Multiple aberrant hormone receptors in Cushing's syndrome

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Abstract

The mechanisms regulating cortisol production when ACTH of pituitary origin is suppressed in primary adrenal causes of Cushing's syndrome (CS) include diverse genetic and molecular mechanisms. These can lead either to constitutive activation of the cAMP system and steroidogenesis or to its regulation exerted by the aberrant adrenal expression of several hormone receptors, particularly G-protein coupled hormone receptors (GPCR) and their ligands. Screening for aberrant expression of GPCR in bilateral macronodular adrenal hyperplasia (BMAH) and unilateral adrenal tumors of patients with overt or subclinical CS demonstrates the frequent co-expression of several receptors. Aberrant hormone receptors can also exert their activity by regulating the paracrine secretion of ACTH or other ligands for those receptors in BMAH or unilateral tumors. The aberrant expression of hormone receptors is not limited to adrenal CS but can be implicated in other endocrine tumors including primary aldosteronism and Cushing's disease. Targeted therapies to block the aberrant receptors or their ligands could become useful in the future.

Introduction

Cushing's syndrome (CS) results from chronic exposure to increased concentrations of circulating free cortisol activating glucocorticoid and mineralocorticoid receptors expressed in most tissues (1, 2). Corticotropin-independent causes account for ∼15–25% of cases of endogenous overt hypercortisolism, which are mainly due to adrenal adenomas and carcinomas (1, 2). Rare causes of CS (<2%) include primary bilateral macronodular adrenal hyperplasia (BMAH) and primary pigmented nodular adrenal disease (PPNAD) either as isolated disease or as part of Carney complex (CNC) (1, 2). Unilateral or bilateral adrenal incidentalomas found in ∼4% of adults frequently secrete dysregulated amounts of cortisol producing sub-clinical CS (3). Regulation of cortisol secretion is

exerted mainly by the binding of adrenocorticotropin (ACTH) to its specific melanocortin type 2 receptor (MC2R), a G-protein coupled hormone receptor (GPCR) expressed on zona fasciculata cells that interacts with MC2R-associated proteins (4); the ensuing dissociation of Gs-α subunit and activation of adenylate cyclase (AC) generates cAMP from ATP (5). The binding of cAMP to specific domains of the regulatory subunits of PKA dissociates the tetramer, releases the catalytic subunit (PRKACA) that phosphorylates different intracellular targets, including the transcription factor CREB; the latter activates the transcription of cAMP-responsive element-containing genes in the nucleus including cholesterol transporters and steroidogenic enzymes (6) (Fig. 1). In primary adrenal causes of CS, the excess secretion of cortisol suppresses the production of corticotropin-releasing hormone (CRH) in hypothalamus and of ACTH by the corticotroph cells (2) (Fig. 1). The mechanisms regulating cortisol production, despite suppression of ACTH, were partially identified two decades ago when it was demonstrated that cortisol could be regulated by the aberrant expression and function of diverse GPCR, particularly in BMAH, or in some unilateral cortisol-secreting adenomas (7). More recently, diverse genetic and molecular mechanisms that lead to increased production of cAMP and activation of steroidogenesis were elucidated (2). In 35–65% of unilateral adrenal adenomas with overt CS, constitutive secretion results from mutations in the catalytic subunit of protein kinase A (PRKACA). Those mutations are rare in adenomas secreting less cortisol (8, 9, 10, 11, 12). Somatic mutations of GNAS (encoding Gαs) and β-catenin (CTNNB1) were found in 5–17 and 16% respectively of cortisol secreting adenomas (10, 11, 12). The mechanisms regulating steroidogenesis in adrenocortical carcinoma remain largely unknown. Isolated or familial PPNAD and CNC result most frequently from inactivating mutations of the gene coding for protein kinase A regulatory-subunit typeI-A (PRKAR1A) leading to increased PKA signalling (13, 14, 15, 16). Inactivating mutations in phosphodiesterase 11A isoform 4 gene (PDE11A) or 8B (PDE8B) have been identified in rare kindreds with non-pigmented micronodular hyperplasia without PRKAR1A mutations (17, 18). In McCune-Albright syndrome, or in rare cases of BMAH, activating mutations of the Gsα subunit of heterotrimeric G protein also termed *gsp* mutations (GNAS) occur in a mosaic pattern in the adrenal during early embryogenesis resulting in constitutive activation of the cAMP pathway (19, 20, 21, 22). In BMAH, activating mutations in MC2R gene have been described in only two cases (23, 24, 25, 26).

