Aberrant expression of multiple hormone receptors in ACTH-independent macronodular adrenal hyperplasia causing Cushing’s syndrome

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Abstract

Objective: Aberrant adrenal expression of various hormone receptors has been identified in ACTH-independent macronodular adrenal hyperplasia (AIMAH) causing cortisol hypersecretion regulated by hormones other than ACTH. We aimed to determine aberrant expression of multiple hormone receptors in vivo and in vitro in adrenal tissue of a patient with AIMAH.

Design: The design of the study includes clinical case description, and biochemical and immunohistochemical analysis to demonstrate aberrant expression of multiple hormone receptors in AIMAH.

Methods: The subject of the study is a male diagnosed with Cushing’s syndrome because of AIMAH. Directly after laparoscopic removal of the adrenals, adrenal tissue was incubated with and without test substances (ACTH, forskolin, arginine vasopressin (AVP), desmopressin, epinephrine, norepinephrine, purified human chorionic gonadotropin (hCG), metoclopramide and the combinations of AVP with ACTH, epinephrine and metoclopramide). LH/hCG-receptor (hCG-R) immunohistochemistry and RT-PCR analyses were performed to demonstrate aberrant expression of LH/hCG-R and V₁–₃-AVPR.

Results: AIMAH was characterized by in vivo cortisol responsiveness to AVP and in vitro cortisol responses to AVP, hCG, epinephrine, and norepinephrine suggesting aberrant adrenal expression of the receptors for AVP (the V₁–₃-AVPRs), catecholamines (the β-AR), and LH (the LH/hCG-R). Incubation with combinations of AVP and ACTH and of AVP with epinephrine induced a stronger cortisol response compared with incubation with the individual agents. Moreover, we demonstrated adrenal V₁–₃-AVPR and LH/hCG-R expression.

Conclusions: AIMAH tissue may simultaneously express multiple aberrant hormone receptors, and individual ligands may potentiate each other regarding cell proliferation and cortisol production.

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Introduction

Primary adrenal causes of Cushing’s syndrome cause ~15–20% of cases of endogenous Cushing’s syndrome in adults (1). Of them, bilateral adrenal diseases such as primary pigmented nodular adrenocortical disease, bilateral adrenomas or carcinomas, or ACTH-independent macronodular adrenal hyperplasia (AIMAH) account for only 10–15% of cases (2, 3). Aberrant adrenal expression of various ectopic or eutopic G-protein-coupled receptors (GPCRs) has been identified in several cases of AIMAH and unilateral adrenal adenomas (2). This aberrant expression of receptors leads to a cortisol hypersecretion regulated by hormones other than ACTH thus escaping the normal cortisol-mediated feedback. We report a male patient with Cushing’s syndrome due to AIMAH. The investigation was rendered more complex because a non-functional pituitary incidentaloma was also present. A plasma cortisol response was found after administration of arginine vasopressin (AVP). Unexpectedly, apart from AVP, epinephrine, norepinephrine, and human chorionic gonadotropin (hCG) also induced an aberrant in vitro cortisol response indicating expression of multiple aberrant hormone receptors.

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Materials and methods

Case report

A 60-year-old male was referred for suspected Cushing’s syndrome, bilateral nodular adrenal hyperplasia, and a pituitary microadenoma of 6 mm. He had been treated for hypertension and diabetes mellitus for 12 years. Furthermore, he was diagnosed with osteoporosis. Two years earlier, he underwent percutaneous transluminal coronary angioplasty because of a myocardial infarction. During the last couple of years, he had noticed rounding of his face, weight gain (8 kg), fatigue, and easy bruising. His medication consisted of metformin, rosiglitazone, glibenpiride, omeprazole, metoprolol, ciprolibrate, amlodipine, furosemide, irbesartan, acetylsalicylic acid, and calcium/vitamin D.

