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**Influence of 17-hydroxyprogesterone, progesterone and sex steroids on
mineralocorticoid receptor transactivation in congenital adrenal
hyperplasia**

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Abstract

Background: CAH due to 21-hydroxylase deficiency leads to accumulation of steroid precursors and adrenal androgens. These steroids may have a biological effect on the steroid receptor with clinical consequences on diagnostics and treatment in CAH patients. Therefore, we analysed the effect of accumulated steroids (17 hydroxyprogesterone (17OHP), progesterone, androstenedione, testosterone) on aldosterone mediated transactivation of the human mineralocorticoid receptor (hMR).

Methods: A transactivation assay using transiently transfected COS7 cells was employed. Cells were co-transfected with hMR-cDNA, MMTV-luciferase and renilla-luciferase expression vectors. Transfected cells were incubated with six different steroid concentrations in addition to aldosterone (10^{-10} mol/l). Luciferase and renilla activities were measured to quantify hMR transactivation.

Results: Linear regression analysis showed statistically significant linear inhibition of transactivation of the hMR by 10^{-10} mol/l aldosterone in the presence of increasing 17OHP ($F(1,5)=11.34$, $p=0.019$) and progesterone ($F(1,5)=11.08$, $p=0.021$) concentrations. In contrast neither androstenedione nor testosterone affected hMR transactivation by aldosterone at a concentration of 10^{-10} mol/l.

Conclusion: Our study shows for the first time that neither androstenedione nor testosterone has a biological effect on aldosterone-mediated transactivation of the hMR. 17OHP and progesterone have an anti-mineralocorticoid effect *in vitro* that may clinically lead to an increased requirement of mineralocorticoids in poorly controlled CAH patients.

Introduction

Congenital adrenal hyperplasia (CAH) is a group of disorders affecting adrenal steroidogenesis. The incidence of classic CAH varies between 1 in 10,000 to 1 in 15,000 live births in most Caucasian populations.[1] In about 95% of the cases CAH is caused by 21-hydroxylase deficiency, [2] resulting in impaired adrenal synthesis of cortisol. Cortisol deficiency triggers a counter-regulatory increase in pituitary ACTH secretion leading to accumulation of adrenal steroid precursors before the deficient enzymatic step and increased adrenal androgen production. 21-hydroxylase converts 17-hydroxyprogesterone (17OHP) to 11-deoxycortisol, the penultimate step in cortisol synthesis. Hence 17OHP accumulates and is used as a marker for 21-hydroxylase deficiency.

Classic CAH is commonly subdivided in the salt wasting (SW) and simple virilizing (SV) forms depending on the residual enzymatic activity. SW patients have no residual 21-hydroxylase activity leading to severe salt loss, typically after the first week of life, and prenatal virilization of the female external genitalia. Patients with the SV form of CAH have a residual enzyme activity of 1-2 % and usually have sufficient aldosterone production to prevent severe salt loss whereas glucocorticoid synthesis is severely impaired. In both SW and SV forms elevated adrenal androgens cause prenatal virilization of the female external genitalia and postnatal androgen excess in both sexes. [2,3] Current treatment of CAH consists of lifelong glucocorticoid and, if necessary, also mineralocorticoid treatment.[4] Treatment with glucocorticoids restores feedback within the hypothalamus-pituitary-adrenal axis, consequently achieving down-regulation of adrenal androgen production. However, in many patients supraphysiological doses of glucocorticoids are needed to normalize androgen levels.

Untreated and poorly controlled CAH patients are characterized by elevated levels of steroid hormone precursors, including progesterone and 17OHP, and androgens such as androstenedione and testosterone.[3,5-8] It has been shown that progesterone and 17OHP have antagonistic properties on the human mineralocorticoid receptor (hMR), and therefore may contribute to the mineralocorticoid deficiency in classic CAH patients. [9] The aim of our study was to evaluate the effects of 17OHP, progesterone, androstenedione and testosterone on the aldosterone mediated transactivation and translocalisation of the hMR. Furthermore, we studied the effect of the frequent mineralocorticoid receptor (MR) p.Ile180Val single nucleotide polymorphism (SNP) on transactivation of the hMR.

