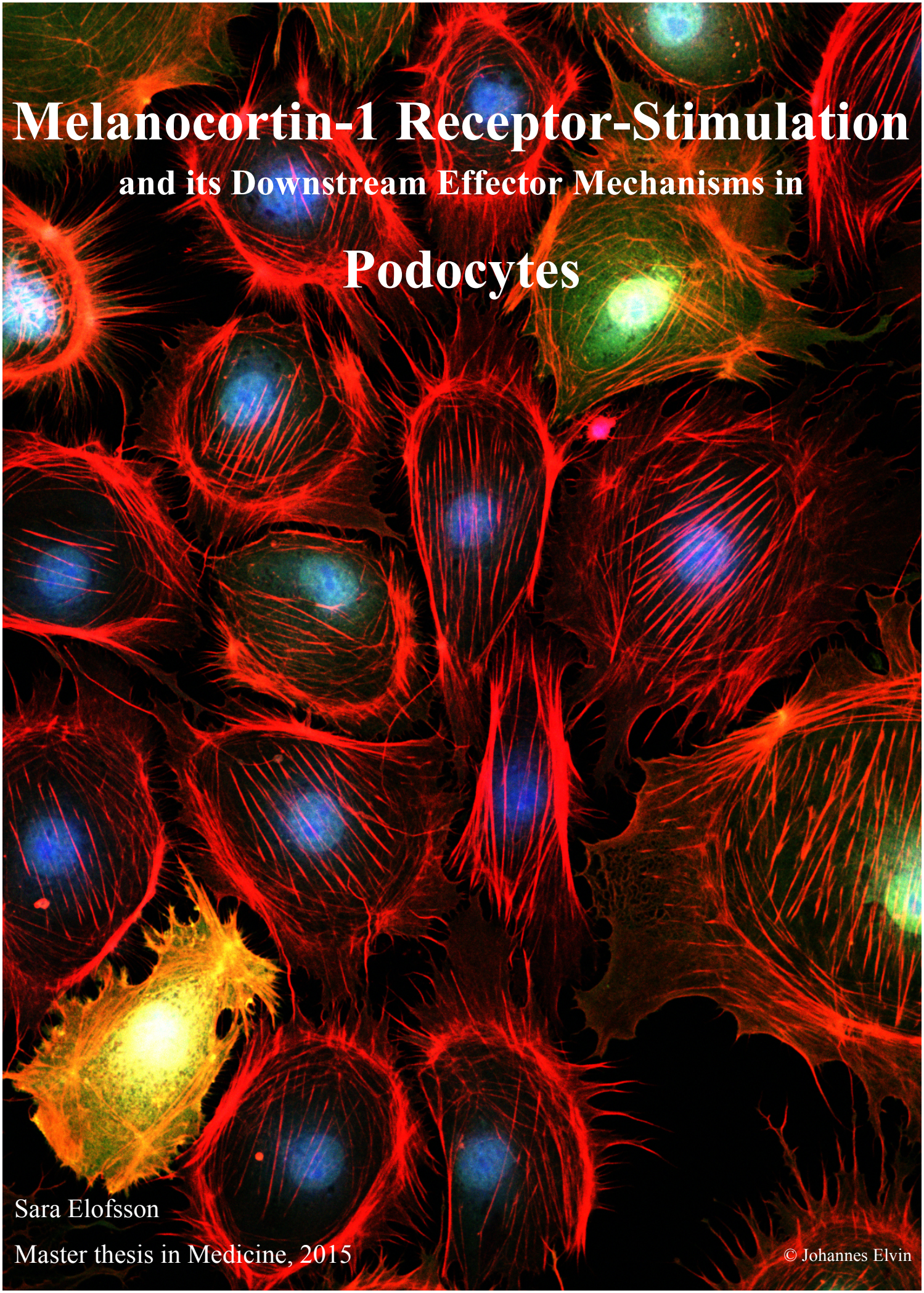
Melanocortin-1 Receptor-Stimulation

and its Downstream Effector Mechanisms in

Podocytes



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Master thesis in Medicine, 2015

Melanocortin-1 Receptor-Stimulation and its Downstream Effector Mechanisms in Podocytes

Master Thesis in Medicine

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Abstract

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Previous clinical studies and *in vitro* experiments have confirmed that treatment with adrenocorticotropic hormone (ACTH) have ameliorating effects on nephrotic syndrome. This action is believed to be mediated by stimulation of Melanocortin-1 Receptor (MC1R) in podocytes. The full extent of MC1R signalling in podocytes is still unknown, but the anti-oxidative enzyme catalase has been found to be one of the downstream effectors. We have studied in what ways catalase is regulated to exert its action and what effect this activity has in podocytes. In addition, we studied the human constitutively active MC1R-mutant, E94K.

Cultured differentiated mouse podocytes were transduced with different lentivirus to overexpress MC1R or the constitutively active MC1R-mutant. The podocytes were subjected to different concentrations of a nephrotoxic agent, puromycin, and/or the known MC1R agonist BMS-470539. In addition, a catalase specific inhibitor, 3-amino-1, 2, 4-triazole, was used to elucidate whether the protective effects of MC1R stimulation is dependent on catalase. The effects due to different treatments on the podocytes were assessed with diverse analytical assays.

The human constitutive MC1R-mutant did not show an effect in the range we expected and the mouse mutant will be tested hereafter. The inhibitor gave a dose-dependent inhibition but had an unsuspected interaction with puromycin.

In summary, catalase still seems to have a possible role in the ameliorating effects of MC1R-stimulation in nephrotic syndrome and these experiments have given new strategies and developed better implements to future experiments.

Introduction

The Kidney

The kidneys are important in the process of regulating the balance and components of the body fluid [1, 2]. By producing 180 litres of primary urine per day: fluid, acid-base and electrolyte levels are regulated at the same time as waste products are being eliminated, through urine excretion. This filtration occurs in the smallest functional unit of the kidney, the nephron. Each kidney has about one million nephrons and one of the important components of each nephron is the glomerulus. In the glomerulus the blood is filtrated over the glomerular filtration barrier, comprised of four layers: endothelial cell surface layer (ESL), endothelial cells, glomerular basement membrane (GBM) and podocytes (see figure 1). These four components form a semi-permeable and selective filtration space based on size, shape and charge of the passing molecule. The glomerulus also contains a structural supporting component, mesangial cells, which have no apparent role in the filtration.

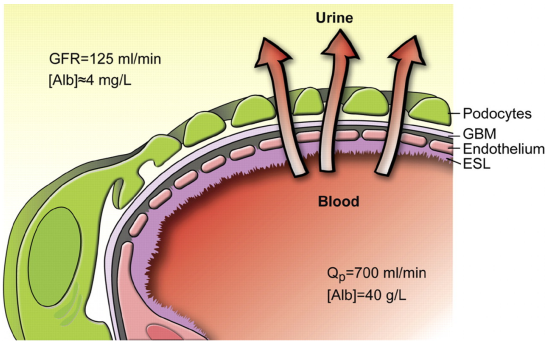


Figure 1 [1]: The layers of the glomerular filtration barrier

Podocytes

The podocytes are highly differentiated and specialized epithelial cells with an important role in the barrier. Between the podocytes are slit diaphragms (SD). The SD is a network of proteins that are connected to the actin cytoskeleton of the podocytes. They have the function to anchor the podocytes together and also to constitute the last barrier between blood and primary urine. The functions of the podocytes are dependent on morphology and require intact actin cytoskeleton to maintain foot processes and SD.

Kidney disease

When the glomerular filtration barrier does not work as it should, a nephrotic syndrome might develop. The nephrotic syndrome includes proteinuria (> 3,5 g protein/day), hypoalbuminemia (< 30 g/L), hyperlipidemia, peripheral edema, increased tendency to create blood clots and increased sensibility to infections. This is a nonspecific disorder with most commonly an idiopathic origin but some are known and further categorized from morphological changes of the kidney, through biopsy. The most common is membranous nephropathy, MN, with an incidence of 5-10 per million population and year [3].

In 80 % of the patients a nephrotic syndrome is present when the diagnosis of MN is defined [3]. With urine samples the presence and level of proteinuria can be clarified along with the degree of hematuria. The kidney function is also measured with blood samples of creatinine, urea and albumin together with calculation of glomerular filtration rate (GFR) through ^{51}Cr-EDTA-clearance, iohexol-clerance or estimated GFR. Other blood samples are also controlled such as haemoglobin, leukocytes, thrombocytes, electrolytes, liver function tests, lipids, circulating antibodies and complement factors.

MN is either idiopathic or secondary due to system diseases, cancer, infections, drugs or toxic agents. Anamnestic questions are important to define which type that is most likely, but the investigations are not just performed to define this but also elucidate the grade of severity. With a biopsy of the kidney the morphology of the glomeruli are visualized, together with subepithelial deposits in electron microscope and IgG-complexes with immunofluorescence.

Pathological components in MN are subepithelial deposits of immune complexes (IgG; IgG4 in idiopathic MN), capillary wall thickening, foot processes effacement and GBM thickening (see figure 2) [2].

70 % of the patients with idiopathic MN have autoantibodies to a receptor, on the cell surface of the podocytes, called phospholipase-A2 (PLA2R) [3]. When the circulating autoantibodies pass from the blood through the endothelial cells to the GBM they bind to PLA2R on the podocytes and form immune complexes. These immune complexes recruit the C5b-9 membrane attack complex (MAC) from the system of complement factors. MAC is inserted into the podocyte cell membrane and this initiates multiple intracellular signalling cascades with production of reactive oxygen species (ROS), alterations of SD-proteins and actin cytoskeleton, activation of nuclear factor kappa B (NF-κB) and effects on mitogen-activated protein kinase (MAPK) pathways including p38, extracellular signal-regulated kinase (ERK) and c-Jun N-terminal kinase (JNK). The result of these processes are foot process effacement and proteinuria [2, 4].