Physical findings revealed facial plethora, ‘moon-face’, truncal obesity (weight, 115 kg; height, 188 cm; body mass index, 33), multiple bruises, and atrophic skin without striae. Blood pressure was 180/80 mmHg while recumbent. On laboratory examination, renal function and electrolytes were within the normal range. Non-fasting glucose was 15.9 mmol/l, and HbA1c was 10.2%. ACTH-independent hypercortisolism was established by an increased urinary cortisol excretion (391 nmol/24 h, normal 20–270 nmol/24 h), insufficient cortisol suppression after administration of 1 mg dexamethasone overnight, and suppressed plasma ACTH concentrations (1.3 ng/l, normal 10–100 ng/l).

In addition, high-dose (7 mg) i.v. dexamethasone did not suppress plasma cortisol and ACTH, and cortisol did not respond to i.v. CRH (100 µg). The pituitary tumor that was diagnosed elsewhere was considered to be a non-functional microadenoma. Evaluation of anterior pituitary function revealed no abnormalities except for secondary hypogonadism, presumably due to chronic hypercortisolism.

To screen for ectopic hormone receptor expression (after cessation of all medication 4 days before testing), the following tests were performed: a standard mixed meal (116 g carbohydrates, 27 g proteins, and 14 g fat), a posture test, tetracosactide (250 µg i.v.), LHRH (100 µg i.v.), TRH (200 µg i.v.), glucagon (1 mg i.v.), cisapride (10 mg orally), AVP (10 IU i.m.), and desmopressin (dDAVP, 2.5 µg s.c.). A positive response was defined by a 50% increase in plasma cortisol levels as defined by Lacroix et al. (4).

Bilateral adrenalectomy was planned for the patient and treated with ketoconazole 2x200 mg daily. However, he had a severe myocardial infarction for which he eventually underwent coronary artery bypass grafting and aortic valve replacement. During this period of over 6 months, he was maintained on ketoconazole, and urinary cortisol levels returned to normal. Eventually, a bilateral adrenalectomy was performed. Both adrenals were diffusely enlarged with multiple nodules. A part of both adrenals was removed for in vitro studies. Microscopic examination revealed nodular hyperplasia extending throughout both glands. Ketoconazole was stopped after surgery. The postoperative course was uncomplicated. The patient was treated with steroid replacement therapy (cortisone acetate 25–12.5 mg and fludrocortisone 62.5 µg daily), metformin, omeprazole, sotalol, alendronic acid, acetylsalicylic acid, and calcium. He remains in followup for his pituitary incidentaloma.

Control patients

Hyperplastic adrenal tissue, serving as control tissue for the in vitro studies, was obtained from two patients who underwent bilateral laparoscopic adrenalectomy; one 62-year-old male with severe Cushing’s syndrome due to ectopic ACTH production (control 1) and one 50-year-old male with persistent Cushing’s disease after pituitary surgery and radiotherapy (control 2). Normal adrenal tissue was obtained from three patients (controls 3, 4, and 5) who underwent nephrectomy due to renal cell carcinoma. Adrenals were removed because of radical tumor resection, but did not contain tumor cells microscopically.

Cell preparation and incubation studies

Directly after laparoscopic removal, adrenal cortical tissue of the major part of the adrenals was minced into small pieces and dissociated with collagenase (type I; Sigma Chemical Co.), as described previously (5, 6). Cell viability was determined by trypan blue staining and was more than 80%. Subsequently, the cells were resuspended in incubation medium (DMEM with 0.2% BSA), and incubated with and without test substances during 2 or 24 h. These incubations were performed in quadruplicate using 500 000 cells/ml. After 2-h incubation, 0.5 ml distilled water was added to each tube, and the resulting suspension was stored at −20°C until the measurement of hormone concentrations, as described previously (5). Cortisol concentrations were measured by a fluorescent immunoassay (Diagnostic Products Corp., Los Angeles, CA, USA). The 2-h incubations were performed with the following substances: ACTH-(1–24) (Synachten, Novartis; concentrations: 31, 62, 125, 250, and 500 pg/ml), forskolin in a concentration of 1 µM, AVP (Pitresmin, Monarch Pharmaceuticals, Bristol, UK; 0.001, 0.01, 0.05, and 0.1 µM), dDAVP (Minrin, Ferring Pharmaceuticals; 0.001 and 0.01 µM), epinephrine (0.1, 1, and 10 µM), norepinephrine (0.1, 1, and 10 µM), purified hCG (Organon, Oss, The Netherlands; 50 mIU/ml), metoclopramide (0.1, 1, and 10 µM), and the combinations of AVP (0.01 µM) with ACTH (62 µg/ml), epinephrine (1 µM), and metoclopramide (1 µM).
Table 1  Peak plasma cortisol levels as percentage of baseline values.