Material and Methods

Construction of plasmids

The hMR cDNA was PCR amplified from the previously used pcDNA3.1-NR3C2 construct[10] using specific primers with *Hind*III and *Eco*RV restriction sites for directional cloning into pcDNA6/V5-His-B vector (Invitrogen Corp., Carlsbad, CA, USA). The p.Ile180Val SNP was recreated in the pcDNA6-hMR construct by site-directed mutagenesis using the QuikChange XL Site-Directed Mutagenesis Kit according to the manufacturer's protocol (Stratagene, Amsterdam, The Netherlands). The correct insertion of the hMR construct and the p.Ile180Val SNP as well as the integrity of the cDNA was checked by direct DNA sequencing. For intracellular localization assays Green Fluorescent Protein (GFP), an autofluorescent genetic reporter, was cloned into pcDNA6. The hMR cDNA and the hMR p.Ile180Val (hMR-I180V) construct were cloned into the pcDNA6-GFP vector using the same restriction enzymes as described above.

In vitro transactivation assays

Transactivation of hMR and hMR-I180V by different concentrations of aldosterone was investigated using a MMTV-luciferase assay. Approximately 2.5×10^4 COS-7 cells were grown in 500 ml of Dulbecco's minimal essential medium (DMEM) High Glucose (4,5 g/l) with L-Glutamine (PAA Laboratories GmbH, Pasching, Austria) supplemented with 10% fetal bovine serum (PAA Laboratories GmbH) and Penicillin/Streptomycin (PAA Laboratories GmbH) in 24-well plates and transiently transfected 24 h after seeding using FuGene® HD transfection reagent (Roche Applied Sciences, Burgess Hill, United Kingdom). Cells were transfected with 300 ng pcDNA6-V5/HisB-hMR or pcDNA6-V5/HisB-hMR variant (p.Ile180Val) in the presence of 300 ng of a mouse mammary tumor virus (MMTV)-luciferase reporter construct (MMTV-luc) driving the firefly luciferase gene. Co-transfection with 50 ng pRL-TK (Promega, Madison, WI, USA), a renilla luciferase vector, was performed to normalize data for transfection efficiency. In each set of experiments 3 wells with COS-7 cells were co-transfected with 300 ng of pcDNA-hMR and 300 ng of pGL3-Basic (Promega) for data normalization and interassay comparison purposes as pGL3-Basic contains a coding region for firefly luciferase for monitoring transcriptional activity in transfected

cells. Two days after transfection cells were treated with aldosterone (Sigma Aldrich, Gillingham, United Kingdom) for 24 hours in different concentrations (final concentrations made up in total of 500 uL full DMEM media: 10^{-6} , 10^{-8} , 10^{-10} , 10^{-12} , 10^{-14} mol/l) , or in a 10^{-10} mol/l concentration in addition to different concentrations of 17OHP (range 5-1000 nmol/l), progesterone (2.5-100 nmol/l), androstenedione (1-250 nmol/l) or testosterone (0.5-60 nmol/l) (Sigma Aldrich). Concentrations of 17OHP, progesterone, androstenedione and testosterone used in the assays are based on biochemical findings in CAH patients.^[5-8]

To evaluate the transactivational potential of 17OHP, progesterone, androstenedione and testosterone on the hMR in the absence of aldosterone, transfected cells were also incubated in 500 uL of full DMEM supplemented with different concentrations of these steroids.