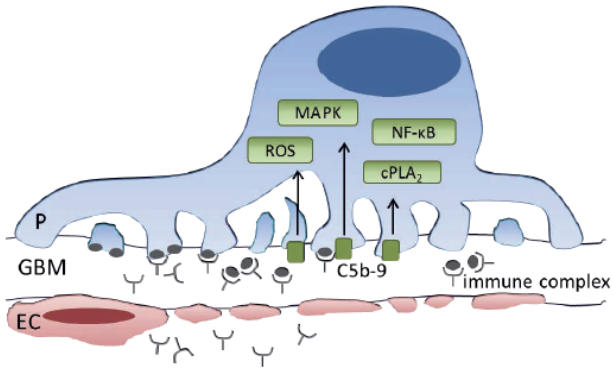


Figure 2 [2]: Circulating autoantibodies against PLA2R passes the endothelial cells (EC) and form immune complexes together with PLA2R. The immune complexes stimulate C5b-9 to be inserted to the podocytes and initiate intracellular cascades with reactive oxygen species (ROS), effect mitogen-activated protein kinase (MAPK), activate nuclear factor kappa-B (NF-κB) and initiate creation of cytosolic phospholipase A_2.

The natural course of the idiopathic disease is highly varied: some have a spontaneous remission and other go quickly to uremia, while some remains with nephrotic syndrome but with preserved GFR through years. Treatments of idiopathic MN without nephrotic syndrome are only symptomatic with efforts to reduce proteinuria

and hopefully take control of the disease. Basal therapy is including ACE-inhibitor (lowering blood pressure and glomerular capillary hydrostatic pressure) and statins. These drugs are also given to the patients without nephrotic syndrome at the time of diagnosis. Further treatment in the aim of reach remission in proteinuria exists of a combination of different immunomodulatory drugs: cyclosporine, glucocorticoids and chemotherapy (primarily alkylating, such as cyclophosphamide or chlorambucil). Glucocorticoids alone do rarely show any effect. Considering the discovery of autoantibodies the treatment with monoclonal antibodies against B-cells (rituximab) has become an alternative. In some cases treatment with adrenocorticotropic hormone (ACTH) is being used with beneficial results [3]. Previous *in vivo* experiments have confirmed that ACTH-treatment reduces proteinuria (50-60%), lowers oxidative stress and leads to improved glomerular morphology by promoting a re-establishment of the podocyte foot processes [2, 4, 5]. Despite the low doses used the stimulation of the adrenal glands by ACTH is enough to release cortisol in such amount that Cushingoid side effects occur, for example osteoporosis, hypertension and edema [2]. The secondary type of MN is treated by targeted treatment against the specific underlying cause.

Melanocortin-1 Receptor and ACTH

ACTH is a known ligand for Melanocortin-1 receptors (MC1R), which is the first of five melanocortin receptor-subtypes [2]. They are G-protein-coupled 7 transmembrane receptors. MC1R is mainly known considering its expression in melanocytes, where it acts in the regulation of skin pigmentation. Stimulation of the receptor is involved in the defence against oxidative stress, due to

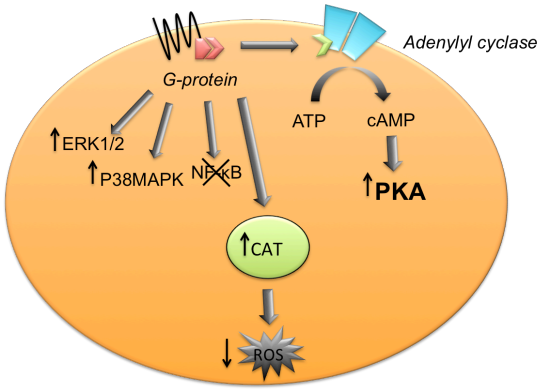


Figure 3: Downstream effects of MC1R

UV-radiation, by activation of the antioxidant enzyme catalase. Sequent, mutations of MC1R are a possible part of an increased risk of melanoma. Furthermore, the MC1R is known to possess anti-inflammatory properties, through its expression on immune cells [2].

Activation of MC1R in the cell membrane of podocytes has in previous studies [2] been shown to involve adenylyl cyclase (AC), activation of MAP kinases p38 and ERK1/2 due to phosphorylation, inhibition of NF-κB and activation of catalase (see figure 3). Increased levels of cAMP activate protein kinase A (PKA), which leads to binding of cAMP responsive element-binding protein (CREB) to cAMP response elements (CRE) in the DNA. This pathway has been shown to be important in melanocytes, but it may not be crucial for normalization of podocyte function. To better understand the ameliorating effects of ACTH it is of great interest to determine the full extent of the signalling pathways and mechanisms of MC1R.

Melanocortin-1 receptor-mutants

The *in vitro* podocyte MC1R-mutants have mutations where residues at specific positions are exchanged to positively charged ones giving them a constitutive MC1R activity, which is not dependent on an activation signal in terms of a ligand. The mouse and human MC1R-mutant are called E92K and E94K, respectively. The mouse mutant has been described in an article by Benned-Jensen et al, 2011 [6], where they analysed the activity initiated from each receptor by measuring the amount of accumulated cAMP in the cells. Like the regular MC1R an accumulation of cAMP is a downstream effect owing to receptor activity and the concentration of cAMP is therefore correlated to the degree of activity. The activity of the mouse mutant is about 50 % of the activity that normally would follow after high stimulation of

MC1R. With the mouse mutant, in contrast to wild-type (wt) MC1R, the accumulation of cAMP increases independently of agonist.

Few published studies have been done on the human constitutive MC1R-mutant, but measurement of CREB activation, which reflects long-term changes in cAMP-levels, has been done [6]. The human constitutive MC1R-mutant had a CREB-activity two and a half times higher than normal MC1R expressing cells. This means that both the mouse and human mutants gives a higher amount of cAMP than the regular MC1R.

Catalase

Catalase is an anti-oxidative enzyme, which increases the intracellular defence against oxygen radical formations formed by ROS (reactive oxygen species). Catalase catalyses the decomposition of hydrogen peroxide to water and oxygen. Stimulation of MC1R has been shown to increase the activity of catalase [5].

Hypothesis

We hypothesize that catalase plays an important role in the downstream pathway following Melanocortin-1 receptor stimulation in podocytes and that catalase is directly involved in the amelioration of nephrotic syndromes.

Research question

With information about the MC1R and its downstream intracellular signalling effects, it might be possible to test new drugs specifically targeting the MC1R as a tool in treating patients with nephrotic syndrome. In this thesis I will focus on two aspects:

- **The regulation of catalase** and if this is through changes in the gene expression or effects on regulation mechanisms in protein level (activation, inhibition or degradation).
- **The possibility of using the constitutively active MC1R-mutant** to simplify future experiments.

Materials and Methods

Experiment made in preparation for this project was not done by me and are marked with an asterisk (*) and not further explained for this reason.

Podocyctes

We used conditionally immortalized mouse podocytes (a kind gift from Dr Mundel at Massachusetts General Hospital, Boston, USA), which were cultured as previously described [7-9]. Differentiation of the podocytes proceeded 8-12 days before experiments and during this time the medium was changed every other or third day. Podocytes cultured for cAMP and catalase experiment were ended after 15-18 days after start of differentiation. The podocytes used in viability experiments with AlamarBlue® were ended at day 25-26 after start of differentiation. Podocytes were used in passages 13-19 at which point they maintained their pheno- and genotype. All studies were performed on differentiated podocytes.

To modify podocytes to overexpress proteins and obtain different characteristics they were transduced with lentiviruses* combined with desired DNA-vector*. The DNA-vector will integrate in the host cells and create an increased expression of this gene. In our experiments we used DNA-vectors to create an overexpression of MC1R,

MC1R-mutant or enhanced green fluorescent protein (EGFP; hereafter referred as virus control). The virus control was used to visualize that the lentiviruses do not have any effect themself.

Pharmacological agents

Among the different identified agonists activating MC1R we used the highly specific and synthetic BMS-470539, described in previous studies [10-12]. BMS was synthetized after inquiry from Enamine (Enamine, Kiev, Ukraine). Henceforth BMS-470539 is referred to as MC1R-agonist. The solvent Dimethyl Sulfoxide, DMSO, was used to solve the MC1R-agonist. To exclude that DMSO itself had any effect on the podocytes it was commonly used as a control in different experiments. Puromycin (puromycin aminonucleoside; Sigma Aldrich Sweden, Stockholm, Sweden) is the aminonucleoside portion of puromycin (antibiotics) and is used both *in vivo* and *in vitro* studies to model nephrotic syndrome [13]. Through actin disruption puromycin rearranges the actin cytoskeleton and thereby induces nephropathy. The medium used to feed the podocytes in the cell culture constitute of RPMI 1640 (Sigma-Aldrich®) Penicillin-Streptomycin, to prevent bacterial contamination, and 10 % Fetal Bovine Serum, FBS.

Catalase experiments

Due to having several kinds of different catalase experiments, I have divided them in four parts (1-4). In the first part, we evaluated the possibility about an increased gene expression (catalase-mRNA) due to MC1R-stimulation. In part two, I searched for orienting background facts through theoretical article researches, to find potential further approach in how we can evaluate possible mechanism behind activation,

inhibition or degradation. In part three, we utilized the information I have found about the previous stated principles of regulation. Finally, in part four, we investigated the properties of the constitutive active MC1R mutant by analysing the catalase activity, the cAMP levels in the podocytes and the cell viability.

1. Catalase mRNA-expression

We used four different treatments on podocytes with MC1R or virus control, to compare the effects of each substance (see table 1). The podocytes representing the virus control have an endogenous (normal) expression of MC1R. The cells overexpressing MC1R has a much higher amount of functional MC1R-protein. The effects of MC1R-stimulation by the agonist will therefore be more distinct. The podocytes were incubated with the different treatments in 6, 24, 48 or 72 hours.

