

Influence of 17-Hydroxyprogesterone, Progesterone and Sex Steroids on Mineralocorticoid Receptor Transactivation in Congenital Adrenal Hyperplasia

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

4
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11
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30 **Abstract**

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

36 *Methods:* A transactivation assay using transiently transfected COS7 cells was employed. Cells were
37 co-transfected with hMR-cDNA, MMTV-luciferase and renilla-luciferase expression vectors.
38 Transfected cells were incubated with six different steroid concentrations in addition to aldosterone
39 (10^{-10} mol/l). Luciferase and renilla activities were measured to quantify hMR transactivation.

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

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

48
49

50 **Introduction**

51 Congenital adrenal hyperplasia (CAH) is a group of disorders affecting adrenal steroidogenesis. The
52 incidence of classic CAH varies between 1 in 10,000 to 1 in 15,000 live births in most Caucasian
53 populations.[1] In about 95% of the cases CAH is caused by 21-hydroxylase deficiency, [2] resulting
54 in impaired adrenal synthesis of cortisol. Cortisol deficiency triggers a counter-regulatory increase in
55 pituitary ACTH secretion leading to accumulation of adrenal steroid precursors before the deficient
56 enzymatic step and increased adrenal androgen production. 21-hydroxylase converts 17-
57 hydroxyprogesterone (17OHP) to 11-deoxycortisol, the penultimate step in cortisol synthesis. Hence
58 17OHP accumulates and is used as a marker for 21-hydroxylase deficiency.

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

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

78 **Material and Methods**

79 *Construction of plasmids*

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

90

91 *In vitro transactivation assays*

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

106 cells. Two days after transfection cells were treated with aldosterone (Sigma Aldrich, Gillingham,
107 United Kingdom) for 24 hours in different concentrations (final concentrations made up in total of
108 500 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
109 addition to different concentrations of 17OHP (range 5-1000 nmol/l), progesterone (2.5-100 nmol/l),
110 androstenedione (1-250 nmol/l) or testosterone (0.5-60 nmol/l) (Sigma Aldrich). Concentrations of
111 17OHP, progesterone, androstenedione and testosterone used in the assays are based on biochemical
112 findings in CAH patients.^[5-8]

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

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

130

131 *Intracellular localization*

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

134 aldosterone. The hMR-GFP construct was used for an intracellular localization assay. Approximately
135 2×10^5 COS-7 cells were grown on glass coverslips in 6-well plates containing 2 mL of DMEM High
136 Glucose (4,5 g/l) with L-Glutamine (PAA Laboratories GmbH) supplemented with charcoal stripped
137 fetal bovine serum (Sigma Aldrich) and Penicillin/Streptomycin (PAA Laboratories GmbH). Twenty-
138 four hours after seeding, cells were transiently transfected using FuGene® HD transfection reagent
139 (Roche Applied Sciences) with 2 µg of hMR-GFP or 2 µg of hMR-I180V-GFP. Forty-eight hours
140 after transfection, cells were treated for 120 min with a combination of 10^{-10} mol/L aldosterone and
141 different concentrations of other steroids (17OHP, progesterone, androstenedione and testosterone) to
142 study the effect of these steroids on the intracellular localization of the receptor. Cells were washed
143 three times in 1x phosphate buffered saline (PBS) and fixed in 1 ml 100% methanol at - 20°C for 15
144 min. Fixed cells were further washed 3 more times in 1xPBS and mounted on Vectorshield with 4', 6-
145 diamidino-2-phenylindole (DAPI; exclusively nuclear staining). Results were obtained from three
146 independent transfection experiments in which 150 transfected cells were classified in 4 categories: 1.
147 Nuclear, 2. Mainly nuclear, 3. Equal nuclear and cytoplasmic, 4. Mainly cytoplasmic. Representative
148 images were taken using confocal microscopy (Nikon Instruments Inc., Melville, NY, USA). To
149 evaluate if treatment causes a difference in the number of cells counted as nuclear, mainly nuclear,
150 equal nuclear or mainly cytoplasmic respectively, a one way ANOVA analysis was performed.
151 Statistical analysis was performed using GraphPad Prism software version 5.0.

152

153 **Results**

154 *Transactivation of the mineralocorticoid receptor by aldosterone*

155 Increasing concentrations of aldosterone caused an increase in potent transactivation of both the hMR
156 and hMR-I180V. The dose dependent effects on the transactivation are shown in a dose response
157 curve (**Figure 1**). An estimated concentration for 50% transactivation (EC-50) of the hMR of around
158 10^{-10} mol/l aldosterone was calculated for both the wild type (2.4×10^{-11} mol/l) and the p.Ile180Val
159 SNP (1.2×10^{-11} mol/l).

