H295R expression of melanocortin 2 receptor accessory protein results in ACTH responsiveness

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Abstract

The H295R adrenocortical cell line is widely used for molecular analysis of adrenal functions but is known to have only modest ACTH responsiveness. The lack of ACTH response was linked to a low expression of its receptor, melanocortin 2 receptor (MC2R). We hypothesized that increasing the MC2R accessory protein (MRAP), which is required to traffic MC2R from the endoplasmic reticulum to the cell surface, would increase ACTH responsiveness. Lentiviral particles containing human MRAP-open reading frame were generated and transduced in H295R cells. Using antibiotic resistance, 18 clones were isolated for characterization. The most ACTH-responsive steroidogenic clone, H295RA, was used for further experiments. Successful induction of MRAP and increased expression of MC2R in H295RA cells was confirmed by quantitative real-time RT-PCR and protein analysis. Treatment with ACTH significantly increased aldosterone, cortisol, and dehydroepiandrosterone production in H295RA cells. ACTH also significantly increased transcript levels for all of the steroidogenic enzymes required to produce aldosterone, cortisol, and dehydroepiandrosterone, as well as MC2R mRNA. Using liquid chromatography/tandem mass spectrometry, we further revealed that the main unconjugated steroids produced in H295RA cells were 11-deoxycortisol, cortisol, and androstenedione. Treatment of H295RA cells with ACTH also acutely increased cAMP production and cellular protein levels for total and phosphorylated steroidogenic acute regulatory protein. In summary, through genetic manipulation, we have developed an ACTH-responsive human adrenocortical cell line. The cell line will provide a powerful in vitro tool for molecular analysis of physiologic and pathologic conditions involving the hypothalamic–pituitary–adrenal axis.

Introduction

The adrenal cortex is composed of three functionally distinct regions: zona glomerulosa, zona fasciculata, and zona reticularis. The zona glomerulosa is regulated by circulating angiotensin II (Ang II), potassium, and adrenocorticotropic hormone (ACTH) and synthesizes mineralocorticoids. The zona fasciculata is regulated by ACTH and produces cortisol. The zona reticularis is mainly regulated by ACTH and produces 19 carbon (C19)
steroids including DHEA and DHEAS. The disruption of adrenal steroid production results in various disorders. *In vitro* cell culture models provide important information for understanding molecular and cellular mechanisms controlling both the normal and pathologic function of the adrenal cortex. The H295R adrenocortical cell line is widely used but is known to have only modest ACTH responsiveness (Bird et al. 1993, Rainey et al. 2004, Wang & Rainey 2012). Therefore, studies directed toward ACTH action have used primary cultures of adrenal cells (Xing et al. 2010, 2011) or Y-1 mouse adrenal cell line, which retains ACTH responsiveness (Schimmer 1979).

ACTH acts through melanocortin 2 receptor (MC2R) to induce the intracellular production of cAMP and stimulate steroidogenesis in the adrenal cortex. For functional expression of MC2R, MC2R accessory protein (MRAP) is required to traffic MC2R from the endoplasmic reticulum (ER) to the cell surface (Metherell et al. 2005, Webb et al. 2009). Mutations in MRAP gene cause familial glucocorticoid deficiency (FGD) type 2, which is an autosomal recessive disorder characterized by severe cortisol deficiency with high plasma ACTH levels (Metherell et al. 2005). In the Y-1 mouse cell line that endogenously express both MC2R and MRAP, knockdown of MRAP by shRNA resulted in a loss of response to ACTH, which was rescued by overexpression of the shRNA-insensitive human MRAP (Cooray et al. 2008). It has also been demonstrated that mRNA levels of both MC2R and MRAP are regulated by ACTH in human adrenal primary cultures (Xing et al. 2010). In the present study, we hypothesized that increasing the expression of MRAP in the H295R cell line would improve ACTH responsiveness.