Overview of the experimental treatments

	Virus control	MC1R
Control 1	PBS	PBS
Control 2	1 % DMSO	1% DMSO
MC1R-agonist	0,1 µM	0,1 µM
Incubation time	6 – 24 – 48 – 72 h	6 – 24 – 48 – 72 h

Table 1: The two controls existed of PBS (Phosphate Buffered Saline), an isotonic water-based salt solution, or 1 % DMSO (Dimethyl Sulfoxide). The experiment podocytes were treated with 0,1 µM MC1R-agonist. One sample represents one well and there was the same amount of podocytes cultured in each well. The samples were analysed in duplicates.

To examine the catalase expression level the amount of catalase mRNA were measured in harvested cell cultures with the different treatments. mRNA-purifications was done with RNeasy® (QIAGEN) and thereafter the RNA-concentrations of every sample were measured by using NanoDrop (Thermo Fisher Scientific Inc.). With these concentrations the volumes needed for cDNA-synthesis of each sample were calculated. cDNA is needed to be able to perform qPCR with Taqman® (Life Technologies Corporation, Thermo Fisher Scientific Inc.), where the gene-expression

of catalase can be analysed. To detect any difference between our samples with different treatments and a normal expression we compared them against the housekeeping gene Glyceraldehyde 3-phosphate dehydrogenase, GAPDH. Housekeeping genes are genes that are stable in their expression at all times and therefore can be used to relate your genes to.

The changes in mRNA-expression between controls and stimulated podocytes were calculated from the results from the qPCR. The Ct (cycle treshold) values define the amount of PCR-cycles necessary to get the fluorescent signal to exceed the background level. The Ct value is inversely proportional to the amount of nucleic acid, which means the lower Ct the higher expression of the gene in terms of mRNA copy number. The differences in Ct values between the gene of interest and the housekeeping gene (GAPDH) give us ΔCt and by using this we can compare different samples to each other normalized to differences in GAPDH. When calculating differences in ΔCt between control and stimulated groups we got ΔΔCt. If this is zero we do not have any differences between the groups and negative ΔΔCt values corresponds to an increase of the gene. Further calculation by using 2^-ΔΔCt visualizes changes of catalase gene expression between samples (fold change).

2. Article research

This was done by going through articles found at the database PubMed, provided by National Center for Biotechnology Information (NCBI). The data about inhibition mechanisms are presented under *Results* but the remaining report is found in *Appendices*.

3. Inhibition of catalase activity

According to the results given from the article research about inhibition mechanisms, we had to evaluate the optimal application conditions of this mechanism in our experiments. To begin with, we had to determine the optimal concentration of the found catalase inhibitor, with regards to administration and ratio between desired effect and damage of the podocytes. With this information we expected to be able to optimize the use of the inhibitor in upcoming experiments. These podocytes were not transduced with any virus and therefore all of them had a normal expression of MC1R and hereafter named wild type (wt). Instead they were exposed to different concentrations of the catalase specific inhibitor (see table 2). By this experiment we analysed the inhibition capacity by comparing the catalase activity between podocytes exposed to different concentrations of the catalase specific inhibitor. The catalase activity was measured in the same procedure as below where the constitutive active mutant is analysed (see part 4).

Overview of the experimental treatments

	wt	wt	wt	wt
Inhibitor	- (Control)	5 mM	10 mM	20 mM
Incubation time	24 - 48 - 72 h	24 - 48 - 72 h	24 - 48 - 72 h	24 - 48 - 72 h

Table 2: Podocytes with normal expression of MC1R were treated with the catalase specific inhibitor in the concentrations of 0, 5, 10 or 20 mM during 24, 48 or 72 hours. Each sample were analysed in triplicates.

4. Evaluation of the constitutive MC1R mutant activity

Catalase activity

Conditionally immortalized mouse podocytes were cultured in 6-well plates and transduced with three different types of lentivirus. The podocytes were made either normal expressive (virus control) or over expressive of MC1R or of the constitutively active MC1R-mutant (see table 3).

To measure the protein concentration in each harvested sample we followed the protocol in BCA (Bicinchonicic acid)-assay Kit (Thermo Fisher Scentific Inc.). Thereafter the samples are visualized by measuring the absorbance at 562 nm on a plate reader (The SpectraMax® Plus 384 Microplate Reader). The results are summarized in the software (SoftMax® Pro). These concentrations were needed to calculate the volume of each sample to Amplex® Red catalase Assay Kit (Molecular Probes®). With the catalase assay we measured and compared the activity of catalase in the differently treated podocytes, normalized to total protein. The measurements were done by reading the fluorescence with a plate reader (Gemini XPS Microplate Reader) and the results were also in this case analysed with SoftMax® Pro.

Overview of the experimental treatments

	Virus control	MC1R	MC1R with inhibitor	Constitutive MC1R-mutant	Constitutive MC1R-mutant with inhibitor
Treatment	10 nM MC1R-agonist	10 nM MC1R-agonist	10 nM MC1R-agonist	DMSO	DMSO
Incubation time	0 – 24 – 48 – 72 – 96 h	0 – 24 – 48 – 72 – 96 h	95 h	0 – 24 – 48 – 72 – 96 h	95 h
Inhibitor	-	-	10 mM	-	10 mM
Incubation time	-	-	1 h	-	1 h

Table 3: Theoretical layout of the different podocytes and their treatments. There were two plates of each podocyte type, which are described above. The low-expressive and MC1R transduced were treated with 10 nM MC1R-agonist during 0-96 hours or 10 nM MC1R-agonist during 95 hours and thereafter 10 mM catalase inhibitor during one hour before harvesting. The constitutive MC1R mutant transduced podocytes were treated with DMSO during 0-96 hours or DMSO during 95 hours and then 10 mM catalase inhibitor during one hour, just like the earlier podocytes. Harvesting was done at the time of 0, 24, 48, 72 and 96 hours. Each sample were analysed in triplicates.

cAMP-level

To evaluate the constitutive MC1R-mutant we compared cAMP-levels in different podocytes, owing to the accumulation of cAMP due to MC1R-activation. The podocytes overexpressing the constitutive active mutant were compared to podocytes

of three other types: MC1R-overexpressive and normal MC1R-expressive with or without virus transduction (virus control and wt, respectively).

The wild type and virus control are both used as controls to show that the podocytes themselves, or the virus do not have any effect on the cAMP-accumulation due to MC1R-stimulation.

The podocytes were cultured in 6-well plates in all experiments and incubated with different treatments (see table 4). Afterwards we ran a cAMP-assay, cAMP-screen® System (Invitrogen®, Thermo Fisher Scientific Inc.) to measure the difference in activity in podocytes transduced with MC1R or the constitutive MC1R mutant.

Overview of the experimental treatments

	wt	Virus control	MC1R	Constitutive MC1R-mutant
Control 1	Medium	Medium	Medium	Medium
Control 2	DMSO	DMSO	DMSO	DMSO
Positive control	Forskolin	Forskolin	Forskolin	Forskolin
MC1R-agonist	0.01 nM	0.01 nM	0.01 nM	0.01 nM
MC1R-agonist	0.1 nM	0.1 nM	0.1 nM	0.1 nM
MC1R-agonist	1 nM	1 nM	1 nM	1 nM
MC1R-agonist	10 nM	10 nM	10 nM	10 nM
MC1R-agonist	100 nM	100 nM	100 nM	100 nM
MC1R-agonist	1000 nM	1000 nM	1000 nM	1000 nM
MC1R-agonist	10 000 nM	10 000 nM	10 000 nM	10 000 nM

Table 4: The experiments were done with culturing of the different podocytes in 6-wells plates. The two controls existed of medium (RPMI 1640, P/S and 0.1 % FBS) or DMSO (Dimethyl Sulfoxide), which is a solvent for MC1R-agonist; Forskolin and IBMX (3-isobutyl-1-methylxanthone). The experiment podocytes were treated with the MC1R-agonist in different concentrations or the cAMP-activator Forskolin (FSK; positive control). In some experiments the concentration range of MC1R-agonist were between 0.01 – 1000 nM and in some 0.1 – 10 000 nM. All podocytes were also treated with the phosphodiesterase inhibitor IBMX which prevent degradation of cAMP. All samples were analysed in duplicates.

Cell viability

To visualize the cell viability AlamarBlue® (LIFE technologies, Stockholm, Sweden) were used. AlamarBlue® create a gradient of higher fluorescence with higher

viability. The active reagent is resazurin, a non-toxic, cell permeable compound which is blue and virtually non-fluorescent. When it has entered cells it will be converted to the fluorescent resorufin, which have an excitation at 530-560 nm and emission at 590 nm. All living cells have a natural capacity of this conversion and the degree of fluorescence stands in proportion to the amount of living cells. By measuring the increase in fluorescence the change in viability can be determined. We did the fluorescent readings day -3, 0, 3 and 8. At day 3 we saw the result of the different experiment mediums added after the reading at day 0. The different podocytes used were MC1R-expressing, constitutive MC1R-mutant-expressing and virus control. These were treated with experiment medium at day 0, just after the reading of fluorescence (see table 5). At day 3 the experiment medium was changed to regular medium and the podocytes had a recovery period of five days until the last

Overview of the experimental treatments

	Virus control	MC1R	Constitutive MC1R-mutant
Control	DMSO	DMSO	DMSO
MC1R-agonist	10 nM	10 nM	10 nM
Puromycin	10 µg/ml	10 µg/ml	10 µg/ml
Puromycin	20 µg/ml	20 µg/ml	20 µg/ml
Puromycin / MC1R-agonist	10 µg/ml / 10 nM	10 µg/ml / 10 nM	10 µg/ml / 10 nM
Puromycin / MC1R-agonist	20 µg/ml / 10 nM	20 µg/ml / 10 nM	20 µg/ml / 10 nM
Incubation time	72 h	72 h	72 h
Inhibitor (Exp. 1+2)	20 nM	20 nM	20 nM
Incubation time	1h	1h	1h
Inhibitor (Exp. 3)	10 nM / 20 nm	10 nM / 20 nm	10 nM / 20 nm
Incubation time	24h	24h	24h

Table 5: The experiment medium contained DMSO 0.1% (control), 10 nM MC1R-agonist, 10 µg/ml puromycin, 20 µg/ml puromycin, 10µg/ml puromycin and 10 nM MC1R-agonist or 20 µg/ml puromycin and 10 nM MC1R-agonist. In the first and second experiment we added 20 nM catalase specific inhibitor to some plates one hour before the regular experiment medium at day 0 (after the reading). In the third experiment the podocytes were incubated with the inhibitor for 24 hours instead, from day 0 to day 1. Thereafter they got the regular experiment medium (without any extra reading at day 1)

reading at day 8. Wells only containing alamar solution were used as blanks. The results will be normalized to the control of day 0 in respective experiment and the analysis will show percental differences between the wells. .

