Expression of vasopressin receptors in ACTH-independent macronodular bilateral adrenal hyperplasia causing Cushing’s syndrome: molecular, immunohistochemical and pharmacological correlates

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Abstract
Cortisol secretion in ACTH-independent macronodular adrenal hyperplasia (AIMAH) causing Cushing’s syndrome can be controlled by illegitimate receptors. The aim of the present study was to characterize the molecular, immunohistochemical, and pharmacological profiles of vasopressin receptors in cells derived from three patients with AIMAH (H1–H3), in order to evaluate the role of ectopic vasopressin receptors in the physiopathology of hypercortisolism. Expression of mRNAs encoding the vasopressin receptor types (V1a, V1b, and V2) were analyzed by RT-PCR in adrenal tissues. The presence of V1a and V2 receptors was studied by immunohistochemistry on adrenal sections. The pharmacological profiles of vasopressin receptors involved in the control of cortisol secretion were investigated using the V1a receptor antagonist SR49059 and the V2 receptor agonist [deamino-Cys1, Val4, d-Arg8]-vasopressin on cultured cells. The V1a receptor protein was present and functional in H1 and H3 tissues, whereas the V1b receptor was not expressed in any of the tissues. RT-PCR experiments revealed that V2 receptor mRNAs were detected in the three tissues. In contrast, immunohistochemical and cell incubation studies showed that the V2 receptor was involved in the stimulatory effect of AVP on cortisol secretion in H1 and H2, but not in H3 cells. Taken together, these data show that expression of functional ectopic V2 receptors and repression of eutopic V1a receptor can coexist in some hyperplastic corticosteroidogenic tissues. They also reveal that immunohistochemical and incubation studies are essential for the characterization of ectopic receptors actually involved in the control of cortisol secretion by AIMAHs.

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Introduction
Expression of ectopic and overexpression of eutopic G-protein-coupled receptors for peptide hormones have been reported in different lesions, including hyperplasia, tumors, and cancers of various tissues (Qu et al. 2004, Havt et al. 2005, Weng et al. 2006). Activation of aberrant receptors initiates intracellular signals responsible for excessive cellular activity and cell proliferation. Characterization of aberrant receptors may be used to help shed light on the pathophysiological mechanism of gain-of-function disorders and may allow the development of therapeutic approaches to reduce the activity of hyperfunctioning cells. In Cushing’s syndrome, due to unilateral adrenal adenoma or adrenocorticotropin (ACTH)-independent bilateral macronodular adrenal hyperplasia (AIMAH), overproduction of cortisol, which leads to suppression of ACTH release, is under the control of illegitimate G-protein-coupled receptors (Lacroix et al. 2004). These receptors include ectopic receptors for gastric inhibitory polypeptide (GIP), luteinizing hormone (LH) or serotonin- (5-HT3) receptors, and abnormally active eutopic receptors like vasopressin V1 and 5-HT4 receptors (Lacroix et al. 2004). Illegitimate receptors were initially detected by clinical studies aimed at evaluating the plasma cortisol responses to various physiological and pharmacological stimuli (Lacroix et al. 1999). In particular, aberrant expression of vasopressin receptors has been described in some cases of AIMAH responding to physiological stimuli of endogenous vasopressin. In these reports, elevation of plasma AVP provoked by upright posture, insulin-induced hypoglycemia, or hypertonic saline infusion test was followed by an increase in plasma cortisol (Lacroix et al. 1997, Daidoh et al. 1998, Miyamura et al. 2003). An abnormal response of cortisol to injection of AVP or lysine vasopressin (LVP) was also observed (Lacroix et al. 1997, Daidoh et al. 1998, Miyamura et al. 2003, Bertherat et al. 2005). It was conceivable that hypersensitivity of cortisol secretion to AVP in these patients might result from activation of illegitimate receptors,
i.e., overexpressed eutopic V1a receptors and/or ectopic V1b and V2 receptors. In agreement with this hypothesis, molecular studies have shown overexpression of V1a receptor and abnormal expression of V1b and V2 receptor mRNAs in whole tissue explants removed from AIMAHs (Miyamura et al. 2002, Mune et al. 2002, Lee et al. 2005). However, RT-PCR data are ambiguous since it has been reported that adrenal chromaffin cells and blood vessels express the V1b and V2 receptors respectively (Aldasoro et al. 1997, Grazzini et al. 1999, Medina et al. 1999). In addition, ectopic vasopressin receptor mRNAs may not be translated into functional proteins in adrenal hyperplastic tissues. In this respect, administration of the V2 receptor agonist desmopressin failed to affect plasma cortisol in a case of vasopressin-sensitive AIMAHs expressing the V2 receptor mRNA (Miyamura et al. 2003, Vezzosi et al. 2007). It can also be noticed that the effect of V2 and V1b receptor ligands on cortisol production has never been studied in vitro in vasopressin-sensitive AIMAHs expressing V1b and/or V2 receptor mRNAs. Thus, there is currently no clear evidence for the involvement of ectopic AVP receptors in the abnormal cortisol response to AVP in AIMAH tissues.