The most important recent finding in this field is the identification of germline mutations of Armadillo Repeat-Containing Protein 5 (ARMC5) in up to 55% of cases (25% in larger recent series) of apparently sporadic and familial BMAH; additional biallelic ARMC5 somatic mutations are found in the adrenal macronodules (27, 28, 29, 30, 31). ARMC5 inactivation decreases the expression of both MC2R and various steroidogenic enzymes although it has no apparent link to the cAMP pathway (30). The possible association between meningioma and BMAH was confirmed by the finding of a somatic frameshift mutation in ARMC5 in the intracranial meningothelial tissues of a patient with familial BMAH and germline ARMC5 mutation (27, 32, 33).
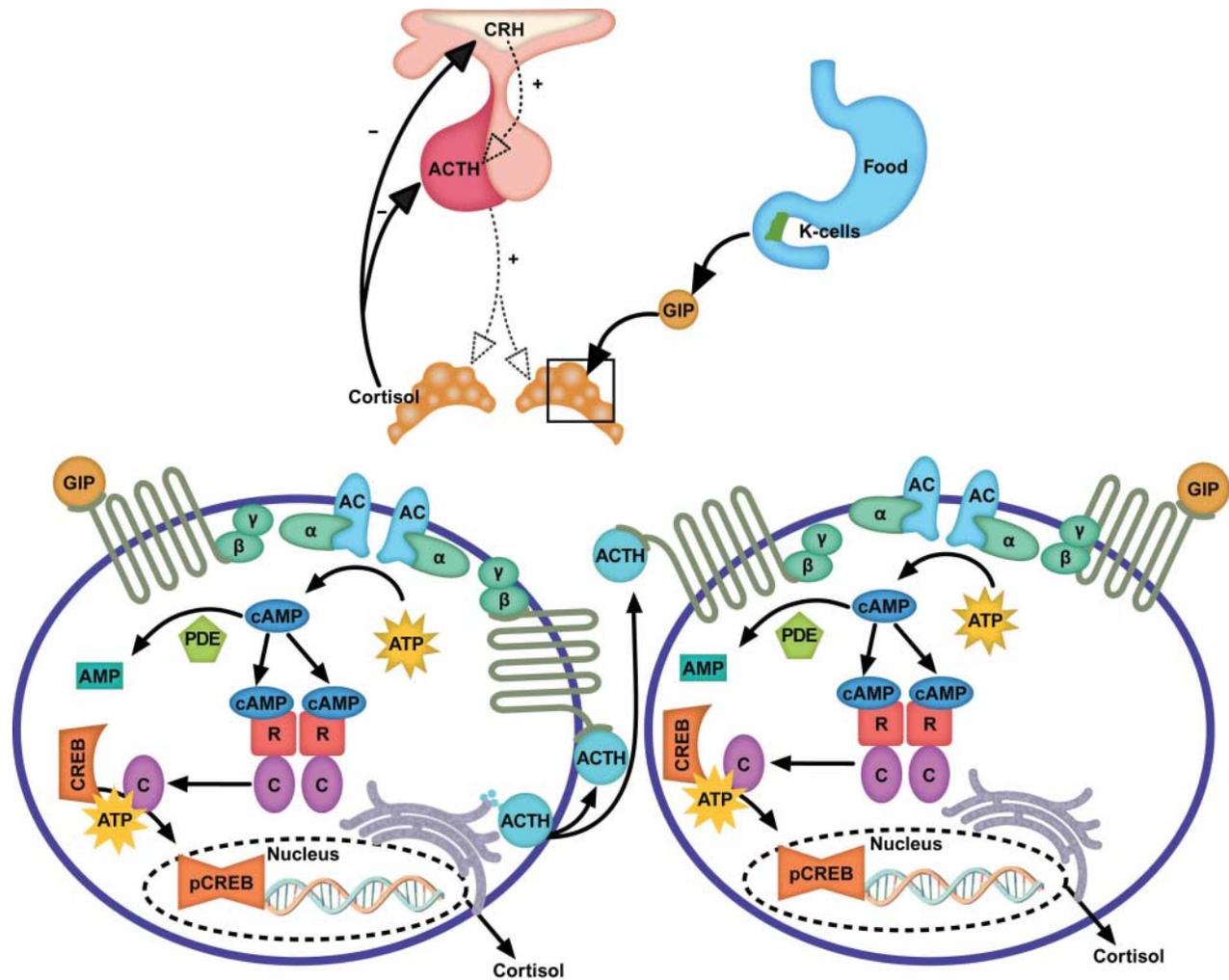
In contrast to mechanisms that would mainly lead to constitutive activation of the cAMP system, abnormal regulation of steroidogenesis can result from the aberrant adrenal expression of several hormone receptors, particularly GPCR and their ligands, which will be reviewed here. Aberrant hormone receptors can also exert their activity by regulating the paracrine secretion of ACTH or other ligands for those receptors in BMAH or unilateral tumors (34, 35).

Methods

For this review, we performed a search in the PubMed database since 1970 using several terms including adrenal gland, CS, AIMAH, BMAH, PMAH, hypercortisolism, Cushing's disease (CD), aberrant expression of GPCR, primary aldosteronism (PA), paracrine, autocrine and the usual denominations of the various regulatory factors identified in the adrenal tissue, such as ACTH, glucose-dependent insulinotropic peptide (GIPR), vasopressin, serotonin or 5-hydroxytryptamine, catecholamine, angiotensin II, luteinizing hormone (LH) and glucagon. We will focus on the studies that examined the diversity, prevalence, potential molecular mechanisms and new pharmacotherapy of aberrant GPCR and their ligands in adrenocortical hyperfunction. Finally, we will briefly discuss the role of aberrant GPCR in other endocrine tumors including in PA and CD.