Statistical methods

All raw data was summarized and gathered in Excel (Microsoft Office, ©Microsoft Corporation) to sort and/or make further calculations. With some raw data from the experiment runs I did graphs in GraphPad Prism® (©GraphPad Software Inc.) to easily visualize the results. Thereafter, to confirm eventual differences and if these were statistically significant I used analytical methods in Excel or GraphPad. Primarily I calculated p-values by using two-tailed type 2 student's T-tests and to do that we assumed a normal distribution in all experiments. When comparing more than two groups, I used one-way ANOVA. I used two-tailed t-tests to not make preconceived assumptions or make reformulations of H_0 (null hypothesis; a statement suggesting that every seen difference is due to chance until they are proven otherwise). With a two-tailed analysis I do not in advance determine which of the comparative groups that have the highest or lowest mean values. Type 2 is used because the comparing values come from different types of podocytes: MC1R-overexpressing, constitutive active MC1R-mutant-overexpressing or normal expressing with or without virus. The different podocytes in comparison got same treatment with damaging or stimulating agents so the assays were to see if the divergent podocytes reacted different to these. The results are presented as mean ± s.e.m. if $n \geq 3$. A calculation of results with $n \geq 3$ and a p-value < 0.05 are considered statistically significant. A p-value < 0.05 is defined as * in the graphs and p-value < 0.01 and <0.001 are defined as ** and ***, respectively.

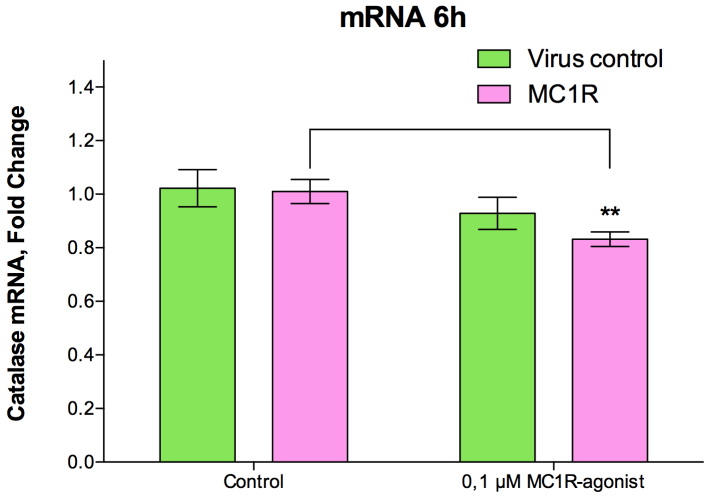
Ethics

All experiments have been performed using mouse podocytes from Dr. Mundel's Lab in Boston, MA. In the isolation and transformation of these cells from mice, the Lab in the US acted after approval of the Local Ethical Committee. The use of these cells, and the viral transfection of mutated or human proteins, does not require ethical permission. It does however require a special permit for laboratory work with virus that the Lab has.

Results

1. Catalase mRNA-expression

The mean fold change after six hours in both virus control and MC1R-podocytes were lower with stimulation by MC1R-agonist; 0.929 ± 0.06 and 0.832 ± 0.027 respectively, compared to the six hours-controls with 1.022 and 1.01 ± 0.045 respectively, (see graph 1). Consequently, the gene expression of catalase has been decreased due to stimulation during six hours. The podocytes overexpressing MC1R decreased even more than the virus controls.



*Graph 1: N = 5. Fold change in Catalase mRNA at the time of 6 hours between virus control and MC1R-podocytes, compared to respectively control at the same time. There is a non-significant decrease of mRNA in virus controls (p=0.3) and a significant decrease in MC1R-podocytes (p<0.01, **).*

2. Article research

Inhibiting pathways

The most interesting facts I found in the articles were about the existence of the catalase specific inhibitor, 3-amino-1, 2, 4-triazole, 3-AT/AMT/ATZ (hereafter referred to as catalase inhibitor). This was the only inhibitor we found to be catalase specific [14].

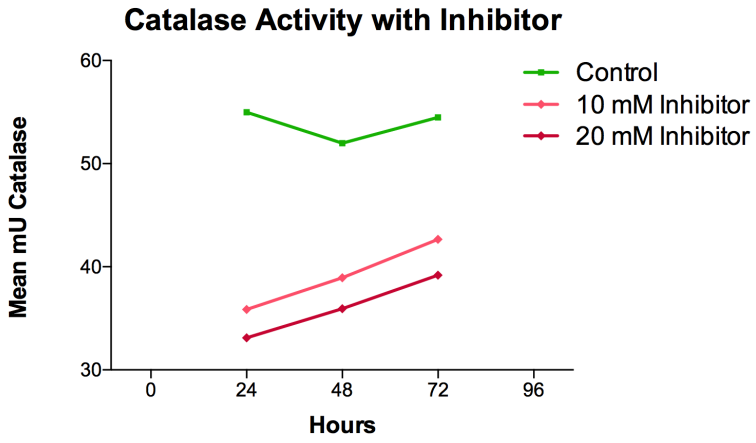
Aragon *et al*, 1991, observed brain and liver catalase in Male Long Evans rats, who got the catalase inhibitor administrated intraperitoneally [15]. They showed a dose- and time-dependent inhibition. The recovery of catalase activity in respective tissues was associated with the disappearance of the catalase inhibitor and consequently this was found to be an irreversible inhibitor. They also investigated the amounts of H_2O_2 in the tissues and concluded a dependence of the catalase inhibitor activity and rate of generated H_2O_2. The catalase inhibitor seems to bind a compound of catalase and H_2O_2. Without H_2O_2 no inhibition is observed, neither does it inhibit in the presence of alcohol, because alcohol compete to bind the same site in the catalase-H_2O_2 compound. The difference between them is that the catalase inhibitor binds irreversibly [16]. Moreover, and on the contrary, they also refer to previous studies, including one by William RN *et al* [17] where they are suggesting the catalase inhibitor to be indirect in action rather than having a direct effect on catalase. According to this the catalase inhibitor would undergo metabolic transformation, in the topic tissue, to become an active inhibitor.

To have guidance in reasonable administration doses of the catalase inhibitor directly to cells in cell culture further studies were investigated. Nenoi M. *et al*, 2001, used HP-100 cells (H_2O_2-resistant variants derived from human leukaemia HL60 cells)

which were incubated during 2 hours with the concentration of 25 mM the catalase inhibitor [18]. HP-100 cells were also used in another study by Hachiya M. *et al*, 2005, and they incubated with 20 mM instead and during one hour [19].

3. Inhibition of catalase activity

We tested four different concentrations and had measure points of catalase activity at the time of 24, 48 and 72 hours. I saw less catalase activity in all inhibitor-exposed podocytes compared to the podocytes not exposed to the inhibitor (control). There was an inhibitory effect with all concentrations in an almost perfect dose-dependent manner, except at 72 hours with 5 mM inhibitor (excluded in the graph). The higher concentration of the inhibitor, the lower level of catalase activity was observed (see graph 2). The lowest activity was seen at 24 hours and with time the catalase activity seems to recover. A later measure point has a mean activity of 109 % compared to the previous one. The activity at 24 hours of podocytes exposed to either 10 mM or 20 mM correspondents to 65.2 % and 60.2 %, respectively, of the activity in controls at the same time.

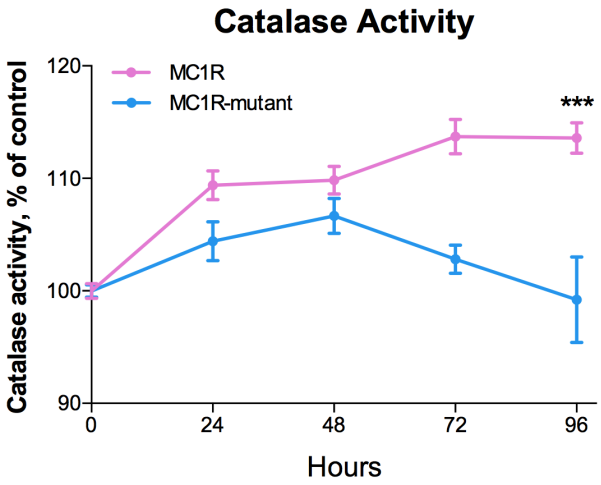


Graph 2: N = 2. The catalase activity decreased as the concentration of the inhibitor increased. At the time of 24 hours the biggest differences between controls and exposed podocytes were seen: 54.999 mU catalase compared to 35.857 and 33.122 mU catalase, respectively. The exposed podocytes had an activity of 65.2 % and 60.2 %, respectively, of the control activity at this time. The recover between two measure points gives an activity of 109 % compared to previous. mU correlates to the degree of catalase activity, as 1 U (unit) is defined as the required amount of catalase to decompose 1 μM H_2O_2 per minute at pH 7 at 25°C.

