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Effects of Melanocortin 1 Receptor Agonists in Experimental Nephropathies

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Abstract

Nephrotic syndrome, characterized by massive proteinuria, is caused by a large group of diseases including membranous nephropathy (MN) and focal segmental glomerulosclerosis (FSGS). Although the underlying mechanisms are beginning to unravel, therapy is unspecific and far from efficient. It has been suggested that adrenocorticotropic hormone (ACTH) has beneficial effects in patients with MN and possibly in other nephrotic diseases. We have previously reported that ACTH may act directly on podocytes through the melanocortin 1 receptor (MC1R). In the present study, we evaluate the effect of highly specific MC1R agonists in two different nephrotic disease models. Experimental MN: Passive Heymann nephritis (PHN) was induced in rats that were treated for four weeks with MS05, a selective MC1R agonist, or saline. The degree of albuminuria was significantly reduced over time and the effect was sustained one week after treatment withdrawal (p < 0.05).

Experimental FSGS: Based on a dose-response study, two doses of adriamycin were used for induction of nephropathy in Balb/c mice. Mice were treated with either a synthetic MC1R agonist (BMS-470539), with α-melanocyte stimulating hormone (α-MSH) or with saline. There was no beneficial effect of treatment. In summary, MC1R agonists reduce albuminuria and improve morphology in experimentally induced MN whereas they have no effect in experimental FSGS. The results illustrate the differences in these podocytopathies in terms of signaling mechanisms underlying proteinuria, and progression of disease.

Introduction

The clinical entity nephrotic syndrome is caused by different morphological diseases such as membranous nephropathy (MN), minimal change disease (MCD), membranoproliferative glomerulonephritis (MPGN), and focal segmental glomerulosclerosis (FSGS). Recent studies have partially revealed the molecular mechanisms underlying these diseases. Thus, antibodies against phospholipase A2 seem to be involved in the pathogenesis of MN [1] as those to megalin are in passive Heymann nephritis in rats [2]. For patients with primary FSGS, soluble urokinase receptor (suPAR) has been reported to be elevated [3]. However, many questions remain [4–7], and treatment options are still far from specific or effective [8].

It was previously reported that treatment with adrenocorticotropic hormone (ACTH) had beneficial effects in patients with a wide range of nephrotic diseases [9,10]. The effect was most prominent in patients with MN, but the treatment was also effective in some cases of MCD, diabetic nephropathy, MPGN and FSGS. The positive effects of ACTH on MN have been confirmed in a randomized controlled trial [11], albeit in low number of patients. In a recent study, we put forward a possible molecular mechanism behind the beneficial effects of the treatment [12]. Firstly, one of the ACTH receptors, melanocortin 1 receptor (MC1R), was expressed in the kidney, and more specifically in the podocytes. Secondly, MC1R agonists ameliorated the nephrotic disease in rats with passive Heymann nephritis (PHN), an experimental model of MN. Thus, ACTH and agonists of MC1R decreased proteinuria, reduced oxidative stress, and improved podocyte morphology.

Podocytes play a crucial role in the normal glomerulus and in the development of many glomerular diseases [13]. Although the onset of disease may vary, the resulting podocyte injury with foot process effacement and proteinuria is common in most proteinuric disorders such as human MN and FSGS. PHN in rats and adriamycin-induced nephropathy in mice are well-characterized models resembling human MN and FSGS respectively. PHN is characterized by immune deposits formed in situ in the glomerulus, triggered by antigen expression on the podocyte surface [14], mainly megalin [2]. Proteinuria reaches a peak level 14 days after disease induction and although immune deposit formation...
eventually ceases, proteinuria persists lifelong [14]. The adriamycin model is characterized by glomerulosclerosis, tubulo-interstitial inflammation and fibrosis [15,16]. Podocytes [17–19], as well as endothelial cells and their surface layer [20], are damaged, leading to proteinuria.

In this study, we used different MC1R agonists to treat two experimental nephrotic diseases, MN and FSGS. The agonists used were: MS05, a selective peptide agonist for MC1R [21]; BMS-470539, a synthetic highly selective MC1R agonist [22–24]; and α-melanocYTE stimulating hormone (α-MSH), an agonist for all melanocortin receptors 1–5 except type 2, which is selective only for ACTH. The aim of this study was to evaluate the effects of MC1R agonists in two experimental nephrotic models, representing two different morphological diseases. Our hypothesis was that MC1R agonists would have similar beneficial effects in both MN and FSGS, and that they would ameliorate proteinuria.