Historical background and evolution of aberrant expression of GPCR in CS

The concept of aberrant expression of hormone receptors in adrenal tumors was proposed initially by Schorr & Ney in 1971 (36); Hamet *et al.* (37) described the first case of food-dependent CS in 1987 in a patient with unilateral

Figure 1

In primary adrenal causes of CS, the abnormal regulation of steroidogenesis can result from the aberrant adrenal expression of several hormone receptors, particularly GPCR and their ligands illustrated here by the ectopic expression of GIP receptor. GIP levels increase after meals. GIP binds to its receptor expressed on zona fasciculata cells and induces the activation of intracellular cascade similar to the one activated normally by the binding of ACTH to MC2R. The ensuing dissociation of Gs-α subunit and activation of adenylate cyclase (AC) generates camp from ATP. The binding of cAMP to specific domains of the regulatory subunits of PKA, dissociates the tetramer, releases the catalytic subunit (PRKACA) which phosphorylates different intracellular targets, including the transcription factor CREB; the latter activates the transcription of cAMP-responsive element-containing genes in the nucleus including cholesterol transporters and steroidogenic enzymes. The excess secretion of cortisol suppresses the production of CRH in hypothalamus and of ACTH by the pituitary corticotroph cells. In BMAH tissues, cortisol production is controlled both by aberrant GPCR and autocrine/paracrine ACTH regulatory mechanisms. ACTH released from zona fasciculata cells binds to MC2R and amplifies the aberrant receptor response.

adenoma in whom the cortisol secretion presented an inverse rhythm with low fasting morning plasma levels that increased postprandially. In 1992 Lacroix *et al*. (38) and Reznik *et al*. (39) identified that food-dependent adrenal CS in two patients with BMAH resulted from aberrant responsiveness of adrenal cells to physiological secretion of GIP presumably resulting from expression of ectopic GIP receptors on adrenal cells. This was followed by an extensive number of publications of case reports and small series of patients during the next three decades as

summarized in previous reviews (7, 40, 41). In most patients with BMAH and a lesser proportion of those with unilateral adrenal adenomas with clinical or subclinical CS, aberrant GPCRs regulate steroidogenesis by mimicking the cellular events that are triggered normally by ACTH receptor (MC2R). Steroidogenesis can be driven by the expression of ectopic receptors that are not expressed at significant levels in normal zona fasciculata cells, such as those for GIPR, beta-adrenergic receptors (β-AR), vasopressin (V_2–V_3-vasopressin receptor), serotonin (5-HT$_7$ receptor), glucagon (GCGR) and probably angiotensin II (AT1R). It can also result from increased expression or increased coupling to steroidogenesis of eutopic receptors such as those for vasopressin (V_1-vasopressin receptor), LH/human chorionic gonadotropin (LHCGR), or serotonin (5-HT$_4$ receptor) (7, 40, 42, 43). The clinical manifestation of aberrant GPCR expression occasionally results in specific patterns of CS, for example in food-dependent CS with fasting hypocortisolism (ectopic GIPR) or pregnancy/menopause induced CS (aberrant LHCGR) (40); in most cases, the presence of aberrant receptor does not modify significantly the clinical presentation of CS. However, the identification of aberrant regulation offers the perspective of potential pharmacological therapeutic options that target GPCR or their ligands in an attempt to avoid bilateral adrenalectomy that is the current standard therapy of BMAH (43, 44).

Systematic screening for aberrant expression of GPCR in overt and subclinical CS demonstrates the frequent co-expression of several receptors

In vivo investigation protocols were designed to systematically evaluate the potential presence of aberrant receptors. An ACTH-independent change of cortisol or other steroids ($>50\%$) in response to physiological (upright posture, mixed meals) and pharmacologic (gonadotropin-releasing hormone (GnRH), thyrotropin-releasing hormone (TRH), vasopressin, glucagon, metoclopramide) tests that modulate the levels of ligands for the potential aberrant receptors was examined (41, 45, 46, 47, 48). Following the initial finding of a positive response, further stimulatory tests can be conducted to precisely define the hormone and the specific receptor type implicated (41); for instance, if a positive response was detected following a mixed meal, the following tests would be conducted to document the presence of a GIP receptor: stimulation by oral glucose, lipids and proteins,

non-stimulation by IV glucose, inhibition by octreotide and finally stimulation with GIP perfusion (38).

Abnormal expression of more than one type of GPCR was demonstrated in many systematic studies of patients with subclinical or overt CS (41, 45, 46, 47, 48, 49, 50, 51, 52). Table 1 summarizes the results of five systematic studies that have screened for aberrant expression of GPCR in overt and subclinical CS. Among patients with BMAH, including 52 with CS and 35 with subclinical Cushing's syndrome (SCS), 80% showed aberrant cortisol responses to at least one stimulus with multiple responses within individual patients occurring for up to four stimuli in 50% of the patients (45, 46, 47, 48, 49). The percentage of patients with aberrant responses was less frequent in patients with unilateral adenomas and overt CS (45), possibly reflecting the higher prevalence of *PRKACA* mutations in this category of patients (8); however in patients with unilateral adenoma and mild CS or SCS the percentage of patients with aberrant responses were similar to those in BMAH patients (46). Vasopressin (AVP) and serotonin HTR4 agonists were the most prevalent hormonal stimulus triggering aberrant responses *in vivo* (45, 46, 47, 48, 49). *In vitro* studies did not always correlate with *in vivo* responses; hyper-responsiveness to AVP was not consistently related to vasopressin receptor overexpression, but may be explained by more efficient coupling pathways or by the indirect action of AVP through an autocrine/paracrine mechanism (53). Hofland *et al*. (48) compared *in-vitro* data to *in-vivo* testing and found some discrepancies, suggesting that the criterion of 25–50% serum cortisol elevation to establish the partial/complete responsiveness should be re-evaluated, at least when AVPRIA and 5-HT$_4$ receptors are tested . Moreover, the pathogenesis of AVP-mediated cortisol overproduction was linked to an aberrant coupling of normal levels of AVPR1A to the induction of CYP11B1 (48).