We have previously investigated in vivo the responsiveness of cortisol secretion to upright and LVP stimulation tests in three patients with AIMAHs and Cushing’s syndrome (Bertherat et al. 2005). The aim of the present study was to determine in vitro the type of receptors mediating the corticotropic action of AVP in the tissues removed from the three patients. For this purpose, expression of AVP receptors has been investigated using RT-PCR and immunohistochemical approaches. Moreover, pharmacological characterization of vasopressin receptors was performed in cultured hyperplasia cells.

Materials and Methods

Patients

Three previously described patients with AIMAH causing overt Cushing’s syndrome were studied (patients 1, 2, and 4 from Bertherat et al. 2005). The diagnosis of ACTH-independent Cushing’s syndrome was based on the results of hormonal investigations. Briefly, increase in 24-h urinary cortisol excretion, alteration of plasma cortisol circadian rhythm, lack of cortisol suppression under a low-dose dexamethasone test (2 mg/day for 2 days), and suppression of basal plasma ACTH levels (below 1 pmol/l (5 pg/ml)) were observed in the three patients. The patients underwent a series of clinical tests aimed at detecting the expression of illegitimate receptors followed by bilateral adrenalectomy. Pathological examination of the adrenal tissues confirmed the diagnosis in all cases. The effect of AVP on cortisol secretion in hyperplasia cells has been investigated in vitro. The clinical presentation of the patients as well as the results of clinical testing and cell incubation studies are summarized in Table 1.

In the present study, normal adrenal tissue explants (control tissues) were obtained from patients undergoing expanded

| Table 1 Clinical characteristics, endocrine evaluation, and radiological findings in the three patients with adrenocorticotropin (ACTH)-independent macronodular adrenal hyperplasia (AIMAH; H1–H3) |
|---|---|---|---|
| **H1** | **H2** | **H3** |
| **Sex** | Female | Female | Female |
| **Age (year)** | 34 | 45 | 54 |
| **Symptoms of hypercortisolism** | Hypokalemia, hypertension, diabetes mellitus, facial plethora, proximal myopathy | Hypertension, ecchymotic lesions, atrial fibrillation, facial hirsutism | Hypertension, ecchymotic lesions, facial hirsutism, proximal myopathy |
| **Maximum diameter of adrenal nodules at CT scan** | Left 6, right 4 | Left 4, right 3.5 | Left 5.4, right 5.4 |
| **Urinary free cortisol (nmol/d)** | 1172 | 1448 | 1951 |
| **Plasma cortisol (nmol/l)** | 0800 h (275!N!550) | 510 | 166 |
| | 2000 h | 466 | 663 |
| **Plasma ACTH 0800 h (pmol/l)** | 11 | 1 | 11 |
| **Plasma cortisol response to upright test** | C: 62% K: 15% | C: 29% K: 15% | C: 15% K: 29% |
| **Plasma cortisol response to terlipressin (0.5 mg i.v.)** | C: 52% K: 10% | C: 126.8% G: 16.2% | C: 47.9% G: 2.7% |
| **In vitro AVP efficiency on cortisol secretion** | C: 373.3% G: 17.8% | C: 126.8% G: 16.2% | C: 47.9% G: 2.7% |
| **N, normal value.** | | | |
| **%** percentage of basal level. | | | |
nephrectomy for kidney cancer. Pituitary corticotropic adenoma tissue was obtained from a patient undergoing hypophysectomy for Cushing’s disease. Fragments of tissue were either transported to the laboratory in culture medium for primary culture, immediately stored at −80 °C until RNA extraction or fixed in formalin and embedded in paraffin for immunohistochemical study. The protocol of collection of the tissues and the experimental procedures were approved by the regional ethics committee and written informed consent was obtained from all subjects.