Materials and Methods

Experimental Protocol

Animals. All animals had free access to standard food and water, and were housed in a room with a 12-hour dark-light cycle. Anesthesia was induced and maintained by inhalation of isoflurane (2–3% v/v, Schering-Plough, Stockholm, Sweden) mixed with air (1 L/min) in an isoflurane vaporizer (Ohmeda Isotec 5, Simtec engineering, Askim, Sweden). Temgesic® (0.1 ml/100 g body weight, Schering-Plough, Stockholm, Sweden) was given as a post-operative pain reliever in all small operative procedures. Gothenburg Ethical Board for Animal Experiments approved the study.

Experimental MN – Passive Heymann Nephritis

The experiments were performed on male Sprague Dawley rats (Charles River, Germany) of initial body weight of 140–163 g. To induce passive Heymann nephritis (PHN), Anti-Fx1A IgG antibody, 30 mg/mL (Probtex Inc., San Antonio, TX), was slowly injected into the tail vein, 1.5 ml at day 0 and 0.5 ml at day 7. Instead of the PHN-inducing antibody, controls received sterile saline. At day 14–15, treatment with MS05 (custom-made peptide from Agrisera, Vännäs, Sweden), 100 µg/day was started, either via osmotic pump (Alzet® Osmotic Pumps, Cupertino, CA) placed subcutaneously in the neck or via subcutaneous injections. The osmotic pump was replaced after two weeks. Total treatment time was four weeks. After two additional weeks, the rats were sacrificed and the kidneys were harvested for further analyses. Weight was followed twice a week. Spot urine samples were collected twice a week and the analyses were based on 1–2 weekly observations per animal: controls, n = 5 (7–10 observations/week); untreated PHN, n = 14 (19–27 observations/week); MS05, n = 17 (19–33 observations/week).

Experimental FSGS – Adriamycin-induced Nephrotic Syndrome

All experiments were performed on male BALB/c mice (Charles River, Germany) with an initial body weight of 22–26 g. To establish a stable nephropathy, a dose-response study using 5, 7, 8, 9 and 10 mg/kg (n = 5–19) was performed in order to find the optimal doses of adriamycin. Treatment, either BMS-470539 (30 µmol/kg, n = 18–19; synthetized by Enamine, Ukraine), or α-MSH (0.020 µmol/kg, n = 9; Sigma, St Louis, MO), diluted in DMSO or 1:1 in water and PEG400 (Sigma, St Louis, MO), was started one day before adriamycin was given, either via osmotic pump (Alzet® Osmotic Pumps, Cupertino, CA) placed subcutaneously in the neck or via daily subcutaneous injections. Control mice, and mice given adriamycin with no subsequent treatment, received vehicle only.

At day 0, FSGS was induced by a single tail vein injection of 8 or 10 mg/kg adriamycin (doxorubicin hydrochloride, Sigma-Aldrich, St Louis, MO). An equal volume of saline was given to controls. To prevent weight loss due to low appetite, mice were given one intraperitoneal injection of a 2 ml glucose-electrolyte solution (16.7 g/L glucose in hypotonic 75 mM NaCl solution) on day 1 to 11. Body weight was recorded and spot urine samples collected daily. At day 7 to 12, the mice were sacrificed and the kidneys were harvested for further analyses.

Urine Analyses

For rats, albumin was analyzed using the Rat Albumin Elisa Quantitation Kit (Bethyl Laboratories Inc., Montgomery, TX) and corrected for the urine creatinine concentration measured with the Jaffé reaction using a creatinine standard solution (Sigma, St Louis, MO). For mice, albumin was analyzed using the Albuwell M ELISA Kit and all urine samples were corrected for the urine creatinine concentration measured with the Creatinine Companion Kit (both from Exocell Inc., Philadelphia, PA). Optical density was measured on a Spectra max plus reader (Molecular Devices, Sunnyvale, CA).

Blood Urea Nitrogen

Blood was taken from the mice through the caval vein at day 10 (8 mg/kg adriamycin) or day 8 (10 mg/kg adriamycin), centrifuged and plasma was immediately frozen and stored at −20°C. Blood urea nitrogen (BUN) was measured with QuantiChrom™ Urea Assay Kit (BioAssay Systems, Hayward, CA) according to manufacturer’s instructions.
**Real-time PCR**

RNA was prepared from sieved isolated glomeruli using the Qiagen Mini Kit with DNase digestion (Qiagen Nordic, Solna, Sweden). RNA quality was confirmed using the Standard Sensitivity Kit on Experion™ (Bio-Rad, Hercules, CA). RNA was quantified using a NanoDrop spectrophotometer (NanoDrop Technologies Inc., Wilmington, DE). Reverse transcription of RNA was performed using the High Capacity RNA-to-cDNA kit at a total volume of 20 μL (Applied Biosystems, Foster City, CA).