Table 2 presents the characteristics of the different aberrant GPCR; ectopic GIP receptor expression was the most studied GPCR in BMAH and unilateral adenomas, reported in more than 30 cases (41). Food ingestion and particularly its lipid and glucose content stimulate the release of GIP, a 42 amino acid peptide hormone from intestinal K cell (Fig. 1). Normal individuals often present a modest elevation in cortisol after mid-day meal following ACTH stimulation by the protein content of the diet (7). In patients with GIP-dependent CS, as their pituitary ACTH levels become suppressed, they can present low fasting plasma cortisol levels in morning, which increase following physiological elevation of GIP after meals. Fasting cortisol levels may not always be suppressed

Table 1 Systematic studies for aberrant expression of GPCR in patients with primary adrenal lesions with overt or subclinical Cushing's syndrome.

References	BMAH				Unilateral adrenocortical tumors			
	n	Aberrant responses (%)	Several aberrant responses (%)	Stimuli (%)	n	Aberrant responses (%)	Several aberrant responses (%)	Stimuli (%)
(45)	6 CS	6/6 (100%)	2/6 (33%)	Posture (33%) AVP 33% Mixed meal (GIP) 33%	14 CS	3/14 (21%)	0/14 (0%)	Posture 7% AVP (7%) Mixed meal (GIP) 7%
(46)	–	–	–	–	21 SCS	21/21 (100%)	18/21 (86%)	Terlipressin 90% cisapride 81%
(49)	14 CS	9/14 (64%)	NA[a]	AVP 45.5% Posture 36% TRH 33%[b]	12 CS	NA	NA	AVP 40% Posture 25% Glucagon 18%
(47)	10 CS 22 SCS	28/32 (87%)	21/32 (66%)	Posture 67% Metoclopramide 56% Glucagon in SCS 58%[c]	–	–	–	–
(48)	22 CS 13 SCS	27/35 (77%)	17/35 (49%)	Posture 50% AVP 52%	–	–	–	–
Total	52 CS 35 SCS	70/87 (80%)	40/73 (55%)		26 CS 21 SCS	17/35 (49%)	18/35 (51%)	

n, number of patients; aberrant responses, frequency of patients who responded to at least one stimulus; several aberrant responses, frequency of patients who responded to several stimuli; stimuli, type of stimuli inducing the highest percentage of aberrant responses; SCS, subclinical Cushing's syndrome; CS, clinical Cushing's syndrome; NA: data not available.

[a]Details on the exact number of patients who responded were unavailable.
[b]5 HT$_4$ agonists stimulatory test was not conducted in this study.
[c]Cortisol inhibition by octreotide in three CS patients who responded to mixed meal, and in 12 of 13 (92%) patients with SCS.

because other aberrant GPCR can be expressed with GIPR in the same tissue such as LHCGR (52, 54, 55) and HTR4 (52). The expression of the GIP receptor can be detected in the early phases of adrenal hyperplasia (56, 57). Rarely, the adrenal nodules in patients with GIP-dependent BMAH can progress asynchronously, first in one adrenal, later in the other (57); the larger nodules were the result of secondary clonal proliferation events in addition to GIPR overexpression (56). GIP increases cAMP production and DNA synthesis in GIP-dependent cortisol-secreting tissue (Fig. 1) suggesting that GIP can be implicated both in steroidogenesis and cellular proliferation (58). It was suggested that chronic stimulation by ACTH could result in increased GIPR expression (59), but other studies did not confirm GIPR overexpression in the adrenal glands of patients with CD or ectopic ACTH syndrome (58, 60, 61, 62). The demonstration that bovine adrenal cells transfected with the GIP receptor and injected under the renal capsule in mice leads to the development of hyperplastic adrenals and hypercortisolism further supported a role of the ectopic receptor in steroidogenesis and cell proliferation (63).

Transient CS appearing during sequential pregnancies with spontaneous resolution after delivery led to the identification of aberrant LH/human chorionic gonadotrophin (hCG) receptors; sustained increase of LH secretion that is the hallmark of the postmenopausal period resulted in persistent CS (64, 65). Other cases of CS from BMAH or adrenal adenoma outside of pregnancy were also found with aberrant cortisol response to injection of GnRH and hCG (55, 66). In one adrenal adenoma, a heterozygous mutation of Gsa at codon 201 was found in addition to the aberrant LH/CG receptors (67). Kero *et al.* (68) used transgenic female mice as a model to demonstrate that chronically elevated serum LH following gonadectomy induced functional LH receptor expression in mouse adrenal cortex, leading to adrenal hyperplasia and LH-dependent hypercortisolism. Other GPCR overexpressed in BMAH tissues were identified by using transcriptome approach, including those for motilin (MLNR), gamma-aminobutyric acid (GABBR1) and a2 adrenergic receptor (ADRA2A) (69). ADRA2A receptors previously found in adrenocortical carcinoma cells of rats (70) were overexpressed in the human NCI H295R adrenocortical carcinoma cells as well as in 13 of 18 patients with BMAH. Furthermore, clonidine led to an increase in basal cortisol levels, which was counteracted by the ADRA2A antagonist yohimbine (69).

Table 2 Types of GPCR in BMAH or unilateral adrenocortical tumors of patients with overt or subclinical Cushing's syndrome. Some pertinent references for each receptor are cited in order to respect maximal number of references allocated.

