and tanning of the skin¹⁰⁵. Most interestingly, ACTH has effects such as cortisol release, and has had beneficial effects on steroid resistant nephrotic syndromes. This provides evidence that the cortisol effect may not be the main pathway of amelioration in the patients, which points to mediation of the beneficial effects primarily through one of the MCRs.

The findings from this thesis indicate that MC1R stimulation protects against different nephrotic models, and the protection is likely mediated through stabilization of the actin stress fibers and other prosurvival effects such as reduction of ROS. Adding to the findings by Berg et al, and the earlier study performed by our group, MC1R has definitely emerged as an interesting drug target.

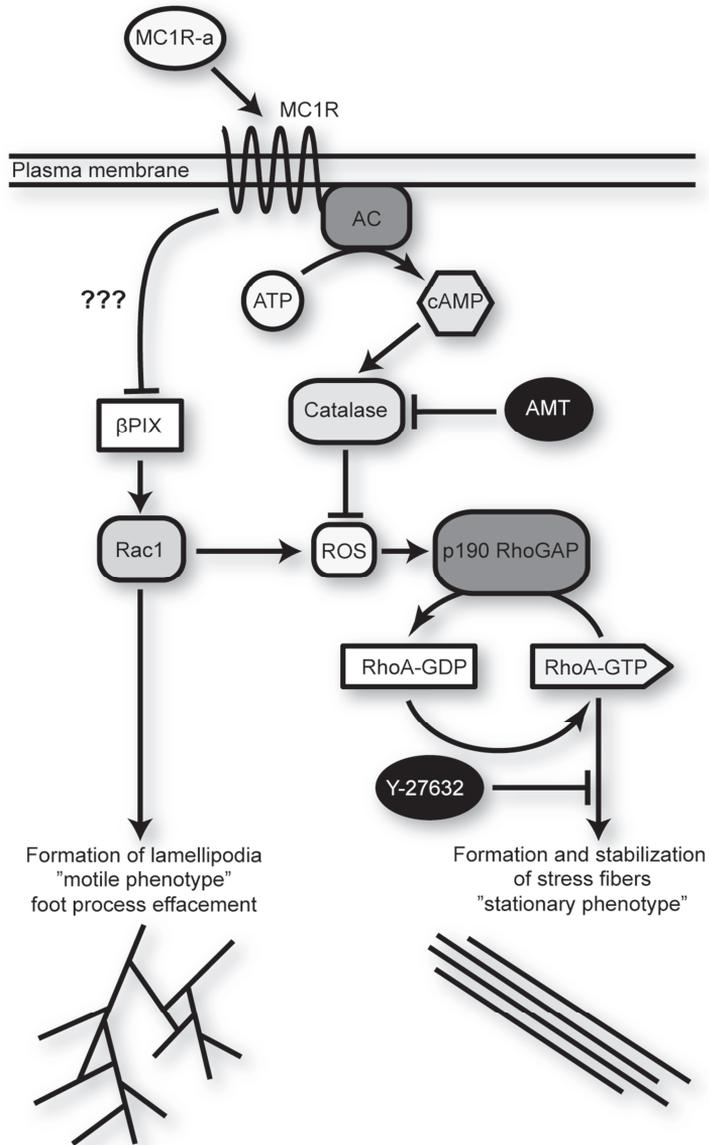


Figure 13. Proposed signaling pathway of MC1R in podocytes. In this thesis we have showed that MC1R stimulation increases catalase activity, decreases reactive oxygen species (ROS), decreases activity of p190RhoGAP, which allows for increased RhoA activity and enhanced stress fiber formation. These effects takes several days to induce and we have used different inhibitors such as AMT and Y-27632 to confirm the findings by blocking the activity of catalase and the RhoA effector ROCK, respectively. In addition, MC1R stimulation decreases βPIX phosphorylation and its ability to activate Rac1, which thereby protects by promoting stress fiber formation.

Regarding the lack of specific treatments for NS, MC1R-a or at least MC1R directed therapy should be a viable target of interest. The selective agonist MC1R-a has some major interesting features and should be considered as a drug candidate. First of all, it is selective against MC1R, which implies that it should not mediate the cortisol release coupled with ACTH treatment. Since it is not a peptide it might also be easily administered, perhaps even orally.

The path to clinical studies should be further examined, more animal studies should be undertaken in order to elucidate any toxic and unspecific effects of MC1R stimulation through the MC1R-a agonist. The E92K mutant could be considered for creation of a transgenic mouse with podocyte-specific inducible expression of E92K MC1R. This could be of value in the unraveling of the MC1R mechanism in kidney diseases. Following additional animal studies the next step could be to synthesize a MC1R-based drug candidate, based on MC1R-a and actually test in the clinic. The data produced in this thesis project and previous studies shows that targeting MC1R might be promising for the treatment of kidney diseases.

In summary, treatment of the underlying condition of NS is usually hard, since many diseases are idiopathic. But there is hope; each little added piece of information helps patients. A lot has happened in the field of renal research. The kidneys have emerged as organs implicated in major health issues, especially with the onset of the obesity epidemic causing type 2 diabetes to explode. DN might not be the same disease as FSGS, MN and MCD, but lessons learned from treating one disease will help future research.

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