<table>
<thead>
<tr>
<th>Stimulation test</th>
<th>Receptor</th>
<th>Peak value (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tetracosactide</td>
<td>ACTH</td>
<td>226</td>
</tr>
<tr>
<td>Posture</td>
<td>V₁,₃-AVP, β-adrenergic, AT-1</td>
<td>8</td>
</tr>
<tr>
<td>Standard mixed meal</td>
<td></td>
<td>6</td>
</tr>
<tr>
<td>LHRH</td>
<td>LH, FSH, GnRH</td>
<td>8</td>
</tr>
<tr>
<td>TRH</td>
<td>TSH, TRH, prolactin</td>
<td>3</td>
</tr>
<tr>
<td>Glucagon</td>
<td>Glucagon</td>
<td>6</td>
</tr>
<tr>
<td>Vasopressin</td>
<td>V₁,₃-AVP</td>
<td>86</td>
</tr>
<tr>
<td>Desmopressin</td>
<td>V₂-AVP</td>
<td>7</td>
</tr>
<tr>
<td>Cisapride</td>
<td>5HT-4</td>
<td>13</td>
</tr>
</tbody>
</table>

*Tests were considered negative if cortisol levels increased by <25%. A partial response was defined as a 25–49% increase, and a positive test was defined as a more than 50% increase in cortisol levels.

RT-PCR of vasopressin receptors

After surgery, residual adrenocortical tissues were snap-frozen and stored at −80 °C. For the purpose of mRNA analysis, samples were homogenized, and subsequently, RNA was isolated using TRIzol reagent (Invitrogen) according to the manufacturer’s instructions. RNA was reverse transcribed into cDNA using previously described methods (7). The following primers were used to detect mRNA expression of vasopressin receptors: F: CGGCTTCATCTGCTACAACATC, R: CGAGTCCCTTCCAACATACCCGC (V₁a-AVPR, 506 bp) (8); F: GTGGCCAAGACTGTGAGGAT, R: CATAGATCATGATCAGGGTTG (V₂-AVP, 181 bp); F: CAGCAGCATCAGTAT, R: TCAAATCCAACAAAGTCTGGCTTATATC. (9).

LH receptor immunohistochemistry

The LH receptor-specific antibody (20C3) used in this study was kindly provided by Dr A Funaro (10). The mouse MAB was used for immunohistochemistry on formalin-fixed paraffin-embedded tissue. Normal adult testis was used as a positive control. Tissue sections of 3 μm thickness were deparaffinized, and incubated with the antibody (dilution 1:1000 in PBS containing 1% BSA) overnight at 4 °C. Rabbit anti-mouse secondary biotinylated antibodies (DAKO, Glostrup, Denmark) were used in a 1:200 dilution and incubated for 30 min at room temperature. Antibody complex was visualized by avidin–biotin conjugated with HRP using 3,3-diaminobenzidine as chromogen and H₂O₂ as substrate. All slides were counterstained with hematoxylin.

Statistical analysis

The effects of various test substances on in vitro cortisol production by cultured adrenal cells were tested by ANOVA followed by Newman–Keuls test.

Results

In vivo studies

After stimulation with 250 μg tetracosactide i.v., plasma cortisol levels increased from 715 nM to peak levels of 2330 nM after 120 min. Administration of 10 IU AVP i.m. was followed by an increase in plasma cortisol levels from 470 to 775 nM after 30 min. Plasma ACTH levels were undetectable during these stimulation tests. No significant cortisol responses were found after a posture test or administration of dDAVP, or after a standard mixed meal and administration of LHRH, TRH, glucagon, or cisapride (Table 1). After administration of LHRH, levels of LH (from 3 to 20 IU/l 90 min after LHRH) and FSH (from 8 to 14 E/l 90 min after LHRH) increased.