50 ng of sample cDNA was used to quantify the mRNA level of each target gene by real-time PCR on the ABI Prism 7900 Sequence Detection system (TaqMan, Applied Biosystems (ABI), Foster City, CA), as previously described [25]. The following primers and probes, all verified by ABI, were used to detect mRNA of the following genes: GAPDH: Mm99999915_g1 (mouse), Rn01775763_g1 (rat), Hs99999905_m1 (human); MC1R: Mm00434851_s1 (mouse), custom made GenBankID AB306978.1 (rat), Hs00267167_s1 (human). All samples were run in duplicates. RNA samples without performing reverse transcription were used as negative controls. The comparative ΔΔCT method of relative quantification was used to calculate the differences in gene expression between the groups. GAPDH was used as endogenous control.

**Electron Microscopy**

Immediately after blood sampling and sacrifice, the kidneys were collected for morphological analysis. The renal artery and vein were clamped and the kidney was fixed by subcapsular injection of Karnovsky's fixative (2% paraformaldehyde and 2.5% glutaraldehyde in 0.05 M Na-cacodylate buffer, pH 7.2). The kidneys were cut into mm-slices and processed by standard procedure as previously described [26]. Slides were taken with a Leo 912AB Omega electron microscope (Leo Electron Microscopy Ltd., Cambridge, England) and examined in a blinded fashion by a pathologist as previously described [12]. Briefly, the number of podocyte foot processes per 10 μm glomerular basement membrane was determined in 2–5 places of each glomeruli, n = 15 glomeruli for all groups.

**Western Blotting**

Protein was prepared from isolated glomeruli and a previously described mouse podocyte cell line [27]. Protein from a human melanoma cell line, A375 (ATCC, Manassas, VA), served as a
positive control for MC1R expression. The protein samples were equally loaded (10 μg each) and western blotting was performed as previously described [12]. The following antibodies and dilutions were used for detection of specific proteins; anti-MC1R antibody 1:500 (AMR-020, Alomone Labs, Ltd., Israel) and anti-actin goat polyclonal antibody 1:1000 (sc-1615, Santa Cruz Biotechnology, Inc., Dallas, TX). An MC1R control peptide antigen (Alomone Labs, Ltd., Israel) was separately added at a 1:1 weight ratio to the MC1R antibody solution before incubation to confirm antibody specificity.

Statistical Analyses
All results are presented as mean ± SEM if not otherwise stated. Differences were determined using the Student’s t-test, or for morphological analyses one-way ANOVA. Differences in albuminuria between groups were determined with the exact Mann-Whitney non-parametric test, p<0.05 was considered statistically significant.

Results
MC1R Agonists Reduce Albuminuria in Experimental MN, but not in FSGS

Rats with PHN. As can be seen in Figure 1, four weeks of treatment with the MC1R agonist MS05, gradually decreased the urinary albumin-to-creatinine ratio (UACR) from 58.7±8.1 to 22.7±8.8 (n = 17), compared to untreated PHN: from 68.0±9.2 to 47.4±15 (n = 14, p<0.01). Control animals remained at low and stable levels throughout the study: with UACR values below 0.4 (n = 5). In addition, one week after treatment withdrawal, the effect was sustained in MS05-treated rats with a UACR of 2.4 (n = 17, p<0.05). There were no differences between the untreated PHN with a UACR of 2.46±0.73 (n = 19, n.s.). For the higher adriamycin dose, 10 mg/kg, that induced a more severe disease, BMS-470539 treatment resulted in a slightly higher UACR, 13.2±2.8 (n = 18) compared to untreated adriamycin mice, 7.34±2.4 (n = 17, p<0.05). Treatment with α-MSH did not significantly increase UACR, 13.6±7.9 (n = 9, n.s.). Controls remained at normal levels, i.e. UACR <0.02 (n = 10). At day 12 after adriamycin injection, the level of UACR had increased further, but with no differences between the groups: untreated adriamycin mice, 12.2±1.9 (n = 7); BMS-470539, 18.7±2.4 (n = 9, n.s.); α-MSH, 15.9±3.2 (n = 6, n.s.).