Cells were lysed in 100 uL of passive lysis buffer (Promega). Consequently 30 uL of cell lysate was used for the measurement of firefly and renilla luciferase activity, with a luminometer (Berthold, Bad Wildbad, Germany), using the Dual-Luciferase ® Reporter Assay System (Promega) according to manufacturer's standard protocol. The hMR transactivation was calculated by the ratio of the steroid dependent (firefly) luciferase and the steroid independent renilla (luciferase). Luciferase/Renilla ratios were normalized for luciferase activity driven by pGL3-Basic. Data were normalized for the transactivation by a 10^{-10} mol/l aldosterone concentration and are presented as fold transactivation compared to the transactivation by 10^{-10} mol/l aldosterone (transactivation by 10^{-10} mol/l aldosterone was set as 1.0 fold transactivation). All assays were performed in triplicate – triplicate. Statistical analysis was performed using GraphPad Prism software version 5.0 (GraphPad Software, San Diego, CA, USA). Results were analyzed by both linear regression analyses and ANOVA with Bonferroni adjustment for multiple comparisons (all possible comparisons were analyzed). Differences between the hMR wild type and the p.Ile180Val construct were analyzed using a t test. A *p* value of < 0.05 was considered significant.

Intracellular localization

The transactivational potential of the hMR-GFP construct was evaluated to ensure comparable transactivational potential to the hMR construct in the presence of 10^{-10} M concentrations of

aldosterone. The hMR-GFP construct was used for an intracellular localization assay. Approximately 2×10^5 COS-7 cells were grown on glass coverslips in 6-well plates containing 2 mL of DMEM High Glucose (4,5 g/l) with L-Glutamine (PAA Laboratories GmbH) supplemented with charcoal stripped fetal bovine serum (Sigma Aldrich) and Penicillin/Streptomycin (PAA Laboratories GmbH). Twenty-four hours after seeding, cells were transiently transfected using FuGene® HD transfection reagent (Roche Applied Sciences) with 2 µg of hMR-GFP or 2 µg of hMR-I180V-GFP. Forty-eight hours after transfection, cells were treated for 120 min with a combination of 10^{-10} mol/L aldosterone and different concentrations of other steroids (17OHP, progesterone, androstenedione and testosterone) to study the effect of these steroids on the intracellular localization of the receptor. Cells were washed three times in 1x phosphate buffered saline (PBS) and fixed in 1 ml 100% methanol at - 20°C for 15 min. Fixed cells were further washed 3 more times in 1xPBS and mounted on Vectorshield with 4', 6-diamidino-2-phenylindole (DAPI; exclusively nuclear staining). Results were obtained from three independent transfection experiments in which 150 transfected cells were classified in 4 categories: 1. Nuclear, 2. Mainly nuclear, 3. Equal nuclear and cytoplasmic, 4. Mainly cytoplasmic. Representative images were taken using confocal microscopy (Nikon Instruments Inc., Melville, NY, USA). To evaluate if treatment causes a difference in the number of cells counted as nuclear, mainly nuclear, equal nuclear or mainly cytoplasmic respectively, a one way ANOVA analysis was performed. Statistical analysis was performed using GraphPad Prism software version 5.0.

Results

Transactivation of the mineralocorticoid receptor by aldosterone

Increasing concentrations of aldosterone caused an increase in potent transactivation of both the hMR and hMR-I180V. The dose dependent effects on the transactivation are shown in a dose response curve (**Figure 1**). An estimated concentration for 50% transactivation (EC-50) of the hMR of around 10^{-10} mol/l aldosterone was calculated for both the wild type (2.4×10^{-11} mol/l) and the p.Ile180Val SNP (1.2×10^{-11} mol/l).

Effect of 17OHP, progesterone, androstenedione and testosterone on hMR transactivation

Increasing concentrations of 17OHP and progesterone inhibited aldosterone mediated transactivation of the hMR in a dose dependent fashion (**Figure 2**). Linear regression analyses showed a linear inhibition of transactivation of the hMR by 10^{-10} mol/l aldosterone in the presence of increasing concentrations of 17OHP ($F(1,5)=11.34$, $p=0.019$) and progesterone ($F(1,5)=11.08$, $p=0.021$). Variable concentrations of 17OHP ($F(6,48)=111.9$, $p<0.0001$) and progesterone ($F(6,48)=62.11$, $p<0.0001$) have a significant effect on transactivation of the hMR by aldosterone in the presence of 10^{-10} mol/l aldosterone, as shown by ANOVA analyses (**Supplementary table 1-2**).