**Materials and methods**

**H295RA cell culture establishment**

The human adrenocortical cell line H295R was cultured in DME/F12 medium (Invitrogen), 10% Cosmic Calf serum (Hyclone, Logan, UT, USA), and antibiotics. The expression of MRAP was enhanced in H295R cells using a pLX304-Blast-V5 vector with a MRAP open reading frame (GE Dharmacon, Lafayette, CO, USA, OHS6085-213581101, Accession #BC062721). Lentiviral particles containing the MRAP open reading frame were produced and transduced into H295R cells with a multiplicity of infection of 5.0 using 6 μg/ml of polybrene (EMD Millipore Corporation, Billerica, MA, USA). Transduced cells were then selected by 1 μg/ml of blasticidin (Life Technologies). The concentration of blasticidin was determined according to the blasticidin selectivity test with H295R cells. After the antibiotic selection, cells were plated at cloning density and isolated using cloning cylinders (Sigma–Aldrich). From 18 isolated clones, the most ACTH-responsive steroidogenic clone (H295RA) was used for this study. The parental H295R cells were used for comparison experiments. For experiments, cells were plated in 24-well plates at a density of 200 000 per well and incubated at 37 °C for 2 days. One day before the experiment, cells were changed to a low serum experimental medium (DME/F12 medium with 1% Cosmic Calf serum and antibiotics). The next morning, cells were treated in the same low serum or serum-free experimental medium for the indicated times. Serum-free medium was used for cAMP measurement to avoid interference of Cosmic Calf serum to the assay.

**Steroid immunoassays**

Aldosterone, cortisol, and DHEA were measured in cell culture medium using ELISA. ELISA kits for cortisol and DHEA were purchased from Alpco (Sakem, NH, USA). MAB against aldosterone was kindly provided by Dr C E Gomez-Sanchez, University of Mississippi Medical Center (Gomez-Sanchez et al. 1987). The results were normalized to protein amounts and shown as fold change over basal condition.

**Steroid quantitation by liquid chromatography-tandem mass spectrometry**

Unlabeled and deuterium-labeled steroid standards were obtained from Sigma–Aldrich, Steraloids, Cerilliant (Round Rock, TX, USA), C/D/N Isotopes (Pointe-Claire, Quebec, Canada), or Cambridge Isotope Laboratories (Tewksbury, MA, USA), or they were synthesized (shown in Supplementary Table 1, see section on supplementary data given at the end of this article for unpublished steroids). Preparation and measurement of Δ⁴ steroids were performed as previously described (Turcu et al. 2015). Steroid sulfates were extracted with 1 ml of 1:1 chloroform:2-butanol from a 50 μl serum aliquot after mixing with 200 μl 1 M ammonium sulfate. The dried extracts were reconstituted with 200 μl of methanol/deionized water (1:1) and transferred to a 0.25 ml vial insert. Steroid quantitation was performed as previously described (Turcu et al. 2015). Supplementary Table 1 gives retention times and precursor/product ion pairs for the targeted steroids not previously published.
RNA isolation and quantitative real-time RT-PCR

Total RNA was extracted from cells using RNeasy mini kits (Qiagen). The purity and integrity of the RNA were checked spectroscopically using a Nano Drop spectrometer (Nano Drop Technologies, Wilmington, DE, USA). Total RNA was reverse transcribed using the High-capacity cDNA Archive kit (Applied Biosystems). PCR were performed in the ABI StepOnePlus Real-Time PCR systems (Applied Biosystems). Primer and probe mixtures for the amplification of the MRAP (Hs1588793_m1), MC2R (Hs00300820_s1), and peptidylprolyl isomerase A (PPIA, Hs99999901_m1) target sequences were purchased from Applied Biosystems. Primer and probe sets for cholesterol side-chain cleavage (CYP11A1), steroidogenic acute regulatory protein (StAR), 3β-hydroxysteroid dehydrogenase type II (HSD3B2), 17α-hydroxylase-17, 20-lyase (CYP17), 21-hydroxylase (CYP21), 11β-hydroxylase (CYP11B1), and aldosterone synthase (CYP11B2) were prepared as described previously (Sirianni et al. 2005, Ye et al. 2009). PPIA transcript was used as a reference gene for sample normalization. The delta-delta threshold cycle (ΔΔCt) method was used to calculate fold changes in mRNA expression (Livak & Schmittgen 2001).