Aberrant receptor	Phenotype	*In vivo* screening protocol	Co-expression	Targeted medical therapy
GIP receptor (ectopic) (37, 38, 39, 45, 52, 54, 55, 57)	Food-induced hypercortisolism	Mixed meal Oral glucose	LH/hCG R 5-HT$_4$ R	Octreotide, pasireotide GIPR antagonist
Vasopressin receptor V$_1$ (eutopic) (32, 45, 49, 50, 53, 78, 79, 84, 105, 144) V$_2$, V$_3$ (ectopic) (32, 79)	Upright posture-related hypercortisolism	Upright posture AVP/desmopressin	β-AR 5-HT$_4$ R	Specific AVP receptors antagonist
β-adrenergic receptor (ectopic) (45, 78, 117)	Upright posture Insulin-induced hypo-glycemia exercise/ stress test hypercortisolism	Upright posture Isoproterenol (β1-agonist) Salbutamol (β2-agonist)	V$_1$ 5-HT$_4$ R	β-blockers
AT-1 receptor (ectopic) (55, 145)	Posture-dependent hypercortisolism	Upright posture Angiotensin II		AT-1 receptor antagonist
LH/hCG receptor (eutopic) (50, 52, 54, 55, 64, 121)	Pregnancy (transient) Postmenopausal (persistent)-depen-dent hypercortisolism	GnRH hCG, Recombinant LH	5-HT$_4$ R GIPR	Long-acting GnRH agonist (leuprolide acetate)
5-HT$_4$ receptor (eutopic) (64, 79, 85) 5-HT$_7$ receptor (ectopic) (71, 87)	Serotonin-dependent hypercortisolism	5-HT4 receptor agonists (metoclopramide, cisapride)	LH/hCG R GIPR β-AR	5-HT$_4$ receptor anta-gonist (GR113808) 5-HT$_7$ receptor anta-gonist
Glucagon receptor (ectopic) (47, 72, 73, 74, 75)	Hypoglycemia ?	Intravenous glucagon	5-HT$_4$ R LH/hCG R	Octreotide

Several *in vitro* studies have further supported the expression of GPCRs in human adrenocortical benign and malignant tumors; these include thyrotropin, follicle stimulating hormone and interleukin 1, in addition to those clearly confirmed *in vivo* (7, 40). Furthermore, 5-HT$_7$ receptor agonists were also found to stimulate cortisol production from an adrenal carcinoma that also produced rennin (71). The presence of ectopic glucagon receptors was demonstrated in patients with subclinical or overt CS (47, 72, 73, 74, 75). Injection of glucagon increased cortisol in two out of 13 patients with BMAH or unilateral adenoma. *In vitro* studies revealed the presence of glucagon receptor-like immunoreactivity in clusters of spongiocytic cells in adrenal tissues from patients who were sensitive *in vivo* to glucagons (75). Expression of somatostatin receptors SSTRs (particularly of SSTR1-3) was increased in PPNAD tissues carrying a PRKAR1A mutation compared to normal adrenal and to tissues from other adrenal diseases. *In vivo* cortisol reduction seen in some patients following octreotide administration (although not significant) indicates that somatostatin analogues may be considered as treatment for PPNAD (76). Despite the initial report of ectopic response of adrenal tumors *in vitro* to thyrotropin (36), no further reports of such a response were identified in the following systematic studies, which

therefore do not appear to justify systematic testing with TRH (45, 46, 47, 48, 49).

Aberrant GPCR in familial cases of BMAH

BMAH was initially believed to be a sporadic disease, suggesting somatic mutations occurring in adrenal pro-genitor cells during embryogenesis. However, several reports of familial cases with autosomal dominant transmission patterns have been published suggesting germline genetic etiology (32, 77, 78, 79, 80, 81, 82, 83). In familial BMAH without other genetic syndrome (such as MEN-1, polyposis coli), specific aberrant GPCR including vasopressin, β-adrenergic and HT4 receptors were found in all members from the same family; in contrast, in one large Brazilian family, the pattern of aberrant receptors varied between the different affected members (27, 32, 77, 78, 79, 80, 81, 82, 83). The relation between *ARMC5* mutation and aberrant GPCR has not been examined in detail yet. Preliminary studies have found that *ARMC5* mutation carriers can present aberrant response to upright posture, vasopressin and metoclopramide tests (30, 31, 32, 36). In contrast, none of the six patients with food-dependent CS carried *ARMC5* mutations (30, 31). Further studies will be necessary to clarify the relation between *ARMC5*

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mutations and aberrant GPCR or autocrine production of ACTH in BMAH tissues.

Cortisol is also regulated by the paracrine/autocrine production of ligands for the aberrant receptors in adrenocortical tumors and hyperplasias

Paracrine regulatory mechanisms of cortisol secretion were proposed following the demonstration of increased adrenocortical expression of pro-opiomelanocortin (POMC)/ACTH, serotonin, vasopressin or glucagon in some affected adrenal tissues (53, 72, 84, 85, 86, 87). A complex paracrine regulation was confirmed by the recent demonstration of expression of POMC in clusters of steroidogenic cells in the majority of 30 tissues studied from patients with BMAH and CS (34). Prohormone convertase 1 was co-expressed in POMC-producing cells allowing maturation to active corticotropin. BMAH tissues released ACTH and cortisol episodically during perifusion studies, but this secretion was not regulated by CRH, dexamethasone or the GR antagonist mifepristone. In contrast, in BMAH tissues expressing aberrant GPCR, GIP, serotonin or hCG led to release of ACTH, and cortisol increase was inhibited 40% by the MC2R antagonist ACTH (7–38) in these tissues (34). Cortisol production in BMAH is thus controlled both by aberrant GPCR and autocrine ACTH, which amplifies the aberrant receptor effects. GIP also promoted the production of ACTH in human adrenocortical carcinoma H295R cells transfected with GIPR (88). BMAH tissues may also produce serotonin, vasopressin, glucagon and other factors that suggest further paracrine regulatory loops of cortisol secretion and cell proliferation. A subpopulation of adrenocortical cells of BMAH tissues was found to produce serotonin; cortisol secretion appeared to be stimulated by two mechanisms including a direct action on steroidogenic cells involving over-expressed 5-HT$_4$ and/or 5-HT$_7$ receptors and an indirect effect through activation of ACTH release by adrenocortical gonadal-like cells (35). The confirmation of a role of autocrine ACTH will await demonstration that blockade of adrenal ACTH receptors *in vivo* will reverse hypercortisolism in affected patients (89).

