Autocrine/paracrine regulatory mechanisms in adrenocortical neoplasms responsible for primary adrenal hypercorticisim

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Abstract

A wide variety of autocrine/paracrine bioactive signals are able to modulate corticosteroid secretion in the human adrenal gland. These regulatory factors, released in the vicinity of adrenocortical cells by diverse cell types comprising chromaffin cells, nerve terminals, cells of the immune system, endothelial cells, and adipocytes, include neuropeptides, biogenic amines, and cytokines. A growing body of evidence now suggests that paracrine mechanisms may also play an important role in the physiopathology of adrenocortical hyperplasias and tumors responsible for primary adrenal steroid excess. These intra-adrenal regulatory systems, although globally involving the same actors as those observed in the normal gland, display alterations at different levels, which reinforce the capacity of paracrine factors to stimulate the activity of adrenocortical cells. The main modifications in the adrenal local control systems reported by now include hyperplasia of cells producing the paracrine factors and abnormal expression of the latter and their receptors. Because steroid-secreting adrenal neoplasms are independent of the classical endocrine regulatory factors angiotensin II and ACTH, which are respectively suppressed by hyperaldosteronism and hypercortisolism, these lesions have long been considered as autonomous tissues. However, the presence of stimulatory substances within the neoplastic tissues suggests that steroid hypersecretion is driven by autocrine/paracrine loops that should be regarded as promising targets for pharmacological treatments of primary adrenal disorders. This new potential therapeutic approach may constitute an alternative to surgical removal of the lesions that is classically recommended in order to cure steroid excess.

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Introduction

Adrenal hyperplasias and tumors responsible for hyperaldosteronism and hypercortisolism are independent of angiotensin II (ANG II) and adrenocorticotrophin (ACTH), which are suppressed by corticosteroid excess. For this reason, their secretory activity has long been considered to be autonomous. A major progress in the comprehension of the physiopathology of these corticosteroid-producing neoplasms was made in the 1990s when several studies showed that primary adrenal hypersecretion of cortisol can be driven by abnormally expressed membrane receptors for circulating hormones, such as glucose-dependent insulinotropic peptide and luteinizing hormone, which normally have no action on the adrenal cortex (1). More recently, further investigation has demonstrated that some of these ‘illicit’ or ‘illegitimate’ receptors bind intra-adrenal stimulatory factors that are also found in adrenocortical neoplasms leading to formation of paracrine stimulatory systems resembling those described in the normal adrenal gland. The aim of the present article is to update the current knowledge on the paracrine control of corticosteroid production in adrenocortical steroid-secreting hyperplasias and tumors and to examine their potential role in the physiopathology of adrenal tumorigenesis and hypersecretion. We will first summarize the main paracrine regulatory loops occurring in the normal adrenal gland. We will then review the pathological intra-adrenal regulatory processes described in hypersecreting adrenocortical neoplasms and, finally, we will discuss the physiopathology mechanisms involved in these systems as well as their potential interest for the clinical management of patients with primary adrenal excess of corticosteroids.