4. Evaluation of the constitutive MC1R mutant activity

Catalase activity

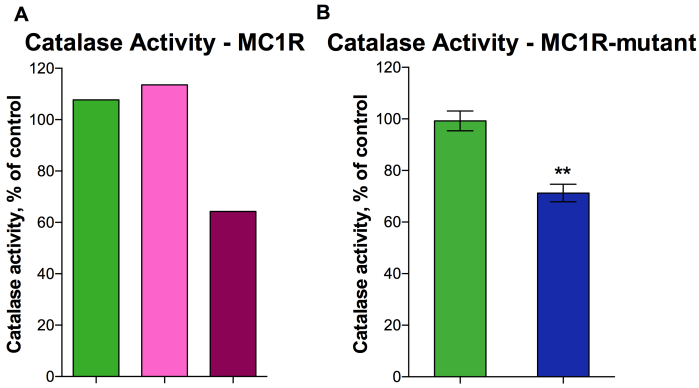
The degree of catalase activity in the different categories of podocytes was normalized to the starting value at zero hours, to show the development of the activity. Certain individual measurements could not be used due to technical problems, but no valid values were discarded. All starting values were correct and the normalization is thereby not impacted.



Graph 3: At the time of 96 hours the stimulated MC1R-podocytes had an activity at 113.6 % ± 1.357 of starting value (n=4), compared to the activity of non-stimulated podocytes with MC1R-mutant at 99.22 % ± 3.819 (p = 0.745, n=3).

In the podocytes overexpressing MC1R, we saw an increased catalase activity due to stimulation with MC1R-agonist (see graph 3). At the time of 96 hours, the activity was 13.6 % higher compared to the starting point ($p<0.001$, ***). Contrary to our hypothesis the catalase activity in podocytes with MC1R-mutant remained unchanged.

Podocytes exposed to catalase specific inhibitor exhibited decreased levels of catalase activity compared to the controls at 96 hours (see graph 4). The MC1R-overexpressing podocytes had an activity of 59.64% and the podocytes with MC1R-mutant had an activity of 71.27% ± 3.392 ($p < 0.003$). Comparison between MC1R and MC1R-mutant shows a trend of less inhibition in the podocytes with MC1R-mutant.

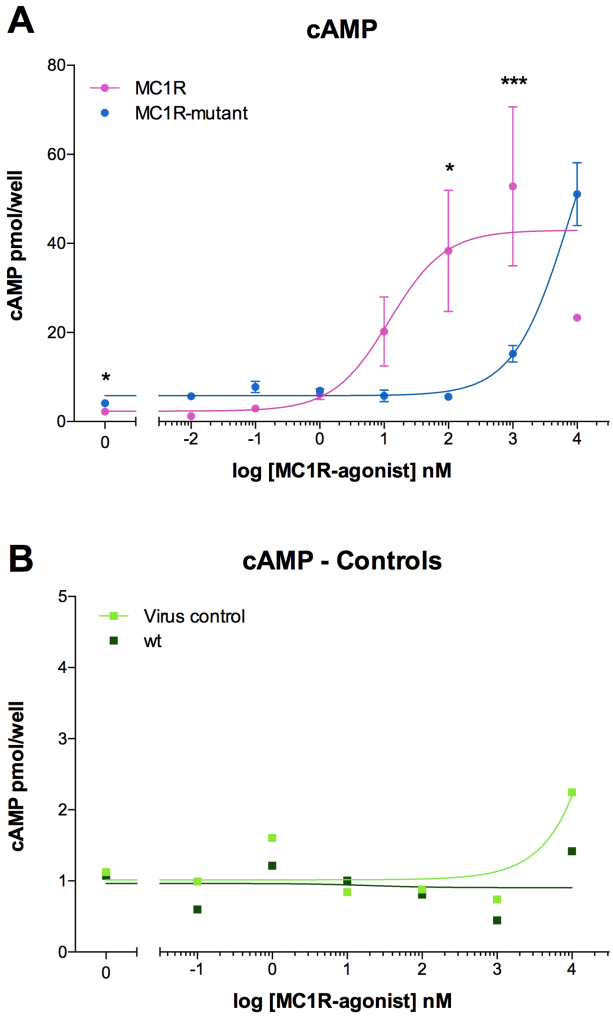


Graph 4: The catalase specific inhibitor decreases catalase activity in both podocytes with MC1R (A) and MC1R-mutant, respectively (B). All values are normalized to the starting value at 0 hours. Activity after exposure to catalase inhibitor compared to control at 96 hours (green bar) were 59.64% in MC1R-podocytes (n=1) and 71.27% ± 3.392, $p < 0.003$, in podocytes with MC1R-mutant (n=4).

cAMP-level

Podocytes overexpressing MC1R had a higher endogenous cAMP activity, and got an even higher concentration, of cAMP in response to MC1R-agonist stimulation with concentrations of 10 – 10 000 nM (see graph 5). The dose-dependent correlation had a statistical significance in the range of >10 nM (n = 4), with an exception at 10 000 nM MC1R-agonist which had to be excluded due to too few repeats (n = 2). The constitutive MC1R-mutant had a constitutive activity and did not answer to stimulation by MC1R-agonist in a range of 0,01 – 100 nM. Hovewer, with a concentration >100 nM even the constitutive MC1R mutant had a higher activity and showed a higher concentration of cAMP in dose-dependent manner.

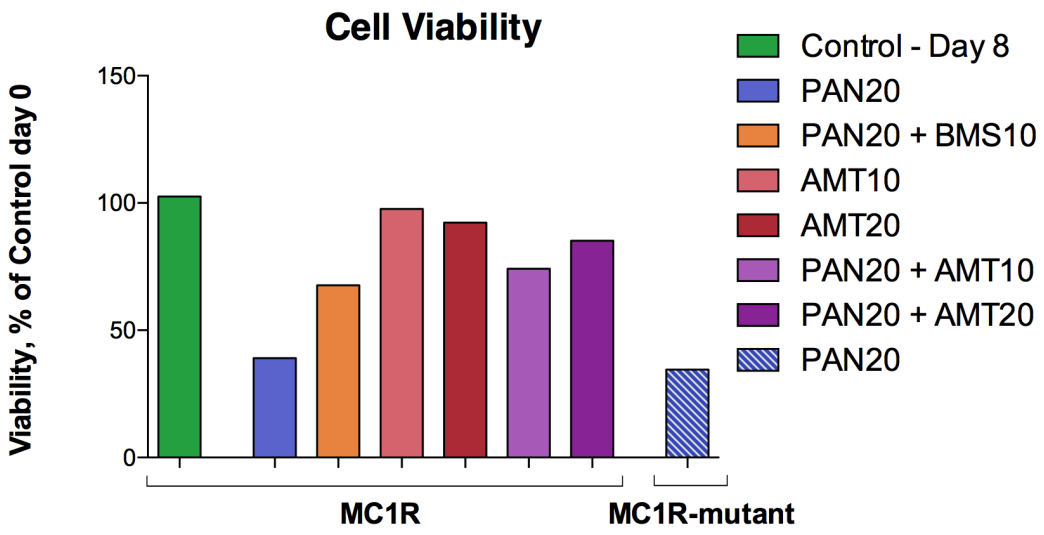
The means of cAMP-concentration in the non-stimulated podocytes of each type had a statistical significant difference with the values of 2.236 ± 0.645 pmol/well (MC1R) and 4.113 ± 0.3894 pmol/well (MC1R-mutant), respectively. A calculation of the best-fit values in the non-linear fit gives starting values of 2.333 pmol/well and 5.818 pmol/well, respectively. These lines are horizontal until the responses on stimulation and additionally, they are closely related to the values of the non-stimulated podocytes and can represent a baseline of non-response (baseline) activity in the podocytes. The baseline of the MC1R-mutant is 2,49 times higher than the baseline of MC1R and this correspondents to 11,01% of the maximal MC1R-activity seen with stimulation of 1000 nM.



Graph 5: The MC1R-mutant had a cAMP-concentration of 4.113 ± 0.3894 pmol/well, which was statistical significant higher than the cAMP-concentration of 2.236 ± 0.645 pmol/well in MC1R-podocytes ($p < 0.05$, n=4). The calculated non-linear fit give a best fitted starting value of cAMP-concentration at 5.818 and 2.333 pmol/well, respectively. With stimulation >1 nM the MC1R-podocytes showed a dose-dependent relationship between MC1R-agonist and cAMP-concentration. The increase showed a statistical significance with stimulation of 100 – 1000 nM ($p < 0.02$* - <0.001***, n=4). The dose of 10 000 nM had a n-number of 2. With a stimulation of >100 nM the podocytes with MC1R-mutant also had a dose-dependent relation to cAMP-concentration, (A). The two controls did not answer to stimulation; mean cAMP-level were 1.217 and 0.915 pmol/well, respectively (n=2), (B).*

Cell viability

We ran three experiments: one in 24-wells plates and two in 12-wells plates. The experiment in 24-wells plate was interrupted and will not be reported. Neither MC1R-agonist nor the concentration of puromycin in 10 µg/ml did show any effect on viability in any direction. According to the trend of data (n=2) in this direction it is uninteresting to do further analysis on the effects of MC1R-agonist itself or if the inhibitor should take away any protecting effects of the same. The parameters with MC1R-agonist (except in combination with puromycin 20 µg/ml) and puromycin 10 µg/ml are therefore excluded in the analysis and any results will not be reported. The exception where MC1R-podocytes were exposed to both MC1R-agonist and puromycin 20 µg/ml, there was shown a higher viability with 173,6 % compared to those only exposed to puromycin 20 µg/ml (see graph 6). The podocytes overexpressing the MC1R-mutant did not show any protection from viability decreased by puromycin and neither further analysis with the MC1R-mutant will be done. Remaining is the effect of the inhibitor and the MC1R-podocytes exposed to the catalase specific inhibitor and puromycin showed in a dose-dependent fashion more viability than the ones exposed only to puromycin. The inhibitor in itself had a weak negative effect on the viability.