In vitro studies

Cultured adrenocortical adenoma cells, obtained from patient and controls 1 and 2, produced 135 ± 2.7, 76 ± 1.9, and 169 ± 5.4 nmol cortisol/tube (mean ± S.E.M.) respectively after 2 h. Incubation with ACTH stimulated cortisol production in a dose-dependent manner (Figure 1).
fashion (Fig. 1). Forskolin, as a stimulator of cAMP production, induced cortisol production by adrenal cells from the patient (Fig. 1) and controls (maximum increase 588 ± 7.0 and 456 ± 6.1% respectively compared to own control).

AVP strongly stimulated cortisol production by cells from the patient in a dose-dependent manner to maximum values nearly reaching values after maximum ACTH stimulation (Fig. 2A). In cells obtained from control patient 1, AVP induced a modest but significant increase in cortisol secretion (Fig. 2B), whereas in control patient 2, AVP only slightly stimulated cortisol production (Fig. 2C). In contrast, dDAVP had no significant effect on cortisol production by cells from the patient or by cells from the controls.

Both epinephrine and norepinephrine had a dose-dependent effect on cortisol production by cells from the patient (Fig. 3A). hCG also induced a significant increase in cortisol production (Fig. 3A). Epinephrine, norepinephrine, or hCG did not affect cortisol secretion by cells obtained from the control patients (data not shown). Figure 3B shows that incubation with the combinations of AVP and ACTH and of AVP with epinephrine induced a stronger cortisol response compared with incubation with the individual agents.

**Vasopressin receptor mRNA expression**

mRNA analysis showed positive expression of all three vasopressin receptors and HPRT1 in adrenal tissue derived from the patient, which is shown in Fig. 4. For V2-AVPR, the amplification of both genomic DNA (286 bp) and cDNA (181 bp) is visible. Controls also showed positive expression of V1 and V2 receptors, but were all negative for V3 receptor mRNA. The expression of V1-AVPR in the patient’s adrenal tissue seems to be higher compared with control adrenal tissues, whereas the expression of housekeeping gene HPRT1 in these samples did not differ.

**LH/hCG receptor expression**

The LH/hCG-receptor (hCG-R)-specific antibody was validated in HEK293 cells, which were transfected with the human LH/hCG-R (data not shown), and in human testis tissue with positive staining of the Leydig cells but not in the somatic component of the seminiferous tubules (Fig. 5, left panel). Hyperplastic adrenal tissue of the patient showed diffuse staining indicating LH/hCG-R expression (Fig. 5, right panel).

**Discussion**

We describe a patient with Cushing’s syndrome based on bilateral AIMAH, and characterized by in vivo cortisol responsiveness to AVP and in vitro cortisol responses to AVP, hCG, epinephrine, and norepinephrine. AIMAH and, to a lesser extent, unilateral adrenal adenomas are associated with aberrant expression of several eutopic and ectopic GPCRs which are functionally coupled to steroidogenesis (2, 3). This includes receptors for AVP (V1, V2-AVPR or AVPR1A, AVPR1B, AVPR2) (11–14), gastric inhibitory polypeptide (GIPR) (15, 16), LH

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**Figure 2** In vitro cortisol response of cultured ACTH-independent macronodular adrenal hyperplasia (AIMAH) cells to AVP and dDAVP (2-h stimulated cortisol values as percentage of patient’s own control values). AIMAH cells were prepared from hyperplastic adrenal glands of a patient with an AVP-responsive Cushing’s syndrome (A), and adrenal adenoma cells of two patients with ectopic ACTH production by a neuroendocrine tumor and pituitary-dependent Cushing’s syndrome respectively served as controls (B and C). Data are expressed as mean (n=4) ± S.E.M. *P<0.001 compared to patient’s own control.