Aberrant regulation by nuclear hormone receptors

An *in vivo* paradoxical increase in urinary free cortisol during the 6-day dexamethasone suppression test was found in 69–75% of two small series of patients with PPNAD (90, 91). In another study, no paradoxical increase in cortisol was seen in nine patients with BMAH, but it was observed in three of 15 patients with a unilateral adenoma (90). Dexamethasone also increased cortisol production in the adrenal tissue dispersed from the PPNAD patients; this response was correlated with a striking glucocorticoid receptor over-expression in the nodules (92). The paradoxical stimulation of cortisol production by dexamethasone was mediated by the glucocorticoid receptor and inhibited by specific inhibitors of PKA (91). In a patient with PPNAD, who increased cortisol secretion during pregnancy and oral contraceptive use (and dexamethasone), β-estradiol (E$_2$) stimulated cortisol secretion in a dose-response manner in the absence of ACTH (93). In PPNAD tissues associated with CS, E$_2$ abnormally stimulated cortisol secretion through activation of overexpressed estrogen receptors ERα and G protein-coupled receptor 30 (GRP30) (94). This finding may explain why the CS of PPNAD is more frequent after puberty in female patients with CNC (94).

Aberrant GPCR is not a unique feature of zona fasciculata cells

The two major causes of PA are aldosterone-producing adenomas (APA) observed in 30–40% of patients and bilateral idiopathic adrenal hyperplasia detected in 60–70% of cases. The mechanisms regulating aldosterone production, while renin and angiotensin II are suppressed, are not fully elucidated. Somatic mutations of genes encoding the potassium channel KCNJ5 (95), L-type calcium channel (96) and sodium/potassium and calcium ATPases (97) were found to stimulate aldosterone synthase expression and aldosterone hypersecretion through calcium influx in ~50% of aldosteronomas; their role in cell proliferation are less well defined, but these mutations may also play a role in the clonal expansion of the larger aldosterone secreting adrenal nodules. Such mutations are not found in PA secondary to idiopathic bilateral hyperplasia except in rare kindreds with familial hyperaldosteronism type III (98). *In vitro* studies have suggested that some GPCRs are abnormally expressed in APA, including ectopic expression of GnRH receptor and TRH receptor (99, 100) and eutopic overexpression of MC2R (100, 101, 102), 5-HT$_4$R (103), AT-1R (100), LH/hCG receptor (104), V$_1$-vasopressin receptor (105, 106), endothelin-1 ET$_A$ and ET$_B$ receptors (107). Administration of 5-HT$_4$ agonists such as metoclopramide, cisapride and tegaserod resulted in higher stimulation of aldosterone (99, 103, 108). Nonspecific inhibitors of 5-HT such as

cyproheptadine and ketanserin produced only minor and transient inhibition of aldosterone secretion in aldosteronomas (109, 110). Specific 5-HT₄R antagonists such as GR 113808 are potent inhibitors of basal and cisapride-induced aldosterone secretion (103). Chromaffin cells, endothelial cells, nerve terminals and cells of the immune system are localized in the immediate vicinity of zona glomerulosa cells and can secrete various factors to control aldosterone secretion (111). Local release of 5-HT by perivascular mast cells (MC) can activate 5-HT₄R expressed in zona glomerulosa cells and consequently stimulate aldosterone production (112). A role of MC in tumorigenesis was proposed (113, 114). The density of MC was found to be increased in APA tissues compared with normal adrenals. Immunohistochemistry showed that adrenal MC were tryptase positive and chymase negative. They were primarily observed in adrenal cortex adjacent to adenomas or in the adenomas themselves (115). Using a microarray approach in ten aldosteronomas compared with five normal adrenals and 13 cortisol-secreting adenomas, the six GCPRs with the highest increase in expression included LHCGR, 5-HT₄R, GnRHR, glutamate receptor metabotropic three, endothelin receptor ET$_B$ receptors and MC2R (101).

Renin-independent stimulation of aldosterone secretion was observed *in vivo* following mixed meal, oral glucose, or administration of GIP, vasopressin and tegaserod in a patient with unilateral source of PA (116). Aldosterone secretion was stimulated by GIP in primary cultures of this patient's aldosteronoma. Real-time RT-PCR and immunohistochemistry found increased expression of GIPR as well as 5-HT₄R and MC2R were also present (116, 117). In a patient with BMAH and β-AR-aberrant expression, isoproterenol stimulated both cortisol and aldosterone production (117). A case of PA diagnosed during pregnancy appeared to correlate with aberrant LHR and GNRHR expression (118). To date, no extensive studies compared aberrant expression of GPCR in patients with APA to those with IHA. However, in the study by Zwermann *et al*. (99), of 12 cases of PA including five aldosteronomas and seven bilateral idiopathic aldosteronism (IHA), both subtypes presented aberrant response as 11 had increased response to metoclopramide, three patients had aberrant response to GnRH, six to vasopressin and one each to TRH and glucagons. The expression levels of several aberrant receptors including AT-1R, MC2R, AVPR1, HTR4, LHCGR, GnRH and TRHR using quantitative PCR were highly variable between each resected adrenal adenoma (99). Aberrant GPCR was also expressed in androgen-producing adrenal tumors and

hyperplasias, mainly GIPR (119), LHCGR (64, 120, 121) and 5-HT₄ R (64).