Reagents
Collagenase (type IA), DNase I, insulin, apotransferrin, l-ascorbic acid, [Arg⁸]-vasopressin (AVP), [deamino-Cys⁴, Val⁴, D-Arg⁸]-vasopressin (dDAVP), and Tri Reagent were purchased from Sigma. The rabbit V₁a and V₂ receptor antibodies were obtained from Santa Cruz Biotechnology (Le Perray en Yvelines, France). The nutrient medium Ham’s F-12 (HAM) and Dulbecco’s modified Eagle medium (DMEM) were obtained from Life Technologies Inc. (Paisley, Scotland, UK). The antibiotic–antimycotic solution and fetal bovine serum (FBS) were from Bio-Whittaker (Walkersville, MD, USA). (2S)-1-[(2R,3S)-5-chloro-3-(2-chlorophenyl)-1-(3,4-dimethoxybenzene-sulfonyl)-3-hydroxy-2,3-dihydro-1H-indole-2-carbonyl]-pyrrolidine-2-carboxamide (SR49059) was supplied by Sanofi Recherche. SuperScript II and DNA Taq Polymerase were from Life Technologies.

RNA extraction and conventional RT-PCR
Expression of genes encoding vasopressin (V₁a, V₁b, and V₂) receptors in adrenal hyperplasia tissues, normal adrenal gland, and pituitary corticotropic adenoma was analyzed by RT-PCR. Amplification of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA was used as a control of quality of reverse-transcribed mRNAs. The RT-PCRs were performed according to the method and experimental conditions previously described (Vezzosi et al. 2007). Briefly, total RNAs were extracted by using Tri Reagent. Total RNA (1 μg) from each preparation was converted to single stranded cDNA by SuperScript II with oligo(dT)₁₂–₁₈ primer. PCRs were carried out using gene-specific primers for each sequence (Table 2) and DNA Taq Polymerase. The PCRs were performed for 40 cycles (94 °C, 40 s; 50 °C, 60 s; 72 °C, 90 s). The PCR products were separated on agarose gels, blotted on nylon membranes, and hybridized with [³²P]ATP-labeled internal gene-specific oligonucleotides (Table 2).

Immunohistochemistry
Deparaffinized sections from the three hyperplasias and three normal adrenal glands were incubated overnight at 4 °C in a humidified atmosphere with rabbit polyclonal antibodies directed against V₁a and V₂ receptors (1:200). The sections were then incubated with a streptavidin–biotin–peroxidase complex (Dako Corporation, Carpinteria, CA, USA) and the enzymatic activity was revealed with diaminobenzidine. The specificities of the immunoreactions were controlled by substituting the primary antisera with non-immune serum. The tissue sections were counterstained with hematoxylin, mounted in Eukitt (Kindler Gmbh & Co., Freiburg, Germany), coverslipped, and examined on an Eclipse E-600 microscope equipped with a CCD DXC950 camera (Nikon, Les Ulis, France).

Cell culture
Cell culture experiments were conducted as previously described (Bertherat et al. 2005). Briefly, hyperplasia and normal adrenal gland fragments free of fat were immediately immersed in DMEM supplemented with 0.5% antibiotic–antimycotic solution. After dissection of medullary tissues with scissors, the adrenocortical fragments were enzymatically dissociated in DMEM containing collagenase (2 mg/ml) and desoxyribonuclease I (70 μg/ml) for 45 min at 37 °C. Adrenocortical cells were cultured at 37 °C in 100% relative humidity in a 5% CO₂–95% (v/v) air atmosphere. Incubation