Blood urea nitrogen (BUN) is commonly used to assess kidney function. As can be seen in Figure 3, there was no difference between mice with untreated adriamycin-induced nephropathy compared with those treated with BMS-470539 (n.s.).

Weight loss in adriamycin-treated mice was low due to daily intraperitoneal injections of a glucose-salt solution. At day 7, adriamycin-treated mice had lost approximately 5% of their initial body weight (p<0.05). There were no differences between the MC1R agonist treated groups and the vehicle-treated mice given adriamycin (n.s.).

Table 1. Gene expression of MC1R in human, rat and mouse kidney tissue.

<table>
<thead>
<tr>
<th>Species</th>
<th>Group</th>
<th>n</th>
<th>MC1R Ct*</th>
<th>GAPDH Ct*</th>
<th>2–(ΔΔCt)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human*</td>
<td>1</td>
<td>28</td>
<td>21.8</td>
<td>21.1</td>
<td></td>
</tr>
<tr>
<td>Rat†</td>
<td>Control</td>
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<td>34.4±0.50</td>
<td>19.8±0.53</td>
<td>1</td>
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<tr>
<td></td>
<td>Untreated PHN</td>
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<td>20.5±0.20</td>
<td>0.94±0.26</td>
</tr>
<tr>
<td></td>
<td>MS05</td>
<td>14</td>
<td>33.1±0.32</td>
<td>20.5±0.24</td>
<td>0.92±0.22</td>
</tr>
<tr>
<td>Mouse*</td>
<td>Control</td>
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<td>33.5±0.37</td>
<td>16.6±0.08</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Adriamycin</td>
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<td>33.2±0.28</td>
<td>16.9±0.21</td>
<td>0.86±0.19</td>
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<tr>
<td></td>
<td>BMS-470539</td>
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<td>16.7±0.18</td>
<td>1.0±0.35</td>
</tr>
<tr>
<td></td>
<td>α-MSH</td>
<td>6</td>
<td>33.3±0.81</td>
<td>16.6±0.07</td>
<td>2.1±1.6</td>
</tr>
</tbody>
</table>

*Whole kidney tissue, previously published in Lindskog et al. [12].
†Glomeruli.

Podocyte Morphology is Improved by MC1R Agonists in MN but not in FSGS

Treatment with the MC1R agonist MS05 improved morphology in the PHN rats [12]. Adriamycin treated mice displayed pathological changes with a focally disrupted glomerular barrier, including pronounced foot process effacement (Figure 4B). This effect was segmental and in line with the disease model, since FSGS nephritic mice presented unaffected glomerular parts as well. Glomerular damage was also quantified by counting the number of foot processes along the glomerular basement membrane. Controls had 22.8±0.90 numbers of foot processes/
Table 1, the CT levels were higher (33) reflecting lower expression in PHN rats was confirmed but, as can be seen in of around 28 [12]. In this study, glomerular MC1R gene expression between the different groups (n.s).

Discussion

Glomerular Expression of MC1R in Rat and Mouse

We have previously shown with real-time PCR that MC1R mRNA is expressed in human and rat kidney tissue with a C_t level of around 28 [12]. In this study, glomerular MC1R gene expression in PHN rats was confirmed but, as can be seen in Table 1, the C_t levels were higher (33) reflecting lower expression levels (Table 1). There was no change in expression between controls, MS05 treated and untreated PHN rats (n.s).

The mRNA expression levels in mice glomeruli were analyzed with real-time PCR (Table 1). The gene was expressed at low levels in mice with a mean Ct level of 33. There was no change in expression between the different groups (n.s.).

Protein expression of MC1R was also confirmed in mouse by western blotting, both in glomerular tissue as well as in a mouse podocyte cell line (Figure 5). A375, a human malignant melanoma cell line was used as positive control.

In PHN rats treated with an MC1R agonist, the amelioration of albuminuria was sustained after treatment withdrawal, which is in line with clinical observations where albuminuria has been further reduced after removal of ACTH treatment [9,10,12]. These results suggest that the disease is reversible and that MC1R agonists affect the podocytes in a beneficial manner. Clinical data suggest that ACTH and melanocortin receptor agonists (MCRs) could ameliorate nephrotic disease [9–11]. Indeed, we have shown that MC1R co-localizes with synaptopodin, indicating specific expression in podocytes [12], which suggests a general role for the receptor in podocytopathic nephrotic diseases such as MN.

In the same study we demonstrated human MC1R mRNA is expressed in human and rat kidney tissue with a C_t level of 33. There was no change in expression between controls, MS05 treated and untreated PHN rats (n.s.).
Acknowledgments

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References