Methods


Autocrine/paracrine control of steroid secretion by the normal adrenal gland

The adrenal cortex is not only composed of steroidogenic cells but also contains various cell types that constitute the histological basis for paracrine regulations of steroid secretion. First, zonation of the two main histological parts of the adrenal gland is not complete and the presence of islets and rays of chromaffin cells in the cortex has been well established in various mammalian species including humans (2). In addition, the adrenocortical tissue is richly innervated by sympathetic fibers originating from extra-adrenal neurones, which reach the adrenal gland with blood vessels and form subcapsular plexus (3, 4, 5). Other nerve fibers originate from cell bodies localized in the medulla and innervate the inner zone of the cortex. Cells of the immune system, including lymphocytes, macrophages/monocytes, and mast cells, have been observed in the three zones of the cortex where they establish close contacts with steroidogenic cells (6, 7, 8, 9, 10). The adrenal cortex also contains a dense network of capillaries, allowing cell-to-cell interactions between endothelial cells and steroid-producing cells (11, 12). Finally, the adrenal gland is surrounded by fat tissue and adipocytes, sometimes arranged in small islets, and can be seen within the cortex (13). All these cell types release bioactive substances capable of influencing corticosteroidogenesis. Several review articles have been dedicated to these autocrine/paracrine processes (14, 15, 16, 17, 18, 19). The regulatory signals released within the adrenocortical tissue, as well as their action on the secretory activity of steroidogenic cells, are summarized in Table 1. Most of these signals stimulate the adrenocortical function while only a few of them, such as adrenomedullin, neuropeptide Y, and dopamine (20, 21, 22, 23), exert an inhibitory action on corticosteroid release. It should also be noticed that the action of the neurocrine/paracrine factors can either principally affect secretion of one category of steroids, such as dopamine that specifically inhibits aldosterone production, or modulate the secretory activity of the three zones of the cortex, like vasoactive intestinal peptide (VIP) that is able to stimulate DHEA, cortisol, and aldosterone secretions.

Chromaffin cells, located in the cortex or at the corticomedullary junction, are able to influence steroid production by adrenocortical cells through secretion of catecholamines and numerous neuropeptides including VIP, calcitonin-related peptide, tachykinins, neuropeptide Y, enkephalins, atrial natriuretic peptides, somatostatin, vasopressin, galanin, corticotropin-releasing hormone, and corticotropin itself (2, 14, 15). The release of these substances is regulated by splanchnic nerves and proinflammatory cytokines, suggesting that chromaffin cells may be important intermediates in the activation of the adrenal cortex during stress and inflammation. However, this complex regulatory system and the physiological relevance of the corticomedullary interaction remain unclear (19).

Intracortical nerve endings establish synapses with steroidogenic cells and, like chromaffin cells, are able to modulate basal and ACTH-induced steroid production
through the release of catecholamines and numerous neuropeptides (3, 24, 25). The sympathetic innervation of the adrenal cortex seems to play a major role in the diurnal rhythmicity of cortisol by synchronizing the central and adrenal clocks as well as in the cortisol response to stress and light through a pituitary-independent suprachiasmatic nucleus adrenal pathway (15, 18, 26, 27).

Several cell types of the immune system are present in the adrenal cortex. Lymphocytes, which establish tight junctions with adrenocortical cells, seem to exert a stimulatory tone on DHEA secretion by zona reticularis cells (28). In addition, they could be able to activate cortisol secretion by the release of pro-opiomelanocortin (POMC)-derived peptides, including ACTH itself, especially during viral infections (10, 29). Macrophages and monocytes secrete various cytokines, like interleukins 1 and 6 (IL1 and IL6), tumor necrosis factor α (TNFα), and transforming growth factor β (TGFβ), and neuropeptides such as VIP, capable of influencing the adrenocortical function (9, 10, 30, 31, 32). This cell-to-cell interaction is multifaceted as the actions of these regulatory signals can result in activation or inhibition of corticosteroidogenic cells, depending on the type of cytokine preferentially released by macrophages. It is made even more complex by the observation that macrophages, which express β-adrenoreceptors, are under the control of the sympathetic system and, in return, cytokine secretion by macrophages is inhibited by cortisol. Mast cells, which are principally located in the subcapsular region of the adrenal cortex (6), are known to release the biogenic amines, histamine, and serotonin (5-HT) and various neuropeptides and cytokines (33). It is now established that mast cells stimulate aldosterone secretion by zona glomerulosa cells through the release of 5-HT and activation of the serotonin receptor type 4 (5-HT4) (7, 34). Like macrophages, adrenal mast cells receive sympathetic innervation, suggesting that they could play the role of local intermediates and amplifiers in the nervous control of aldosterone production (35).