Table 2 RT-PCR analysis of vasopressin (V₁a, V₁b, and V₂) receptor mRNAs

<table>
<thead>
<tr>
<th>Receptor</th>
<th>Accession number in Genbank</th>
<th>Primer sequence (5’–3’)</th>
<th>Primer position</th>
</tr>
</thead>
<tbody>
<tr>
<td>V₁a</td>
<td>AF 101725</td>
<td>s: CGGCTTCATCTGCTACAACATC</td>
<td>731–752</td>
</tr>
<tr>
<td></td>
<td></td>
<td>as: CGAGTCCTTCCATACCCGT</td>
<td>1215–1235</td>
</tr>
<tr>
<td></td>
<td></td>
<td>p: ATGTGGTCTGTCTGGC</td>
<td>957–977</td>
</tr>
<tr>
<td>V₁b</td>
<td>AF 101726</td>
<td>s: CAGCAGCATCAACCATCT</td>
<td>921–940</td>
</tr>
<tr>
<td></td>
<td></td>
<td>as: CCATGTAGATCCAGGGT</td>
<td>1122–1141</td>
</tr>
<tr>
<td></td>
<td></td>
<td>p: CAGCAGCATCAAACACTCC</td>
<td>1063–1083</td>
</tr>
<tr>
<td>V₂</td>
<td>NM 000054</td>
<td>s: GTGGCCAAGACTGTAAGGAT</td>
<td>1031–1050</td>
</tr>
<tr>
<td></td>
<td></td>
<td>as: CATAGTACCAGGGGTG</td>
<td>1192–1211</td>
</tr>
<tr>
<td></td>
<td></td>
<td>p: GATCCCAACATGCTGCTTTC</td>
<td>1157–1177</td>
</tr>
<tr>
<td>GAPDH</td>
<td>M17701</td>
<td>s: TGCTGATCTGCTGAGATC</td>
<td>297–317</td>
</tr>
<tr>
<td></td>
<td></td>
<td>as: TGCTGATCTGCTGAGATC</td>
<td>467–488</td>
</tr>
</tbody>
</table>

Oligonucleotide sequences for sense (s), antisense (as), and hybridization probes (p).

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experiments of cells were conducted after 2 days in culture with fresh DMEM (control experiments) or DMEM with either AVP or dDAVP. AVP was incubated in the absence or presence of SR49059. Cells were incubated with each secretagogue for 24 h at 37°C. Following each incubation period, aliquots of the culture medium were taken and immediately frozen at −20°C until cortisol RIA (Lefebvre et al. 1992). Results are expressed as mean ± S.E.M. from four experiments and statistical significance was assessed by Bonferroni test after one-way ANOVA. The dose–response curves were generated using the Prism software (GraphPad Software, San Diego, CA, USA).

Results

Molecular characterization of AVP receptors in AIMAH tissues

Expression of genes encoding V1a, V1b, and V2 receptors in adrenal hyperplasia, normal adrenal gland, and pituitary corticotrophic adenoma tissues were determined by RT-PCR. V1a receptor mRNAs were detected in normal adrenal gland (NA), and in both adrenal hyperplastic tissues from patients 1 and 3 (H1 and H3), but not in H2 tissue (Fig. 1). V1b receptor mRNA was not present in any of the adrenal tissues, but its occurrence was observed in the pituitary corticotrophic adenoma (control experiment). PCR products corresponding to V2 receptor mRNA were visualized in hyperplastic tissues from the three patients.

Immunohistochemical localization of V1a and V2 receptors in AIMAH tissues

Presence of V1a and V2 receptors in normal adrenal gland and hyperplasia tissues was examined by immunohistochemistry. Incubation of normal adrenal tissue slices with anti-V1a receptor antibodies produced intense labeling of the zona fasciculata (Fig. 2A and B). V1a receptor-immunoreactive cells were also observed in hyperplasia H1 and H3 (Fig. 2C and G).

Pharmacological properties of AVP receptors in AIMAH tissues

AVP receptors expressed in AIMAH tissues and normal adrenal glands were characterized by a pharmacological approach. As previously reported (Perraudin et al. 1993), AVP stimulated cortisol production in cultured cells derived from normal adrenal gland (Fig. 4A). The stimulatory effect of AVP was blocked by the V1a receptor antagonist SR49059 (10−6 M). Application of graded concentrations of AVP (10−12–10−7 M) to cultured H1 cells induced a dose-related increase in cortisol secretion with high potency (mean pEC50 = 10.1 ± 0.1; n = 4) and efficacy (Emax = 37.3 ± 17.8%; n = 4; Fig. 4B). SR49059 (10−7 M) shifted the AVP response curve to the right yielding a mean pEC50 of 8.9 ± 0.3 (n = 4; P < 0.001; Fig. 4B). AVP (10−12–10−7 M) also stimulated cortisol production in a dose-dependent manner (pEC50 = 10.2 ± 0.4; Emax = 126.8 ± 16.2%; n = 4) in cultured H2 cells (Fig. 4C). However, SR49059 (10−7 M) did not significantly modify the stimulatory effect of AVP in H2 cells (pEC50 = 9.9 ± 0.4; n = 4; P > 0.05; Fig. 4C). In cultured H3 cells, AVP (10−12–10−7 M) provoked an increase in cortisol release (pEC50 = 10.9 ± 0.2; Emax = 47.9 ± 2.7%; n = 4; Fig. 4D). The V2 receptor agonist dDAVP (10−12–10−7 M) had no effect on cortisol production (Fig. 4D). The stimulatory effect of AVP on H3 cells was inhibited by SR49059 (pEC50 = 7.5 ± 0.3; n = 4; P < 0.0001; Fig. 4D).