Protein extraction and western analysis

Cells were lysed in mammalian protein extraction reagent (Thermo Scientific, Rockford, IL, USA). The protein content of samples was determined by the bicinchoninic acid protein assay using the micro bicinchoninic acid protein assay kit (Thermo Scientific, Rockford, IL, USA). The protein concentration and shown as fold change over basal condition.

Immunocytochemistry

Cells were grown on the glass slides covered with poly-β-lysine. Cells were washed with PBS and then fixed with 100% methanol for 20 min. After washing, slides were incubated with MC2R antibody (rabbit, 1:50; H-70, sc-13107, Santa Cruz) (Table 1) overnight at 4 °C followed by secondary antibody incubation (goat-anti rabbit IgG Alexa Fluor 488, Jackson Immunoresearch, Inc., West Grove, PA, USA). Fluorescence was imaged using an Olympus Fluo-View 500 confocal microscope system.

Statistical analysis

Results are given as means ± S.E.M. unless noted otherwise. The data were analyzed and compared with control values using the t test with the SigmaPlot 12.5 software package (Systat Software, Inc., San Jose, CA, USA). One-way ANOVA methods were applied for samples with variables totaling three or more groups. Results were considered to be significantly different when the P<0.05.

Table 1 List of primary antibodies used in the present study

<table>
<thead>
<tr>
<th>Target</th>
<th>Manufacturer or individual providing the antibody</th>
<th>Species raised</th>
<th>Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>MRAP V5</td>
<td>Abcam, ab168120 Thermo Scientific, MAS-15253</td>
<td>Mouse Mouse</td>
<td>1 μg/ml (WB) 1:1000 (WB)</td>
</tr>
<tr>
<td>MC2R StAR</td>
<td>Santa Cruz, sc-13107 Dr D B Hales (Southern Illinois University School of Medicine)</td>
<td>Rabbit Rabbit</td>
<td>1:50 (ICC) 1:2500 (WB)</td>
</tr>
<tr>
<td>P-StAR</td>
<td>Dr D M Stocco (Texas Tech University Health Science Center)</td>
<td>Rabbit</td>
<td>1:1000 (WB)</td>
</tr>
<tr>
<td>β-actin</td>
<td>Sigma–Aldrich, A5441</td>
<td>Mouse</td>
<td>1:10000 (WB)</td>
</tr>
</tbody>
</table>

WB, western blotting; ICC, immunocytochemistry.
Results

Expression of MRAP in H295RA cells

Phase contrast photomicrographs of the parental H295R cells and H295RA cells are shown in Fig. 1A. Successful induction of MRAP was assessed by quantitative PCR (qPCR) and western blotting. MRAP mRNA was significantly upregulated in H295RA cells compared to regular H295R cells (8638-fold vs H295R cells, \( P < 0.001 \); Fig. 1B). H295RA MRAP tagged protein was detected between 30 and 40 kDa in western analysis using a V5 antibody (Fig. 1C, upper panel). Western analysis with an MRAP antibody showed similar results as seen with the V5 antibody, detecting double bands only in H295RA cell lysate between 30 and 40 kDa (Fig. 1C, lower panel). Neither endogenous nor tagged MRAP was detected in the parental H295R cells (Fig. 1C). We further compared basal MC2R expression between H295R and H295RA cells. In qPCR, MC2R mRNA expression was significantly higher in H295RA cells than in H295R cells (4.7-fold vs H295R cells, \( P = 0.01 \); Fig. 1D). Confocal microscopic images showed increased MC2R protein expression in H295 RA cells in immunocytochemistry (Fig. 1E).