In contrast, treatment with increasing concentrations of androstenedione and testosterone did not have any measureable effect on hMR transactivation (**Figure 2**). No linear effect of increasing concentrations of androstenedione ($F(1,5)=0.709$, $p=0.438$) or testosterone ($F(1,5)=1.57$, $p=0.265$) on transactivation of the hMR by aldosterone was found.

In addition, ANOVA analyses showed that different concentrations of androstenedione or testosterone did not affect transactivation of the hMR by aldosterone (**Supplementary table 3-4**).

The effect of three different concentrations of 17OHP on the aldosterone mediated transactivation of the hMR was also evaluated in the p.Ile180Val SNP construct (**Figure 3**). The inhibitory effect of 17OHP on hMR-I180V was found to be similar to its effect on the wild type hMR ($p>0.05$).

Intracellular localization of the hMR

The transactivation potential of both the hMR-GFP and the hMR construct were compared to assess that the GFP has not altered transactivational properties of the construct prior to performing an intracellular localization assay. The hMR-GFP construct showed to have equal transactivational properties as the hMR construct (**Supplementary Figure 1**).

In untreated cells, the hMR was localized only in the cytoplasm or equally distributed in nucleus and cytoplasm (**Figure 4A**). Treatment with aldosterone for 120 minutes resulted in a clear translocation of the hMR with a predominantly nuclear localization.

17OHP and progesterone did not influence the translocation of the hMR to the nucleus in the presence of aldosterone (**Figure 4B**). Treatment with 17OHP, progesterone, androstenedione or testosterone did not result in significant differences in the intracellular localization of the hMR.

In the presence of aldosterone, the hMR-I180V-GFP was also mainly localized in the nucleus. 17OHP did not inhibit the translocation of the hMR-I180V-GFP to the nucleus in the presence of aldosterone (**Figure 4C**).

Discussion

We studied the effects of different adrenal steroid hormone precursors and androgens on the transactivational potential and localization of the human mineralocorticoid receptor. Our study shows for the first time that excess concentrations of androstenedione and testosterone do not have a biological effect on the aldosterone mediated transactivation of the hMR *in vitro*. Furthermore, 17OHP and progesterone have a strong anti-mineralocorticoid effect *in vitro*, which confirms previous findings.[9] This study highlights the anti-mineralocorticoid effect of elevated 17OHP concentrations as found in poorly controlled CAH patients.

These findings may have important implications for the clinical care provision. Based on our results, it can be suggested that elevated 17OHP and progesterone concentrations are likely to have an adverse effect on the mineralocorticoid effect in untreated and poorly treated CAH. This may potentially lead to increased requirement of mineralocorticoids and sub-optimal control. In contrast, elevated androgens did not influence the mineralocorticoid transactivation *in vitro*. We therefore hypothesize that elevated androgens per se do not have a clinical relevant effect on mineralocorticoid treatment in the clinical care of CAH.

The current treatment strategy is based on normalizing of adrenal androgens to prevent adverse effects of hyperandrogenism. Slightly elevated 17OHP concentrations are generally accepted because of the possible side effects of high dosages of glucocorticoids needed to achieve physiological 17OHP concentrations. Based on our results it can be suggested that lowering of highly elevated 17OHP concentrations may also have an additional positive effect on the dosage of mineralocorticoid treatment and consequently decrease the potential risk of adverse effects of mineralocorticoid treatment such as hypertension. Unfortunately, supraphysiological doses of glucocorticoids are generally necessary to lower 17OHP levels that may lead to adverse effects and long term complications. Therefore, the treatment goal in CAH patients is normalization of adrenal androgens with slightly elevated 17OHP levels. [4] Elevated renin levels may indicate the need of higher mineralocorticoid doses. However, based on our data elevated renin concentrations may also reflect the anti-mineralocorticoid effect of elevated 17OHP concentrations. A fine balance between the use of supraphysiological dosages of glucocorticoids, mineralocorticoid treatment and normalizing 17OHP

levels has to be achieved to prevent long-term complication of overtreatment with glucocorticoids on one hand and overtreatment with mineralocorticoids on the other hand.