Aberrant receptors in CD and other endocrine tumors

The concept of aberrant hormone receptors has become quite familiar in primary adrenal tumors, but in fact modifications of expression of several receptors and their effects on the regulation of secretion exist in many other endocrine tumors. In CD, a high proportion of corticotroph tumors express the vasopressin-3 receptor (AVPR1b) ectopically and overexpress AVPR2 and respond to desmopressin (DDAVP) *in vitro* and *in vivo* (122). In 29 patients with CD, most of the tumors exhibited abundant expression of AVPR1a, AVPR1b and CRHR, whereas the expression of AVPR2 varied greatly and was lower in macroadenomas than in microadenomas. After adjustment for tumor volume, a positive correlation was found between the percentage increment of ACTH and the degree of AVPR2 expression, but not between that of AVPR1 or AVPR1b. No relationship was observed between the level of CRHR expression in tumor tissues and the percentage increment or net AUC of ACTH to CRH administration in CD patients (123). Luque *et al*. evaluated a total of eight normal pituitaries (NPs), 23 corticotropinomas, 14 non-functioning pituitary adenomas, 17 somatotropinomas and three prolactinomas for AVPR expression; corticotropinomas variably expressed all AVPR subtypesAVPR1b being the predominant subtype followed by AVPR2 > AVPR1a. AVPR1b was significantly more expressed in corticotropinomas than in NPs. The use of an AVPR1b antagonist completely blocked DDAVP stimulatory effects. Remarkably, only AVPR1b expression was positively correlated with elevated plasma ACTH levels in corticotropinomas (124). Patients with CD respond aberrantly to ghrelin and hexarelin with increased ACTH secretion (125); the expression of ghrelin receptors, GHSR1a and GHS-R1b, in tumor tissue samples from two patients with CD were demonstrated. Ghrelin was found to be expressed in this same tissue and its ultrastructural immunolocalization within secretory granules supports the hypothesis that ghrelin can influence corticotropinoma cell function through an autocrine/paracrine mechanism (126).

Epidermal growth factor receptor (EGFR) is frequently overexpressed in CD tumors, and in animal models the EGFR tyrosine kinase inhibitor gefitinib inhibited corticotroph tumor growth and clinical or biochemical features of CS (127). Recently, the mechanism of EGFR

overexpression in CD was identified as somatic mutations in ubiquitin-specific peptidase 8 gene (USP8) were found in 35–62% of corticotroph adenomas from patients who had smaller but relatively more active tumors than those without USP8 mutations (128, 129). Inactivation of USP8 led to enhanced EGFR deubiquitination, impairing its downregulation, increasing epidermal growth factor signaling and POMC/ACTH synthesis that was suppressed by the EGFR inhibitor gefitinib (128, 129).

Other endocrine tumors harbored ectopic GPCR, including pheochromocytomas in which ectopic expression of glucagon receptor and HTR4 were demonstrated (130, 131). A female patient with pheochromocytoma experienced a life-threatening hypertension crisis after metoclopramide administration. Her tumor as well as 17 additional pheochromocytomas (nine benign and eight malignant) were studied *in vitro*. Metoclopramide and cisapride were found to activate catecholamine- and granin-derived peptide secretions by cultured tumor cells. These secretions were inhibited by the HTR4 antagonist GR 113808. HTR4 mRNAs were detected in the patient's tumor and the series of 17 additional pheochromocytomas (131). Based on a microarray analysis of phechromocytoma subtypes, both adrenal cortex and medullary tumor tissues and two adrenal tumor cell lines (PC-12 and SW-13) expressed receptors for somatostatin, GHRH, or the SV-1 splice variant, as well as for GnRH. The immunohistochemical staining of adrenal tissue showed strong staining for sst2 in normal adrenal cortex, adrenocortical adenoma and carcinoma, as well as in the SW-13 adrenocortical cell line. In adrenal medulla tumors, both benign and malignant specimens were moderately positive for sst2, as were PC-12 cells; however, no sst2 staining could be detected in the normal adrenal medulla (132). A somatostatin octapeptide RC-160 significantly reduced growth and survival of SW-13 human adrenocortical cells under proliferative culture conditions. Similar results were seen with the use of GHRH antagonist MZ-4–71 and LHRH antagonist Cetrorelix (132). In acromegaly the presence of ectopic TRH, GnRH or GIP receptors explains the frequently observed paradoxical increase in GH after administration of TRH, GnRH or oral glucose respectively (133, 134, 135, 136, 137, 138). GIPR expression was investigated in 43 somatotropinomas and 12 normal pituitary glands by quantitative RT-PCR, western blotting and immunohistochemistry. GIPR was expressed at higher levels compared to NPs in 13 GNAS1 mutation-negative somatotropinomas. GIP stimulated adenylyl cyclase and GH-promoter activity in GIPR-transfected GH3 cells, confirming a correct coupling of