Discussion

Both clinical and molecular studies have demonstrated the presence of multiple illegitimate receptors including AVP receptors in AIMAHs causing subclinical and/or overt Cushing’s syndrome (Lacroix et al. 2004). In the present study, we have investigated the expression of AVP receptor subtypes in normal adrenal gland and three hyperplasia tissues. We have also examined the pharmacological profile of AVP receptors in cultured cells derived from the three AIMAHs.
Figure 2  Immunohistochemical localization of V1a receptor in AIMAH causing Cushing's syndrome. V1a receptor-immunoreactive cells in the zona fasciculata of two normal adrenal glands (NA1, A; ×260 and NA2, B; ×280). V1a receptor-immunopositive cells in H1 and H3 tissues (H1, C; ×220 and H3, G; ×220). Higher magnification view of hyperplasia sections showing the presence of V1a receptor immunoreactivity in the cytoplasm and at the periphery of spongiocytic cells (H1, D; ×440 and H3, H; ×360). Absence of V1a receptor immunoreactivity in H2 tissue (E; ×220) and (F; ×220). Scale bars: 50 μm.
Figure 3  Immunohistochemical localization of V₂ receptor in AIMAH causing Cushing’s syndrome. Absence of V₂ receptor-immunoreactive cell in zona fasciculata of two normal adrenal glands (NA1, A; ×220 and NA2, B; ×220). Clusters of V₂ receptor-immunopositive cells in H1 and H2 tissues (H1, C; ×320 and H2, E; ×220). Higher magnification view of hyperplasia sections showing the presence of V₂ receptor immunoreactivity in the cytoplasm and at the periphery of spongiocytic cells (H1, D; ×380 and H2, F; ×440). Absence of V₂ receptor immunoreactivity in H3 tissue at low (G; ×100) and high (H; ×340) magnifications. Scale bars: 50 μm.
It is well documented that AVP stimulates cortisol secretion, through activation of V1a receptors, in normal adrenal gland (Perraudin et al. 1993, Gallo-Payet & Guillon 1998) and some AIMAH tissues (Lacroix et al. 1997, Daidoh et al. 1998). In agreement with these data, our RT-PCR experiments revealed the presence of V1a receptor mRNA in both normal tissue and hyperplasias H1 and H3. In addition, V1a receptor-like immunoreactivity was visualized in the normal cortex and a subpopulation of steroidogenic cells in H1 and H3 tissues. The stimulatory effect of AVP on cortisol production from cultured cells, derived from either normal adrenal gland or hyperplasia H3, was blocked by the V1 receptor antagonist SR49059, indicating that the corticotropic action of the nonapeptide was mediated by eutopic V1a receptors. SR49059 exerted a weaker inhibitory effect on H1 cells, suggesting that eutopic V1a receptors partially relayed the AVP response in this lesion. By contrast, the presence of V1a receptor mRNA or protein was not detected in the H2 tissue. Consistent with the lack of V1a receptor, our in vitro experiments revealed that SR49059 failed to inhibit AVP-induced cortisol secretion in H2 cultured cells. These data demonstrate the absence of eutopic V1a receptor in an adrenal hyperplastic lesion and indicate that the stimulatory effect of AVP is actually mediated by ectopic receptors in H2 tissue. Such loss of eutopic receptor and gain of function due to abnormal expression of another receptor subtype in AIMAH have already been reported for 5-HT receptors but have never been described for AVP receptors (Louïset et al. 2006). This observation is also consistent with the previously shown underexpression of the melanocortin type 2 receptor in AIMAH tissues overexpressing the GIP receptor (Lampron et al. 2006). It has been proposed that these combined molecular defects may be related to abnormal activity or mutations of transcription factors involved in the regulation of the expression of multiple membrane receptors (Lacroix et al. 2001).