The antagonistic properties of progesterone on the human, rat and sheep mineralocorticoid receptor have been previously described. [9,11-15] A 50% inhibition of the maximum transactivation of the mineralocorticoid receptor is caused by progesterone concentrations between 2 to 11 nmol/l.[9,16-18]

The inhibitory effect of progesterone described in our study is in line with those described in the studies mentioned above. Minor differences between the results of those studies may be explained by different cells and different luciferase constructs used.

The effect of slightly elevated 17OHP concentrations on the hMR have been studied previously.[9]

The previously reported concentration of 135 nmol/l, causing a 50% inhibition of transactivation of the hMR by a 10^{-9} mol/l aldosterone, is in line with the antagonistic effect of 17OHP on aldosterone mediated transactivation described in our study. In our study we evaluated the effect of even higher 17OHP concentrations, as found in untreated or poorly controlled CAH patients.

In contrast to the effect on transactivation the translocation to the nucleus seems not to be affected by 17OHP or progesterone. The physiological human ligand of the hMR is aldosterone. After binding to aldosterone the hMR undergoes a conformational change and partial dissociation of the ligand binding complex occurs, leading to translocation of the hMR to the nucleus. Within the nucleus the activated receptors regulate transcription by different pathways including transactivation of target genes [19-23]

Intracellular localization studies on the hMR have shown that in the absence of steroids the hMR is localized in the cytoplasm and in the nucleus, aldosterone causes a rapid nuclear accumulation of the hMR.[19,24-27] Binding of aldosterone to the hMR causes dissociation of several associated proteins from the receptor, followed by dimerization and finally nuclear translocation of the activated receptor.

The translocation assay performed in this study shows a similar subcellular localization with a predominant localization of the hMR in the cytoplasm in the absence of steroids. Treatment of the COS-7 cells expressing the hMR-GFP construct with aldosterone causes a quick translocation of the hMR to the nucleus of the cells. However, different concentrations of 17OHP and progesterone in addition to a 10^{-10} mol/l aldosterone concentration do not have an impact on the translocation of the

hMR to the nucleus. This finding is in contrast to the described effects of hMR antagonists, such as spironolactone and eplerenone, which inhibit the translocation of the hMR to the nucleus.[19]

The mechanism of the inhibition of the aldosterone mediated transactivation of the hMR by progesterone and 17OHP remains unclear. It has been shown that 17OHP has a relatively high binding affinity for the hMR.[9] Therefore, competitive binding of the hMR between 17OHP and aldosterone, such as in patients with poorly controlled CAH, is very likely. We showed that 17OHP does not inhibit the translocation of the hMR to the nucleus. We, therefore, hypothesize that the anti-mineralocorticoid effect of 17OHP on the hMR is not due to an effect on the translocation of the hMR but might be caused by effects on the transcription after translocation to the nucleus. It has been suggested by Hellal-Levy *et al.* that binding of an antagonist to the hMR leads to an inactive conformation of the hMR. Due to instability this complex of the MR and its antagonist will not be converted into a transcriptionally active conformation. [20] This hypothesis may explain the antagonistic properties of 17OHP and progesterone on the hMR

The MR p.Ile180Val SNP (rs5522) is one of the most frequent SNPs in the hMR with a frequency of 10.2 % of the G allele in a European population (HapMap project, www.hapmap.org). The MR p.Ile180Val SNP has been associated with an increased hypertension risk. [28] As CAH patients have a tendency to develop elevated blood pressure, [29,30] the role of this SNP in CAH patients might be important with respect to their cardiovascular risk profile. We showed that the hMR p.Ile180Val SNP does not affect transactivation of the hMR by aldosterone. These findings are in line with the results by De Rijk *et al.*[31] In addition 17OHP has the same antagonistic effect on the hMR-I180V SNP as on the on the wild-type hMR. Thus, the results of this study do not explain the increased hypertension risk in p.Ile180Val.