Graph 6: (n=2) Day 8. The podocytes with MC1R-mutant exposed to puromycin 20 µg/ml showed less viability, 34.51 % of control at day 0. MC1R-podocytes exposed to puromycin or puromycin and MC1R-agonist had a viability of 39.05 % and 67.66 %, respectively. MC1R-podocytes exposed to the inhibitor showed a weak dose-dependent decrease in viability: 97.72 % and 92.31 %, respectively. The MC1R-podocytes exposed to both the inhibitor and puromycin showed reversed dose-response with higher viability; 74.14 % and 85.20 %, respectively. PAN20 = puromycin 20 µg/ml, BMS10 = MC1R-agonist 10 nM, AMT10/20 = inhibitor 10 mM/20 mM.

Discussion

On the whole my work evolved to be a development of experiment types and methods to get new approaches to future research. With this we have been able to dismiss some research questions and evaluate our strategies to examine the hypothesis about the role of catalase in the downstream mechanisms of MC1R-stimulation.

From our examination of the regulation of catalase we could exclude a transcriptional dependence on and continued to examine catalase by regulation on protein level. Here we characterized a catalase specific inhibitor to evaluate the connection between

MC1R-activation and catalase activity. In other words, we intended to show that the mediation of the positive effects seen with MC1R-activation occurs through catalase, because podocytes with inhibited catalase will be more affected by damaging agents, despite the fact that MC1R is active. Our experiments showed that the inhibition of catalase was dose-dependent, meaning that the higher concentration of the inhibitor the podocytes were exposed to the less activity seen by catalase. However, to fully evaluate the relationship between inhibitor dose and catalase activity in podocytes, an increased number of repeats is required. This is also preferable to get higher strength to the results. Further, we also saw a recovery of the catalase activity over time and as referred to before, in previous experiments done by Aragon *et al*, 1991 [15], has shown a recovery in activity dependent on the disappearance of inhibitor in rat brain and rat liver. The concentrations used in the experiments were hereafter 10 or 20 mM.

Further we analysed the constitutively active E94K MC1R-mutant and compared it to regular MC1R. As expected, MC1R showed a dose-dependent activity of intracellular cAMP increase and the mutant a constitutive cAMP accumulation, but not in the range we had predicted. According to previous studies done by Benned-Jensen *et al* [6], the activity of the mouse constitutive MC1R-mutant lies in a range around 50 % of stimulated MC1R-maximum, but our data of the previously untested human mutant were just showing an constitutive activity of 11 %. We saw an activity independent on stimulation (until doses ≥ 100 nM) that was 2,49 times higher than baseline in MC1R overexpressing podocytes, but not as high as we had expected. We also compared the catalase activity in podocytes overexpressing MC1R or MC1R-mutant, respectively. Contrary to our hypothesis, we were unable to see an increased catalase activity in the podocytes with MC1R-mutant over time. However, we saw the expected up-regulation of catalase activity in podocytes overexpressing MC1R, stating the

connection we already knew from earlier experiments [2]: induced MC1R-activity leads to increased catalase activity. The inhibition of catalase by the specific inhibitor did affect both podocyte types with a decreased activity and with this we also got further evidence of its inhibition capacity.

To summarize the different reasons behind the vague results and difficulties in drawing new conclusions from the catalase activity, we had technical problems, which lead to an inability to reproduce the experiments as we wanted. We identified unusable measurements to be either due to a recurrent fault in the concentration calculations or to an administration mistake concerning the standards in an Amplex Red® assay. The miscalculations of the concentrations were caused by a setting error in the software that summarized the protein concentrations (SoftMax® Pro) during the BCA-assay.

To conclude the catalase activity experiments, the human mutant did not completely have the effect we wanted and future experiments are probably better done with mouse mutant, E92K. If this works better it could also be interesting for future mouse models. With genetically engineered mouse models it might be possible to analyse differences in downstream effects using constitutionally active MC1R.

In addition, we tried to visualize the viability of podocytes overexpressing MC1R or MC1R-mutant, respectively and also in relation to catalase activity interfered with inhibitor. We assumed that the podocytes with the highest catalase activity should be most viable. With the use of the catalase inhibitor the role of catalase in podocyte protection could be further investigated. Through this we wanted to get better preconditions in the process to elucidate whether the protective effects of MC1R is dependent on catalase. Unfortunately, these experiments were followed by technical

problems and therefore a very low number of repeated experiments were performed. According to the data we got, we did not see a higher viability in podocytes with MC1R-mutant exposed to puromycin compared to MC1R-podocytes with same exposure. This can be explained by the results from the experiment, previously discussed, with measurements of catalase activity in both kinds of podocytes. The MC1R-mutant did not show a higher catalase activity and the results of the viability assays do according to this make sense. The mutant did neither show increased catalase activity, nor increased protection against puromycin induced reduction of viability. The catalase inhibitor did not in itself induce significant loss of viability nor stronger effects together with puromycin compared to podocytes only exposed to puromycin. On the contrary, the podocytes with a combined treatment had higher viability than the other ones. The protection against puromycin induced viability loss is hard to explain, but it might be due to cross-reaction between the inhibitor and puromycin, despite studies suggesting otherwise [20]. In summary, our experiments does not provide enough evidence that catalase is the reason for the protection of podocytes against the harmful nephrotoxin puromycin, but we did gain knowledge regarding the regulation of catalase in podocytes following MC1R stimulation.

Conclusion and Implications

We are in the process of investigating the signaling pathways activated by MC1R-stimulation in podocytes. We believe that increased catalase activity plays an important role and we have been able to make some progress in examining the effects of catalase in podocytes.

In this thesis, we observed that catalase does not seem to be transcriptionally regulated by MC1R, and we assume the regulation to be at the protein level. We were able to dose-dependently inhibit the activity of catalase by using a specific inhibitor. In addition, we also overexpressed and characterized the human MC1R-mutant, E94K, which were shown to exhibit a low constitutive MC1R-activity.

However, we still have a long way to go and we need to reach additional insights before understanding the bigger picture. If we can verify catalase to be the mediator of the ameliorating effects of MC1R-stimulation in nephrotic syndrome, this information can be used to plan new treatment strategies in future medical studies. Development of new treatments is desirable, since the treatment options available are scarce, ineffective and non-specific. MC1R-mediated changes in catalase activity might be a key to reach this goal.

Populärvetenskaplig sammanfattning

Vid njursjukdom med skador på beståndsdelar i njurens filtrationsenhet (exempelvis den specifika celltypen podocyter) utsöndras ämnen, som egentligen ska stanna kvar i blodet, till urinen. Det är främst ett proteinläckage som sker och därmed fås protein i urinen. Det finns många njursjukdomar av detta slag och en av dem är Membranös Nefropati (MN), med en incidens på 5-10 per miljon invånare per år och som kan leda till njuren helt slutar att fungera. MN saknar botande behandling men man har sett att behandling med ett visst hormon (signalsubstans), kallat adrenokortikotropt hormon (ACTH) ger ett minskat proteinläckage. Vi tror att behandling med ACTH påverkar de specifika filtrationscellerna (podocyterna) positivt genom att aktivera en signalmottagare (receptor) på deras cellyta, kallad Melanocortin-1 receptorn (MC1R). Denna receptor har setts leda till en ökning av aktiviteten hos det reaktionseffektiviserande proteinet (enzym) katalas som finns inne i filtrationscellerna och vi tror att detta är en viktig del av den positiva och förbättrande effekten man ser av ACTH. Katalas omvandlar skadliga föreningar till de oskadliga ämnena vatten och syre. Detta kan rädda en skadad cell från att dö och istället återhämta sig och återfå sin funktion.

Genom att sätta in en förändrad variant av MC1R som alltid har en aktivitet (oberoende av signalsubstans) i podocyter som vi har i odling, ville vi kunna förenkla framtida experiment genom att slippa stimulera receptorn för att den ska fungera. Experiment visade dock att den alltid aktiva varianten av MC1R bara uppvisade 11 % av full aktivitet och den var därför inte intressant för fortsatta experiment.

Vi analyserade även katalas roll i podocyterna och för att specifikt utreda det tittade vi på olika möjligheter till aktivitetsreglering av katalas. Först tittade vi på om

aktiviteten styrs via nyskapande av katalas eller om det finns regleringsmekanismer som påverkar redan existerande mängd av katalas. Dessutom tittade vi på hur podocyter mådde i relation till aktiviteten av katalas. Vi såg ingen ökad nyproduktion av katalas vid stimulering av MC1R och regleringen bör således inte bero på detta. Vi gick vidare till att studera om vi kunde påverka aktiviteten hos redan befintligt katalas. Genom att minska katalasaktiviteten kan man sammankoppla MC1R-aktiveringen med katalas. Med andra ord ville vi testa om förmedlingen av den positiva effekten av MC1R-aktivering sker via katalas eftersom mindre katalasaktivitet leder till att cellen skadas/dör trots att MC1R är aktiverat. Våra experiment visade att aktivitetsminskningen av katalas är beroende av dos, vilket betyder att ju mer av aktivitetsminskaren vi tillförde desto svagare aktivitet uppvisade katalas.

Den fulla omfattningen av MC1R-signalering och effekterna av katalas i podocyter är fortfarande okänd. Mer kunskap kan möjliggöra utformningen av nya läkemedel specifikt inriktade mot aktivering av MC1R eller påverkan på katalas. Positiva resultat skulle bli ett betydande bidrag till de få behandlingsalternativen som idag finns till patienter med proteinläckande njursjukdom.

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Appendices

Activating pathways

We mainly found four different activating pathways of catalase and three of them were mediated on protein level by; 1) protein kinases, 2) Sirt1 – FoxO3a or 3) c-Abl and Arg. It also exist regulation at 4) transcriptional level.

1. Protein kinases

Protein kinase A and C (PKA resp. PKC) and Casein kinase II (CKII) have been shown to increase catalase activity in human erythrocytes [21]. PKC requires longer time than PKA (rapid change) to reach the plateau where catalase activity was increased but there is also a difference between different PKC isozymes. PKC consists of several isozymes and their ability of modulation is due to the presence or absence of PKC activators. The most effective in both presence and absence of these is PKCζ. Because of the ability to modulate in the absence of activators PKCζ is likely to act as a constitutive modulator of catalase activity in most cell types. PKA and PKCζ have also a synergistic modulation effect.