Steroidogenic ability of the H295RA cell line

As observed in the previous results using human adrenal primary cells (Xing et al. 2010), treatment with ACTH for 24 h significantly increased the MC2R mRNA in H295RA adrenal cells compared to that observed in untreated cells (12.0 ± 1.5-fold; \( P < 0.001 \) (Fig. 2A). On the other hand, the parental H295R cells showed only a modest increase in MC2R mRNA after ACTH treatment (2.0 ± 0.2 fold vs basal). H295RA cells also significantly increased aldosterone production (3.8 ± 0.5-fold vs basal; \( P = 0.001 \), and DHEA accumulation (2.6 ± 0.08-fold vs basal; \( P < 0.001 \) after treatment with ACTH for 24 h (Fig. 2B, C, and D). Of note, the H295RA cells also retained Ang II activation of aldosterone production (9.6 ± 0.2-fold vs basal; \( P < 0.001 \); Fig. 2B). Compared to H295RA cells, the parental H295R cells showed little or no increase in these steroids after 24 h.
Steroidogenic responsiveness of H295RA cells to agonist treatment.

(A) Expression of MC2R mRNA after treatment of 10 nM ACTH. The experiments were performed in quadruplicate and results represent the mean ± s.d. of data from a single experiment. Statistical analysis was performed from three independent experiments of basal vs 24 h treatment using t test (P<0.001 vs basal). The labeled solid gray circle indicates the fold increase vs basal condition in the parental H295R cells after 24 h treatment with 10 nM ACTH. (B, aldosterone; C, cortisol; D, DHEA) Steroid accumulation in cell culture medium following treatment with 10 nM ACTH or 10 nM angiotensin II (Ang II) for 24 h. Experiments were performed in quadruplicate. Results were normalized to protein concentration and shown as fold change over basal conditions. Results represent means ± S.E.M. of data from three independent experiments. Statistical analyses were performed using one-way ANOVA followed by Dunnett’s post hoc test for aldosterone and t test for cortisol and DHEA. **P<0.001 vs basal, *P<0.001 vs basal.

Table 2 Steroids produced under basal and ACTH treatments in H295R and H295RA cells. H295R cells and H295RA cells were incubated with or without ACTH (10 nM) for 24 h. Concentrations of steroids were measured using LC-MS/MS and normalized with cellular protein content. Results represent the mean ± s.d. from three independent experiments. P values were calculated using t test.