GIPR to Gas (134). A 39-year-old woman with a confirmed diagnosis of acromegaly had an ACTH response to DDAVP (plasma ACTH levels increased from 13.9 to 50.4 pg/ml at 90 min). After transsphenoidal resection of the pituitary tumor, immunohistological examination confirmed a GH and PRL-producing adenoma, whereas ACTH was negative. ACTH response to DDAVP disappeared after tumor removal. To determine the cause of preoperative ACTH response to DDAVP, the expression of corticotropin-releasing factor (CRF) family peptides and vasopressin V1b receptor in the pituitary adenoma was examined by immunohistochemistry. Immunohistochemistry revealed positive immunostaining for CRF, urocoritn1 (Ucn1), urocortin3 (Ucn3) and vasopressin V1b receptor in the adenoma. These observations raised the possibility that DDAVP caused an ACTH response, perhaps via the paracrine effects of tumor-derived CRF and Ucn1 (139).

Could targeted medical therapy become useful for adrenal tumors and hyperplasia?

Surgery is the current recommended therapy for BMAH: bilateral adrenalectomy for severe disease with lifelong cortisol/fludrocortisone replacement vs unilateral adrenalectomy for milder disease (urinary free cortisol less than two times the upper limit of normal) with resection of the larger gland or the functionally more active. For non-surgical candidates, inhibiting adrenal steroidogenesis by ketoconazole or metyrapone remains a valid option with a drawback of many adverse events such as liver toxicity and hypertension. Annual evaluation for subclinical disease with close monitoring of blood glucose, blood pressure control and osteoporosis is indicated (44). To date, no large prospective randomized trials followed patients with SCS with aberrant hormone responses to evaluate the rate of progression to overt CS. Recently, Albiger *et al*. reported on the follow-up of 16 patients with BMAH and CS: one case with food-dependent CS was treated with octreotide LAR and two patients with a positive postural test were treated with propranolol. Limited clinical response was noted despite marked improvement in biochemical values in all three patients. Twelve patients underwent unilateral adrenalectomy, with long-term remission in three, recurrence in eight and persistence in one other. Four patients subsequently needed contralateral adrenalectomy for overt CS, one received ketoconazole and four other patients remain under surveillance for SCS (140).

The concept of regulation of steroidogenesis by aberrant GPCR expression and by the paracrine/autocrine

production of their ligands offered the possibility of targeted therapy using specific receptor-targeted peptide antagonists (Table 2). β-blockers and leuprolide acetate achieved long-term control of BMAH in β-AR and LH/hCGR dependent hypercortisolism respectively (41, 64, 117, 141). Octreotide or pasireotide resulted in short-term control in a BMAH patient with aberrant expression of GIPR presumably because GIP suppression escapes as downregulation of somatostatin receptors in K cells occurs during chronic administration of the long-acting agonists (41, 142, 143). Administration of antagonists of V1aR, AT1R or β-AR reduced cortisol levels in patients with aberrant response to upright posture (144, 145, 146). ACTH-receptor antagonists significantly inhibited cortisol secretion *in vitro* in perifused BMAH tissues from patients with abnormal plasma cortisol responses to GIP because GIPR activation partially stimulated cortisol secretion by stimulating ACTH secretion in a paracrine way to control cortisol synthesis (34). As CS is relatively rare, the development of specific antagonists for the more frequent aberrant GPCR such as AVPR1 or HTR4 have been lacking; considering the high prevalence of SCS cases with BMAH or unilateral incidentaloma, or of PA with their increased cardiovascular morbidity, it is hoped that the development of antagonists for the aberrant receptors could become more appealing to the pharmaceutical industry.

Conclusions and Perspectives

In recent years, growing evidence has shown that in a significant proportion of adrenal tumors or hyperplasia steroidogenesis is not constitutive but is rather regulated by aberrant GPCR and their ligands. Whether the aberrant GPCR expression is the initiating event or the consequence of a proliferative event leading to adrenal hyperplasia or tumorigenesis is still debatable. It was shown that a single genetic event, inappropriate expression of a nonmutated GPCR gene, is sufficient to initiate the complete phenotypic alterations that ultimately lead to the formation of a benign adrenocortical tumors in animal models (63). The recent demonstration of *ARMC5* mutations in BMAH rather supports the notion that aberrant expression of one or several GPCR and of their ligands in adrenal tissues is a secondary rather than a primary event (27, 30, 147). Similarly, in a subset of CD patients, USP8 mutations are responsible for the aberrant overexpression of EGFR (128). Rare intriguing cases, however, support their initiating role in tumorigenesis as illustrated by the study of a 22-year-old woman who presented with two transient pregnancy-induced severe CS and spontaneous resolution of CS and adrenal hyperplasia after preterm parturition. Stimulation with exogenous hCG induced hypercortisolism. Bilateral adrenalectomy showed normal adrenal histology. *In vitro* adrenal LHR-expression and activation was found (148).

Further studies are needed to uncover the link between aberrant receptors and germline mutations of *ARMC5* in BMAH or somatic mutations of ion channels in PA or of *PRKACA* in cortisol secreting adenomas. Future work will probably identify a larger diversity of aberrant GPCRs not only in adrenal tumors, but also in other endocrine and non-endocrine tissues. Autocrine/paracrine secretion of peptides and aberrant receptors in adrenocortical tissues/tumors/hyperplasia or other endocrine tumors offer promising specific targeted medical options as well as potential utility in imaging with specific ligands as suggested by the use of serotonin receptor-based PET scan to identify lateralization of dominant tumor in PA (149).

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