Catalase is constituted of four identical 60 kDa subunits and several minor components. These minor components are probably phosphorylated and are proposed to be one of the reasons behind the protein kinase-dependent activity increase. This leads to changes in electrostatic folding of pre-phosphorylated 60 kDa subunit which affects catalase's active center.

A potent PKA activator is forskolin and it has shown a proportional increase in the expression of one of the minor components, the immunoreactive 110 kDa component. The major 60 kDa subunit did only show a weak increase in expression. Forskolin-

dependent increase of catalase is therefore likely to be associated with an expression increase of 110 kDa component and maybe together with the phosphorylation by PKA.

PKA regulation

PKA itself is an ATP-dependent kinase and is allosterically activated by cAMP, which is formed through cyclization of ATP (see figure I) [22]. Conversion of ATP is done by activation of a G-coupled receptor, where the activated G-protein in turn activates adenylate cyclase. Adenylate cyclases can also be activated by e.g. Forskolin [23]. Down-regulation of PKA is due to a negative feedback mechanism on cAMP. cAMP is hydrolyzed, in the action of phosphodiesterase, to become 5'-AMP [24].

PKC regulation

PKC is activated by a phospholipid as phosphatidylserine, diacylglycerol (DAG) and Ca^{2+} (see figure I) [25].

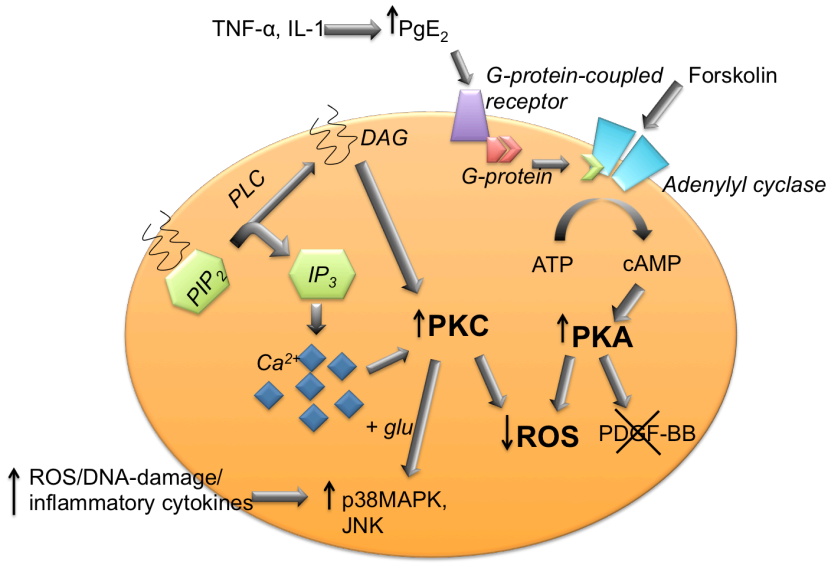

Figure I: Overview of intracellular mechanisms activated due to stimulation of G-coupled receptor or adenylyl cyclase.

MAPK-pathway

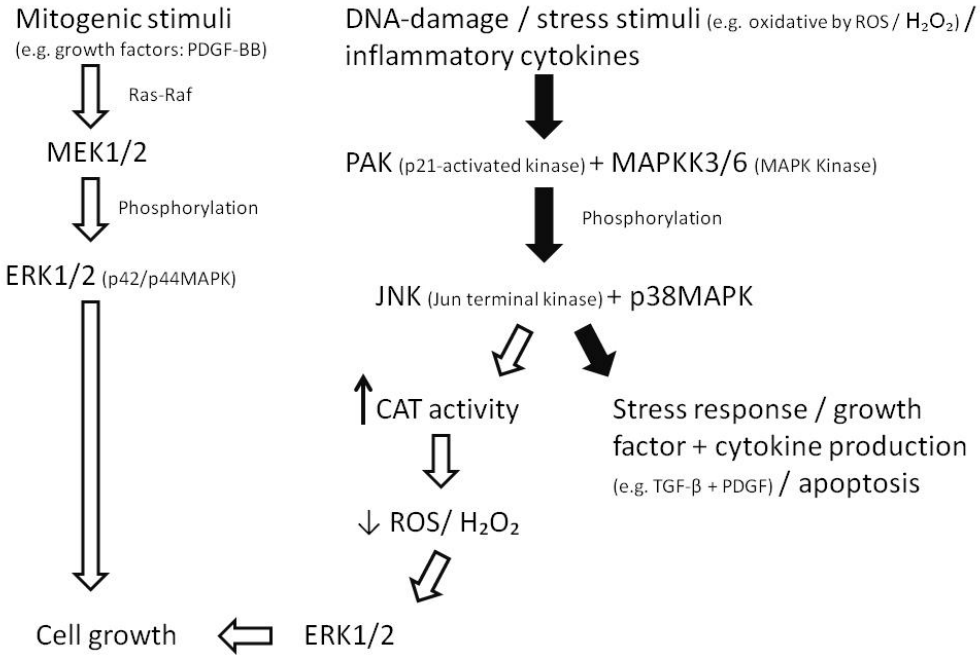

Figure II: Overview of intracellular mechanisms of the MAPK-pathway activated due to mitogenic or damaging stimulation.

There are two different Mitogen-Activated Protein Kinase, MAPK-pathways (see figure II) [19, 26, 27]. p38MAPK increase the catalase activity and protein amount as well as mRNA levels. Growth factor PDGF-BB binds to the PDGF-receptor. This receptor or the creation of Ras-Raf complex are inhibited by PKA and through this PKA decrease cell growth.

The cells used in these studies of the MAPK-pathway were; a) HP100 (Hydrogen Peroxide-resistant cells) as well as the cells they are derived from, human leukemia HL60 cells, b) V79 lung fibroblasts, c) aortic smooth muscle cells and d) cultured mesangial cells exposed to high glucose (probably from humans with diabetic nephropathy).

2. Sirt1 – FoxO3a

Sirt1 – stands for sirtuin, Silent Information Regulator Two (Sir2) protein – is a NAD^+-dependent protein deacetylase which deacetylate many target proteins, e.g. p53 and forkhead (FoxO) transcription factors [28, 29]. These proteins provide protection against apoptosis and play an essential role in mediating the survival of a lot of cell types. When Sirt1 is inhibited in human renal tubular cells under normal conditions apoptosis was induced and the expression of catalase was up-regulated. An overexpression of Sirt1 down-regulates catalase-expression. H_2O_2-treatment induces acute oxidative stress and the Sirt1-expression was up-regulated at the same time as the catalase-expression also was up-regulated, in contrary to the results from previous experiment. This leads to the conclusion that Sirt1 regulates the catalase-expression depending on the underlying conditions; if the cell is affected with H_2O_2 catalase expression is increased via Sirt1 but the other way around decreased.

- H_2O_2-affected cells → ↑ catalase via Sirt1
- Non H_2O_2-affected cells → ↓ catalase via Sirt1

This situation is due to FoxO3a (deacetylated by Sirt1), which differs in localization in the cell dependent on H_2O_2-levels:

- Absence of H_2O_2→ dominantly in the cytoplasm
- Presence of H_2O_2→ translocation to the nucleus

On the contrary Sirt1 is localized in both cytoplasm and nucleus under basal or H_2O_2-treated conditions. When H_2O_2 induces translocation of FoxO3a to the nucleus it co-localizes and forms a complex with Sirt1, which coincidently deacetylates FoxO3a.

This complex tips the cell away from apoptosis towards higher stress resistance with induced cell cycle arrest and an up-regulated catalase expression.

The cell cycle arrest gives the cell more time to detoxify ROS and repair damaged DNA. Accordingly, the stress resistance effects of FoxO3a are Sirt1-dependent and therefore the regulation of catalase expression is dependent on the presence or absence of FoxO3a in the nucleus during conditions of oxidative stress.

Sirt1 regulation

Sirt1 is encoded by the SIRT1 gene (see figure III) [30]. Lamin A binds to and activates Sirt1 [31]. Resveratrol enhances the activity indirectly by increasing the expression of SIRT1 and enhances the binding between Sirt1 and Lamin A.

AROS, active regulator of SIRT1, binds directly to the N-terminus of Sirt1. AROS enhances Sirt1-mediated deacetylation of p53, which thereby inhibits p53 transcriptional activity. Overexpression of AROS represses the p53 response and favours cell survival.

Stress activates p53 protein which through other gene expressions down-regulates SIRT1 and thereby forming a regulatory feed-back loop.

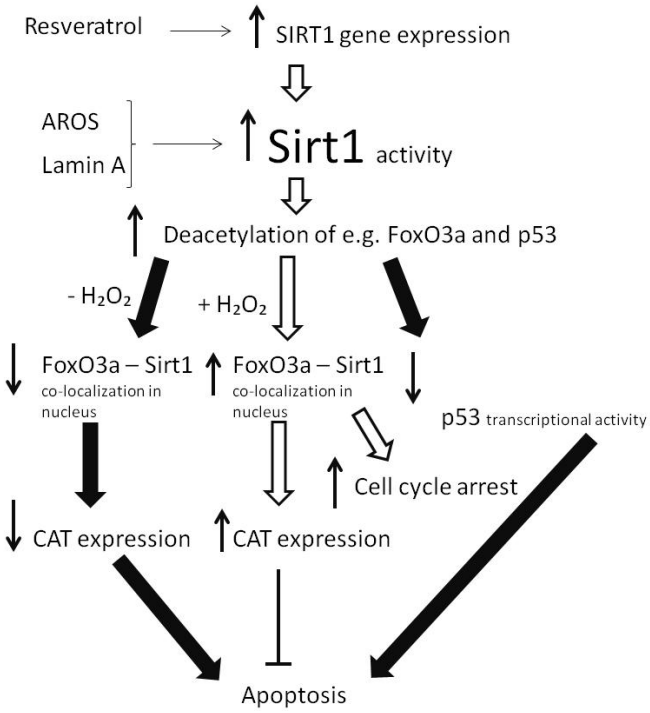

Figure III: Overview of the Sirt1-regulation

3. c-Abl and Arg tyrosine kinases

The activity of c-Abl and Arg tyrosine kinases are up-regulated due to oxidative stress and H_2O_2 induces the binding of them to catalase (see figure IV) [20]. The SH3 domain of respective kinase binds to catalase at PFNP (proline-rich) site and phosphorylate catalase at Y231 and Y386. c-Abl and Arg are required for catalase activity and both c-Abl- and Arg-deficient cells are hypersensitive to H_2O_2-induced apoptosis. By contrast there is also evidence indicating that c-Abl- and Arg-activation by H_2O_2 is necessary for induction of apoptosis and this is described further under the part about degradation pathways.