160

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

162 Increasing concentrations of 17OHP and progesterone inhibited aldosterone mediated transactivation
163 of the hMR in a dose dependent fashion (**Figure 2**). Linear regression analyses showed a linear
164 inhibition of transactivation of the hMR by 10^{-10} mol/l aldosterone in the presence of increasing
165 concentrations of 17OHP ($F(1,5)=11.34$, $p=0.019$) and progesterone ($F(1,5)=11.08$, $p=0.021$).
166 Variable concentrations of 17OHP ($F(6,48)=111.9$, $p<0.0001$) and progesterone ($F(6,48)=62.11$,
167 $p<0.0001$) have a significant effect on transactivation of the hMR by aldosterone in the presence of
168 10^{-10} mol/l aldosterone, as shown by ANOVA analyses (**Supplementary table 1-2**).

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

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

175 The effect of three different concentrations of 17OHP on the aldosterone mediated transactivation of
176 the hMR was also evaluated in the p.Ile180Val SNP construct (**Figure 3**). The inhibitory effect of
177 17OHP on hMR-I180V was found to be similar to its effect on the wild type hMR ($p>0.05$).

178

179 *Intracellular localization of the hMR*

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

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

187 17OHP and progesterone did not influence the translocation of the hMR to the nucleus in the presence
188 of aldosterone (**Figure 4B**). Treatment with 17OHP, progesterone, androstenedione or testosterone
189 did not result in significant differences in the intracellular localization of the hMR.

190 In the presence of aldosterone, the hMR-I180V-GFP was also mainly localized in the nucleus.
191 17OHP did not inhibit the translocation of the hMR-I180V-GFP to the nucleus in the presence of
192 aldosterone (**Figure 4C**).

193

194

195 **Discussion**

196 We studied the effects of different adrenal steroid hormone precursors and androgens on the
197 transactivational potential and localization of the human mineralocorticoid receptor. Our study shows
198 for the first time that excess concentrations of androstenedione and testosterone do not have a
199 biological effect on the aldosterone mediated transactivation of the hMR *in vitro*. Furthermore,
200 17OHP and progesterone have a strong anti-mineralocorticoid effect *in vitro*, which confirms previous
201 findings.[9] This study highlights the anti-mineralocorticoid effect of elevated 17OHP concentrations
202 as found in poorly controlled CAH patients.

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

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

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

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

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

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

232 The previously reported concentration of 135 nmol/l, causing a 50% inhibition of transactivation of
233 the hMR by a 10^{-9} mol/l aldosterone, is in line with the antagonistic effect of 17OHP on aldosterone
234 mediated transactivation described in our study. In our study we evaluated the effect of even higher
235 17OHP concentrations, as found in untreated or poorly controlled CAH patients.

236 In contrast to the effect on transactivation the translocation to the nucleus seems not to be affected by
237 17OHP or progesterone. The physiological human ligand of the hMR is aldosterone. After binding to
238 aldosterone the hMR undergoes a conformational change and partial dissociation of the ligand binding
239 complex occurs, leading to translocation of the hMR to the nucleus. Within the nucleus the activated
240 receptors regulate transcription by different pathways including transactivation of target genes [19-23]

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

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

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

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

263

264 The MR p.Ile180Val SNP (rs5522) is one of the most frequent SNPs in the hMR with a frequency of
265 10.2 % of the G allele in a European population (HapMap project, www.hapmap.org). The MR
266 p.Ile180Val SNP has been associated with an increased hypertension risk. [28] As CAH patients have
267 a tendency to develop elevated blood pressure, [29,30] the role of this SNP in CAH patients might be
268 important with respect to their cardiovascular risk profile. We showed that the hMR p.Ile180Val SNP
269 does not affect transactivation of the hMR by aldosterone. These findings are in line with the results
270 by De Rijk *et al.*[31] In addition 17OHP has the same antagonistic effect on the hMR-I180V SNP as
271 on the on the wild-type hMR. Thus, the results of this study do not explain the increased hypertension
272 risk in p.Ile180Val.

273

274 In conclusion, our study shows for the first time that neither androstenedione nor testosterone have a
275 significant biological effect on the aldosterone-mediated transactivation of the hMR. In contrast,
276 increased 17OHP and progesterone concentrations have an anti-mineralocorticoid effect due to an
277 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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288

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387 **Legends to figures and tables**

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

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

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

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

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

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

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

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

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

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

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

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