**Figure 3** (A) In vitro cortisol response of cultured ACTH-independent macronodular adrenal hyperplasia (AIMAH) cells from a patient with an AVP-responsive Cushing’s syndrome to epinephrine, norepinephrine, and hCG (2-h stimulated cortisol values as percentage of patient’s own control values). Data are expressed as mean (n=4) ± S.E.M. *P<0.001 compared with patient’s own control. (B) In vitro cortisol response of cultured AIMAH cells obtained from the same patient to AVP (10 nM), ACTH (62 pg/ml), epinephrine (1 μM), and to co-incubation of AVP (10 nM) with ACTH (62 pg/ml) and epinephrine (1 μM) respectively. Data are expressed as mean (n=4) ± S.E.M. **P<0.001 compared to incubation with individual agents.
also showed positive expression of V1 and V2 receptors, but were all negative for V3 receptor mRNA. The expression of V1-AVPR in the patient’s adrenal tissue seems to be higher compared with control adrenal tissues.

(LH/hCG-R) (17, 18), catecholamines (β-AR) (19, 20), serotonin (5HT-4R or HTR4) (21, 22), and angiotensin II (AT-1R or AGTR1) (23). AIMAH-dependent Cushing’s syndrome can be accompanied by expression of a single GPCR, but often aberrant co-expression of several GPCRs is found, in particular V1-AVPR and 5HT-4R (3, 24). In this study, we demonstrate simultaneous aberrant responses suggestive of adrenal expression of the V1-AVPR, V3-AVPR, β-AR, and LH/hCG-R, a combination which, to our knowledge, has not previously been reported. The V1-AVPR is overexpressed in combination with ectopic expression of V3-AVPR in this patient with AIMAH, whereas V2-AVPR seems to be expressed in normal adrenals as well. In addition, we found that co-incubation of ligands for these GPCRs potentiated the cortisol response of hyperplastic adrenal tissue \textit{in vitro}. Moreover, we demonstrated adrenal expression of LH/hCG-R immunohistochemically and V1-AVPR by RT-PCR.

The V3-AVPR is physiologically expressed in the adrenal gland, and activation of the V1-AVPR leads to a modest increase in steroidogenesis. This is illustrated by the dose-dependent \textit{in vitro} cortisol responses to AVP by adrenal cells obtained from control patient 1. V3-AVPR expression has been reported in AIMAH, but the effects of the V3-preferential AVPR agonist dDAVP have not been extensively examined \textit{in vivo} (2, 3). The profound cortisol responses, both \textit{in vivo} and \textit{in vitro}, to AVP but not to dDAVP in our patient point to a V1-AVPR- or V3-AVPR-mediated effect. The undetectable ACTH levels after AVP administration exclude an indirect effect via the V3-AVPR expressed in the pituitary. Since we found increased expression of V1-AVPR and ectopic expression of V3-AVPR in our patient, the observed embroidered cortisol response to AVP therefore could represent an abnormal response V1- or V3-AVPR stimulation (3). Although mutations in the V1-AVPR gene or its promoter have not been demonstrated in patients with AIMAH and AVP-responsive Cushing’s syndrome (3), AVP expression in the adrenal cortex has been reported in AIMAH and may as such regulate cortisol production in an auto- or paracrine manner (25). AVP also increases proliferation of different cell types, including rat adrenocortical cells, through activation of V1-Avpr (26), and V2-AVPR may also have contributed to the physiopathology of AIMAH by stimulating cell steroidogenesis and mitogenesis. On the other hand, \textit{in vivo} and \textit{in vitro} responses to dDAVP were absent, and it may be that V2- and V3-AVPRs are not involved in the vasopressinergic control of steroidogenesis and that V2- and V3-AVPR mRNAs present are not translated into functional proteins in AIMAH, as previously observed for the 5HT-4R (27).

Besides AVP responsiveness, our \textit{in vitro} studies also demonstrated cortisol responses to epinephrine, norepinephrine, and hCG, although these abnormal responses were not observed \textit{in vivo}.

A pharmacological agent may stimulate cortisol secretion through either a direct action on adrenocortical cells or an indirect effect on other intra-adrenal cells that, in turn, may modulate the synthesis of glucocorticoids via a cell-to-cell type of communication. Nonetheless, ectopic expression of β-ARs in the adrenal cortex has been reported \textit{in vitro} in several cases of adrenal Cushing’s syndrome, whereas an \textit{in vivo} cortisol response to catecholamines has been demonstrated in few patients with Cushing’s syndrome due to AIMAH (19, 20). An explanation for the absence of an \textit{in vivo} cortisol response on posture in our patient could have been the use of metoprolol, irbesartan, and amlodipine. Although the medication was stopped 4 days before testing, it might have had an effect on the posture test. In addition, the patient used this medication for more than 4 years while symptoms of Cushing’s syndrome worsened. Therefore, we do not believe that aberrant
expression of the β-AR contributed significantly to the clinical picture.