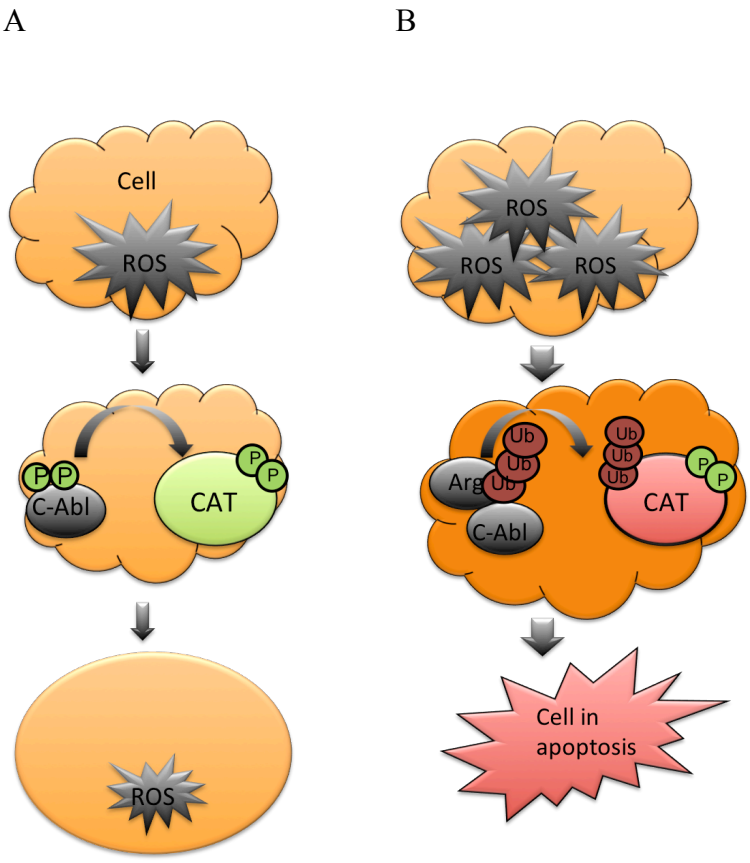

Figure IV: Overview of the C-Abl (A) and the Arg-C-Abl-regulation (B), respectively

c-Abl and Arg regulation

ROS-induced apoptosis activates them both (see figure IV). In the case of the activation of c-Abl, it is PKCδ-dependent and associated with the translocation of c-Abl to mitochondria and the release of cytochrome c. Arg interacts with the pro-apoptotic Siva-1 protein, which is phosphorylated, and is also translocated to mitochondria.

4. Transcriptional

The stimulating protein 1 (Sp1) and CCAAT-recognizing factors regulates catalase at the transcriptional level and EgrSp-70 (overlapping recognition sequence for Egr-1 and Sp1) have critical roles in the regulation of catalase gene transcription (see figure V) [18, 32].

HP100 (Hydrogen Peroxide-resistant, HP100, cells are derived from human leukemia HL60 cells) got much higher levels of nuclear Sp1 and NF-Y than HL60. Sp-1 and WT1/Egr-related factor have low binding in HL60 cells. WT1 (Wilm's tumor suppressor 1) is a negative transcription regulator of catalase.

Sp1 associates with the EgrSp-70 and binds to the GC box (core promoter element) here. This leads to the recruiting of RNA-polymerase II. Sp1 is expressed in high levels in HP100 nuclei and this activates core catalase promoter in cooperation with CCAAT-recognizing factors (CCAAT-92 is a transcriptional enhancer in HP100 cells). NF-Y associates with the CCAAT element.

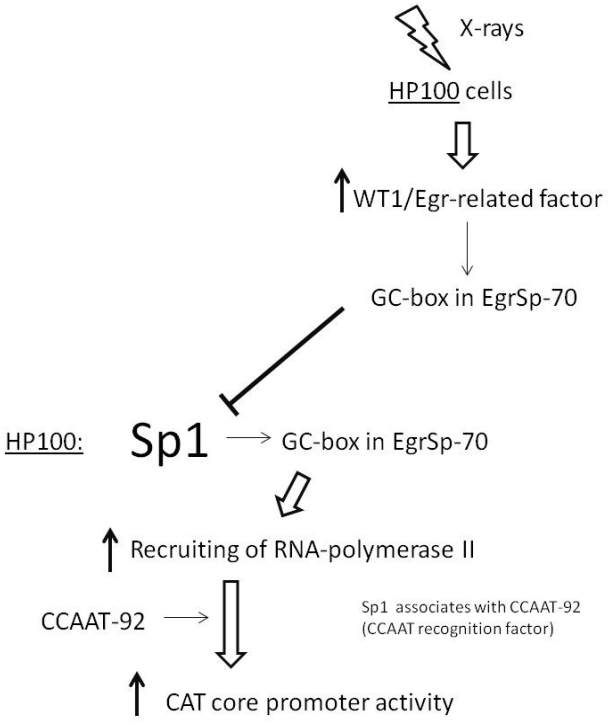

Figure V: Overview of the regulation of Sp1.

X-ray irradiation in HP100 cells leads to reduction in catalase mRNA levels by inducing association of WT1/Egr-related factor with the EgrSp-70 at the core promoter of the catalase gene. The X-ray inducible WT1/Egr-related factor may disturb or compete with the trans-activating ability of Sp1, which leads to inactivation of the core promoter.

Inhibiting pathways

The only inhibitor we found to be catalase specific was 3-amino-1, 2, 4-triazole, 3-AT/AMT/ATZ [14]. Hereinafter referred to as catalase inhibitor.

Aragon *et al*, 1991, observed brain and liver catalase in Male Long Evans rats, who got the catalase inhibitor administrated intraperitoneally [15]. They showed a dose- and time-dependent inhibition. The recovery of catalase activity in respective tissues was associated with the disappearance of the catalase inhibitor and consequently this was found to be an irreversible inhibitor. They also investigated the amounts of H_2O_2 in the tissues and concluded a dependence of the catalase inhibitor activity and rate of generated H_2O_2. The catalase inhibitor seems to bind a compound of catalase and H_2O_2. Without H_2O_2 no inhibition is observed, neither does it inhibit in the presence of alcohol, because alcohol compete to bind the same site in the catalase-H_2O_2 compound. The difference between them is that the catalase inhibitor bind irreversibly [16]. Moreover, and on the contrary, they also refer to previous studies, including one by William RN *et al* [17] where they are suggesting the catalase inhibitor to be indirect in action rather than having a direct effect on catalase. According to this the catalase inhibitor would undergo metabolic transformation, in the topic tissue, to become an active inhibitor.

To have guidance in reasonable administration doses of the catalase inhibitor directly to cells in cell culture further studies were investigated. Nenoi M. *et al*, 2001, used HP-100 cells (H_2O_2-resistant variants derived from human leukemia HL60 cells) which were incubated during 2 hours with the concentration of 25mM the catalase inhibitor [18]. HP-100 cells were also used in another study by Hachiya M. *et al*, 2005, and they incubated with 20 mM instead and during one hour [19].

Degradation pathways

ROS-induced apoptosis is p53-dependent [20, 33].

Previously c-Abl- and Arg-mediated phosphorylation has been mentioned to be necessary in stimulation of catalase activity and now this has also been shown to be of importance in the degradation process of catalase (see figure VI). This is partly described by the level of ROS; low levels leads to phosphorylation of catalase but if the levels continue to increase c-Abl and Arg dissociate from catalase and stimulate an apoptopic response instead.

This means that the anti-apoptopic response of catalase is due to the ROS-levels.

When c-Abl and Arg dissociate, the catalase activity is decreased either by:

1) Tyrosinephosphatases that desphosphorylate Y231-P and/or Y386-P → inactivation

or

2) Targeting of phosphorylated catalase for ubiquitination → degradation

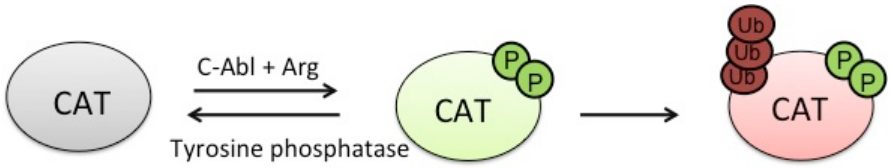

Figure VI: Overview of the degradation pathway of catalase.

catalase has been shown to be degradated by the 26S proteosome and the targeting for the Ub-proteosome pathway is regulated by c-Abl and Arg through tyrosine phosphorylation of the same sites as in activation (Y231 and Y386). C-Abl and Arg regulates catalase ubiquitination and degradation as well as its activation.

H_2O_2 -induced response of catalase levels is attenuated with Lactacystin (proteosomal inhibitor) and leads to less catalase degradation, which in turn results in lower ROS. In turn proteosomal degradation of catalase results in increasing ROS and thereby cell death by apoptosis or necrosis.

c-Abl and Arg are protecting the cell from oxidative stress by stimulating catalase activity but when the ROS-levels goes unmanageable they induce catalase degradation instead which results in cell death. The degradation of catalase might increase the speed of the apoptotic process.

By this they function as a potential switch, which controls the catalase levels and redox balance.