Expression of the V1b receptor gene has been shown in some AIMAH tissues (Mune et al. 2002, Miyamura et al. 2003, Lee et al. 2005). The fact that V1b receptor mRNA cannot be detected in present RT-PCR experiments excludes the involvement of ectopic V1b receptors in AVP-induced cortisol production in the three AIMAH tissues.

As previously reported in some cases of AVP-sensitive AIMAH (Mune et al. 2002, Miyamura et al. 2003, Lee et al. 2005, Vezzosi et al. 2007), we found that V2 receptor mRNA was produced in the three hyperplastic tissues. Immunohistochemical experiments revealed the presence of V2 receptor protein in steroidogenic cells.

Figure 4 Effect of AVP and vasopressinergic ligands on cortisol secretion from cultured AIMAH cells. Effect of the V1a receptor antagonist SR49059 (10\(^{-7}\) M) on cortisol secretion induced by 10\(^{-8}\) M AVP from cultured normal adrenocortical cells (NA, A). Effect of graded concentrations of AVP (from 10\(^{-12}\) to 10\(^{-7}\) M) on cortisol secretion from H1 (B) and H2 (C) cells in the absence (●) or the presence of SR49059 (10\(^{-6}\) M) (○). Effect of graded concentrations (from 10\(^{-12}\) to 10\(^{-7}\) M) of AVP and the V2 receptor agonist dDAVP (△) on cortisol production from H3 cells (D). AVP was administered alone (●) or in combination with SR49059 (10\(^{-6}\) M) (○). Data values represent mean ± S.E.M. from four experiments.

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of H1 and H2 tissues. The AVP-induced cortisol secretion observed in cultured H2 cells, which did not possess V1a, and V1b receptors, provides the first evidence for the occurrence of functional illegitimate V2 receptors in an adrenocortical hyperplasia causing Cushing’s syndrome. The V2 receptor gene is also expressed in hyperplasia H3. However, several data indicate that V2 receptors are not involved in the vasopressinergic control of steroidogenesis in this tissue: i) immunohistochemical studies failed to detect any V2 receptor-like immunoreactivity in steroidogenic cells; ii) the stimulatory effect of AVP on corticosteroidogenesis was suppressed by SR-49059; and iii) the V2 receptor agonist dDAVP had no influence on cortisol secretion. These results suggest that V2 receptor mRNA present may not be translated into functional proteins in AIMAH, as previously observed for the 5-HT4 receptor (Louiset et al. 2006). The lack of V2 receptor protein in H3 tissue may result from decreased mRNA stability. Various molecular partners, such as RNA-stabilizing proteins and small RNAs, may potentially be involved in this process (Chu & Rana 2007, Eberhardt et al. 2007).

We have previously demonstrated the presence of numerous cells producing AVP in the hyperplastic cortex of patients with AIMAH (Bertherat et al. 2005). Thus, it can be speculated that eutopic V1a and ectopic V2 receptors mediate a direct intra-adrenal vasopressinergic tone involving paracrine and autocrine mechanisms. Our observations may be pathophysiologically relevant since it has been reported that AVP increases proliferation of different cell types, including rat adrenocortical cells, through activation of V1a receptors (Chiu et al. 2002, Lagundzija et al. 2004, Trejter et al. 2005). These data suggest that the nonapeptide, locally produced in AIMAH tissues, may stimulate proliferation of steroidogenic cells via activation of V1a receptors. Moreover, expression of ectopic V2 receptors, which are classically coupled to adenylyl cyclase (Birnbaumer 2000), indicates that V2 receptors may also have contributed to the pathophysiology of AIMAH by stimulating cell steroidogenesis and mitogenesis. In support of this hypothesis, it has been recently described that transfer of adenylyl cyclase-coupled GIP and LH receptors in bovine adrenocortical cells causes cortisol hypersecretion and adrenomatous hyperproliferation in a mouse xenotransplantation model (Mazzuco et al. 2006a,b).

In conclusion, the present study provides the first immunohistochemical characterization of AVP receptors in AIMAH tissues. Combined with molecular and pharmacological approaches, immunohistochemistry revealed that the steroidogenic response to AVP can be mediated by ectopic V2 receptor alone or in association with eutopic V1a receptor. Our data also clearly indicate that the sole RT-PCR data are not sufficient for the determination of the receptor subtype(s) actually involved in the steroidogenic effect of a regulatory factor in hyperplastic adrenocortical tissues.

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