<table>
<thead>
<tr>
<th>Steroid (nmol/mg protein)</th>
<th>Basal</th>
<th>ACTH</th>
<th>Fold change</th>
<th>Basal</th>
<th>ACTH</th>
<th>Fold change</th>
</tr>
</thead>
<tbody>
<tr>
<td>21-carbon steroids</td>
<td></td>
<td></td>
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<tr>
<td>17OHP</td>
<td>22.0 ± 1.6</td>
<td>25.7 ± 1.0*</td>
<td>1.2</td>
<td>23.7 ± 3.2</td>
<td>40.6 ± 3.9*</td>
<td>1.7</td>
</tr>
<tr>
<td>11-DOC</td>
<td>9.5 ± 2.1</td>
<td>14.6 ± 1.0*</td>
<td>1.6</td>
<td>11.4 ± 1.2</td>
<td>73.3 ± 18.8*</td>
<td>6.4</td>
</tr>
<tr>
<td>Corticosterone</td>
<td>11.2 ± 2.0</td>
<td>15.9 ± 1.1*</td>
<td>1.4</td>
<td>17.2 ± 5.1</td>
<td>82.8 ± 27.2*</td>
<td>4.8</td>
</tr>
<tr>
<td>Aldosterone</td>
<td>0.22 ± 0.08</td>
<td>0.32 ± 0.13</td>
<td>1.5</td>
<td>0.33 ± 0.16</td>
<td>0.77 ± 0.078*</td>
<td>2.9</td>
</tr>
<tr>
<td>11-Deoxycorticosterone</td>
<td>204.1 ± 3.6</td>
<td>275.7 ± 9.8*</td>
<td>1.4</td>
<td>245.9 ± 27.7</td>
<td>717.7 ± 191.3*</td>
<td>2.9</td>
</tr>
<tr>
<td>Cortisol</td>
<td>41.2 ± 5.2</td>
<td>54.5 ± 2.3*</td>
<td>1.3</td>
<td>83.4 ± 23.9</td>
<td>132.9 ± 15.8*</td>
<td>1.6</td>
</tr>
<tr>
<td>19-carbon steroids</td>
<td></td>
<td></td>
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<tr>
<td>Androstenedione</td>
<td>99.3 ± 13.1</td>
<td>102.8 ± 7.7</td>
<td>1.1</td>
<td>110.2 ± 20.8</td>
<td>157.4 ± 28.3</td>
<td>1.4</td>
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<tr>
<td>Steroid sulfates</td>
<td></td>
<td></td>
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<tr>
<td>Preg-S</td>
<td>196.9 ± 12.6</td>
<td>255.1 ± 23.8*</td>
<td>1.3</td>
<td>86.6 ± 15.8</td>
<td>251.3 ± 44.8*</td>
<td>3.3</td>
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<tr>
<td>17OHPreg-S</td>
<td>18.6 ± 1.9</td>
<td>23.5 ± 3.9</td>
<td>1.3</td>
<td>7.7 ± 0.8</td>
<td>16.0 ± 0.6*</td>
<td>2.3</td>
</tr>
<tr>
<td>DHEAS</td>
<td>48.5 ± 12.7</td>
<td>41.0 ± 5.1</td>
<td>0.9</td>
<td>19.0 ± 3.5</td>
<td>22.1 ± 2.4</td>
<td>1.3</td>
</tr>
</tbody>
</table>

*P<0.05 vs basal. 17OHP, 17α-hydroxyprogesterone; 11-DOC, 11-deoxycorticosterone; Preg-S, pregnenolone sulfate; 17OHPreg-S, 17α-hydroxypregnenolone sulfate.

mRNA expression of steroidogenic enzymes in H295RA cell line

To evaluate the adrenal differentiated function in H295RA cells, the expression of mRNA-encoding enzymes involved in steroid hormone biosynthesis was measured (Fig. 3A, B, C, D, E, and F; Supplementary Figure 1, see section on supplementary data given at the end of this article). Treatment with 10 nM ACTH for 24 h increased all of the mRNA of steroidogenic enzymes required for aldosterone, cortisol, 11-deoxycortisol, pregnenolone sulfate, and 17α-hydroxyprogrenolone sulfate as well as aldosterone and cortisol (P<0.05; Table 2).
Acute response to ACTH in H295RA cell line

To evaluate the acute response to ACTH in H295RA cells, we investigated cAMP production and StAR expression after 10 nM ACTH treatment in H295RA cells. Treatment with ACTH significantly increased cAMP accumulation in cell culture medium after 3 h treatment with ACTH (2.0 ± 0.07 (P < 0.001 vs basal), 1.6 ± 0.08 (P = 0.002 vs basal), 1.6 ± 0.04 (P < 0.001 vs basal), 3.6 ± 0.2 (P < 0.001 vs basal), 3.0 ± 0.08 (P < 0.001 vs basal), 13.7 ± 0.9 (P < 0.001 vs basal), and 3.9 ± 0.07 (P < 0.001 vs basal), respectively), whereas only small effects were observed in the parental H295R cells (each fold increase vs basal condition after 24 h treatment with ACTH is indicated as gray solid circle in Fig. 3A, B, C, D, E, and F; Supplementary Figure 1).