We also found an in vitro cortisol response to hCG and LH/hCG-R protein expression in the patient’s adrenal tissue. The LH/hCG-R is thought to be constitutively expressed in the normal adrenal gland, presumably in the zona reticularis, and may regulate synthesis of dehydroepiandrosterone, at least in human fetal adrenal cells (18, 28). LH-responsive Cushing’s syndrome could result from an increased LH/hCG-R expression and/or an abnormal coupling between LH/hCG-R and steroidogenesis (2, 18). In AIMAH, co-expression of the LH/hCG-R with other GPCRs (5HT-4R, V1–3-AVPR, or GIPR) is observed in a majority of described cases (18, 21, 25, 27). However, this mainly involves detection of LH/hCG-R mRNA. One study demonstrated LH/hCG-R protein expression in a case of AIMAH, but in this report, no data were provided on antibody validation (29). We show, with a well-validated LH/hCG-R antibody, LH/hCG-R protein expression in hyperplastic adrenal tissue.

LH has been postulated to play a role in the pathogenesis of AIMAH. Transgenic mice with constitutive LH overproduction develop both polycystic ovaries and bilateral adrenal hyperplasia with an increased corticosterone production (30). Furthermore, LH/hCG-dependent Cushing’s syndrome caused by AIMAH has primarily been reported in female patients. The initial pathophysiological concept was that LH/hCG levels should reach a certain threshold to exert stimulatory effects on cortisol synthesis as occurs during pregnancy or the postmenopausal state. However, LH/hCG-dependent Cushing’s syndrome has also been described in premenopausal women with AIMAH, indicating that LH levels in the lower range may also regulate cortisol production (18). In addition, Mazzuco et al. have recently demonstrated that transplanation of normal adrenal cells with retrovirus-mediated LH/hCG-R expression into adrenalectomized mice is followed by the development of a hyperplastic adrenal mass with concomitant ACTH-independent hypercortisolism, responsive to hCG (31). Thus, supraphysiological LH/hCG levels are not a prerequisite for the development of LH/hCG-dependent AIMAH in the presence of an enhanced adrenal LH/hCG-R expression. Aberrant cortisol responses to LH/hCG in male patients with AIMAH, however, have only been reported in two patients with an overt and subclinical Cushing’s syndrome respectively (21, 32). The absence of a cortisol response to LHRH in vivo in our patient may be explained by the concentration and time of exposure to LH/hCG. In one patient with LH/hCG-responsive Cushing’s syndrome, the in vitro cortisol response was attenuated with high concentrations of hCG suggesting the occurrence of a desensitization phenomenon of the receptor, as previously shown in Leydig cells (25, 30).

The aberrant expression of multiple GPCRs makes it difficult to draw any conclusions on the relative importance of each receptor or the possible co-operation between aberrant GPCRs. Nevertheless, we show for the first time that aberrant stimuli synergize in the stimulation of cortisol production in AIMAH, at least in vitro. AVP-stimulated cortisol production was potentiated by co-incubation with epinephrine or with ACTH. However, the exact mechanism is presently unknown. In this respect, it remains intriguing that the hyperplastic adrenal tissue retains its sensitivity to ACTH, both in vivo and in vitro. Indeed, to date, no (loss-of-function) mutations of the ACTH receptor gene have been found in patients with AIMAH (18, 33).

In conclusion, the present study additionally demonstrates that AIMAH tissue may simultaneously express multiple aberrant GPCRs. We describe for the first time AIMAH responding to both AVP, (nor)epinephrine, and gonadotropins. The synergizing effects of aberrant stimuli on cortisol production observed in vitro may translate into the pathophysiological mechanism in vivo, with potentiating interactions of these ligands on cell proliferation and cortisol production.

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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