In conclusion, our study shows for the first time that neither androstenedione nor testosterone have a significant biological effect on the aldosterone-mediated transactivation of the hMR. In contrast, increased 17OHP and progesterone concentrations have an anti-mineralocorticoid effect due to an inhibition of aldosterone-mediated transactivation of the hMR. However, unlike hMR blockers,

278 neither 17OHP nor progesterone inhibits the translocation of the hMR to the nucleus. Further studies
279 are needed to explain the mechanism of this inhibition of transactivation by 17OHP.
280

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Legends to figures and tables

Figure 1. Dose response curves showing the transactivation of the hMR (wild type) and the hMR-I180V SNP by different concentrations of Aldosterone using a luciferase assay. The results are expressed as the ratio of (firefly) luciferase and renilla (luciferase) activity. Data are means $\pm$ S.E.M for each concentration (n=9).

Figure 2. The effect of different concentrations of 17OHP (A), progesterone (B), testosterone (C) and androstenedione (D) on the transactivation of hMR by 10^{-10} M aldosterone concentration. The transactivation activity of 10^{-10} M aldosterone was set as 1.0. Results are expressed as x fold transactivation of MMTV (firefly) luciferase (MMTV-luc). Data are means $\pm$ S.E.M for each concentration (n=9). Significant differences in transactivation between two concentrations closest to each other are indicated by an asterisks ($p < 0.05$).

Figure 3. The effect of different concentrations of 17OHP on the transactivation of hMR by 10^{-10} M aldosterone concentration compared to the effect of different concentrations of 17OHP on the transactivation of the hMR-I180V SNP. The transactivation activity of 10^{-10} M aldosterone on the hMR (wild type) was set as 1.0. Results are expressed as x fold transactivation of MMTV (firefly) luciferase (MMTV-luc). Data are means $\pm$ S.E.M for each concentration (n=9).

Figure 4 A. Cellular localization of the hMR without the presence of aldosterone and in the presence of aldosterone with or without different concentrations of 17OHP and progesterone. Cells were localized using confocal microscopy as 1. nuclear (black bars), 2. mainly nuclear (dark gray bars), 3. equal nuclear – cytoplasmic (light gray bars) and 4. mainly cytoplasmic (white bars)

Figure 4 B. Images showing the four possible cellular localizations of the hMR: 1. nuclear, 2. mainly nuclear, 3. equal nuclear and cytoplasmic, 4. mainly cytoplasmic. Images are taken using a confocal microscope. Different images were taken showing DAPI staining, GFP and a merged image.

Figure 4C. Cellular localization of the hMR-I180V without the presence of steroids and in the presence of aldosterone with or without different concentrations of 17OHP. Cells were localized using confocal microscopy as 1. nuclear (black bars), 2. mainly nuclear (dark gray bars), 3. equal nuclear – cytoplasmic (light gray bars) and 4. mainly cytoplasmic (white bars).

Supplementary figure 1. Transactivational potential of the hMR construct versus the hMR-GFP construct evaluated by a luciferase assay. The results are expressed as the ratio of (firefly) luciferase to renilla (luciferase) activity corrected for pGL3 (transfection efficiency). Data are means $\pm$ S.E.M. (n=9).

Supplementary table 1. Results of Bonferroni's Multiple Comparison Test for all comparisons in the experiment evaluating the effect of different concentrations of 17OHP on the aldosterone mediated transactivation of the hMR

Supplementary table 2. Results of Bonferroni's Multiple Comparison Test for all comparisons in the experiment evaluating the effect of different concentrations of progesterone on the aldosterone mediated transactivation of the hMR

Supplementary table 3. Results of Bonferroni's Multiple Comparison Test for all comparisons in the experiment evaluating the effect of different concentrations of testosterone on the aldosterone mediated transactivation of the hMR

Supplementary table 4. Results of Bonferroni's Multiple Comparison Test for all comparisons in the experiment evaluating the effect of different concentrations of androstenedione on the aldosterone mediated transactivation of the hMR