Disscusion

The H295R cell line is widely used to study steroid hormone production and to define the molecular mechanisms regulating transcription of steroidogenic enzymes (Rainey et al. 2004, Wang & Rainey 2012). Although the H295R cells increase aldosterone production in response to Ang II and potassium treatment (Bird et al. 1993, Clyne et al. 1997, Wang et al. 2012), the cells have only moderate responsiveness to ACTH. Therefore, most experiments designed to examine the cAMP-dependent pathway require the addition of either forskolin or cAMP analogues (Rainey et al. 1993, 2004). The low response to ACTH has been a drawback of this cell model and linked to the existence of an MC2R accessory factor (Metherell et al. 2002). In 2005, MRAP was identified to cause FGD type 2 (Metherell et al. 2005). MRAP was shown to directly interact with MC2R and is essential for the trafficking of cortisol and DHEA production, namely, CYP11A1, StAR, CYP17, HSD3B2, CYP21, CYP11B1, and CYP11B2 (means of fold increase vs basal ± S.E.M. after 24 h treatment with ACTH were 2.0 ± 0.07 (P < 0.001 vs basal), 1.6 ± 0.08 (P = 0.002 vs basal), 1.6 ± 0.04 (P < 0.001 vs basal), 3.6 ± 0.2 (P < 0.001 vs basal), 3.0 ± 0.08 (P < 0.001 vs basal), 13.7 ± 0.9 (P < 0.001 vs basal), and 3.9 ± 0.07 (P < 0.001 vs basal), respectively).
MC2R from the ER to the cell surface (Metherell et al. 2005). The MRAP gene is encoding a protein of 172 residues and predicted molecular weight of 19 kDa. MRAP forms a stable antiparallel homodimer at the plasma membrane and in the ER and shows glycosylation of the protein (Sebag & Hinkle 2007, Cooray et al. 2008). In the present study, both anti-V5 antibody and anti-MRAP antibody detected the bands in H295RA cells between 30 and 40 kDa on western blotting. The discrepancy in protein size might be due to dimerization and posttranslational modification of the protein. In fact, a previous study demonstrated that endogenously expressed Y-1 adrenal cell MRAP was immunodetected as a band of more than 30 kDa despite the predicted molecular mass of 14 kDa (Cooray et al. 2008).

H295RA cells showed higher basal MC2R expression levels and greater response to ACTH stimulation compared to the parental H295R cells in terms of steroidogenesis and mRNA expression of steroidogenic enzymes. As observed in a previous report using primary cultures of human adrenal cells (Xing et al. 2010), the mRNA level of MC2R was upregulated by ACTH stimulation. It is not clear why H295RA cells exhibited higher basal levels of MC2R compared to the parental cell; however, based on the above the presence of low levels of ACTH in the serum used for growth medium may be responsible. LC-MS/MS analysis revealed that the most abundant unconjugated steroid hormones produced under basal conditions by the H295RA cells were 11-deoxycortisol, followed by androstenedione and cortisol. These results are consistent with previous LC-MS/MS steroid profiles reported in H295R cells (Xing et al. 2011). The reasons for the relatively mild increase in cortisol after ACTH stimulation in our studies may relate to the low basal expression of CYP11B1 and slow ACTH-induction of the enzyme. Whereas the aldosterone production in H295RA cells was very low under basal conditions, the cells showed significant increase in aldosterone after ACTH stimulation. Nonetheless, the concentration of aldosterone produced after ACTH treatment is still much lower than that of cortisol (Table 2). Our previous studies have shown that aldosterone regulation is predominantly regulated by the Ang II pathway as opposed to ACTH/cAMP signaling (Denner et al. 1996). H295RA cells also acutely increased cAMP production and the cellular protein level of total StAR as well as phosphorylation of StAR at serine 195, which is known to approximately double the StAR activity (Arakane et al. 1997). These findings strengthen the functional evidence on the ACTH responsiveness of the H295RA cells.

In conclusion, we have developed an ACTH-responsive cell line, H295RA, from ACTH nonresponsive cells. The cell line will provide an improved in vitro model for molecular analysis of physiologic and pathologic actions involving hypothalamic–pituitary–adrenal axis.

**Supplementary data**
This is linked to the online version of the paper at http://dx.doi.org/10.1530/JME-15-0230.

**Declaration of interest**
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.
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