The adrenal cortex is a highly vascularized organ so that each steroidogenic cell is in close contact with at least one cortical sinusoid (11). This remarkable histological organization allows paracrine regulation of adrenocortical cells by endothelial cells through release of endothelins, adrenomedullin, nitric oxide, and prostacyclin. In particular, endothelial cells seem to be important regulators of aldosterone secretion and may therefore influence arterial blood pressure by this way (36, 37, 38). Endothelin release by adrenocortical sinusoids is also involved in the modulation of corticosteroid secretion by the adrenal blood flow (39).

Adipocytes have been shown to stimulate aldosterone production by releasing soluble bioactive factors that have not yet been characterized (13, 40, 41). In fact, the control of mineralocorticoid secretion by fat tissue may involve two potential mechanisms. Peri- and intra-adrenal adipocytes may be responsible for paracrine regulation of the secretory activity of zona glomerulosa (13). An alternative more complex endocrine pathway involving intra-abdominal adipocytes and oxidized derivatives of fatty acids has also been proposed (42). Reciprocally, aldosterone is able to favor adipocyte differentiation and growth leading to bidirectional cell-to-cell communication (43).

Finally, adrenocortical cells themselves can synthesize and secrete cytokines (30, 31, 32), insulin-like growth factors (IGFs) (44), and inhibit/activin (45) and express the diverse components of the renin–angiotensin system (RAS) (46) resulting in autocrine regulations of the adrenocortical function.

### Table 1 Intra-adrenal autocrine/paracrine factors involved in the control of corticosteroidogenesis in humans.

<table>
<thead>
<tr>
<th>Substance</th>
<th>Origin</th>
<th>Effects on secretion of Cortisol</th>
<th>Effects on secretion of Aldosterone</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACTH</td>
<td>Chromaffin cells</td>
<td>+</td>
<td>+</td>
<td>(104)</td>
</tr>
<tr>
<td>Acetylcholine</td>
<td>Neurons</td>
<td>+</td>
<td>-</td>
<td>(257)</td>
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<tr>
<td>Adrenomedullin</td>
<td>Chromaffin cells</td>
<td>0</td>
<td>-</td>
<td>(23)</td>
</tr>
<tr>
<td>Angiotensin II</td>
<td>Zona glomerulosa cells</td>
<td>+</td>
<td>0</td>
<td>(46)</td>
</tr>
<tr>
<td>AVP</td>
<td>Chromaffin cells</td>
<td>+</td>
<td>+</td>
<td>(113, 114, 258)</td>
</tr>
<tr>
<td>Dopamine</td>
<td>Chromaffin cells</td>
<td>0</td>
<td>-</td>
<td>(20, 21, 259)</td>
</tr>
<tr>
<td>Endothelin</td>
<td>Endothelial cells</td>
<td>0</td>
<td>+</td>
<td>(36, 174, 175, 179)</td>
</tr>
<tr>
<td>Interleukin 1</td>
<td>Immune system</td>
<td>+</td>
<td>+</td>
<td>(10, 30, 66)</td>
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<tr>
<td>Interleukin 6</td>
<td></td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>Noradrenaline</td>
<td>Chromaffin cells</td>
<td>0</td>
<td>+</td>
<td>(90, 260)</td>
</tr>
<tr>
<td>Neuropeptide Y</td>
<td>Chromaffin cells</td>
<td>-</td>
<td>0</td>
<td>(22)</td>
</tr>
<tr>
<td>Serotonin</td>
<td>Mast cells</td>
<td>+</td>
<td>+</td>
<td>(6, 7)</td>
</tr>
<tr>
<td>Vasoactive intestinal peptide</td>
<td>Chromaffin cells</td>
<td>+</td>
<td>+</td>
<td>(261, 262)</td>
</tr>
<tr>
<td></td>
<td>Neurons</td>
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Autocrine/paracrine regulatory factors in adrenocortical hyperplasias and tumors

Several paracrine regulatory loops have been shown to play a role in the physiopathology of steroid hypersecretion associated with adrenocortical hyperplasias and tumors responsible for aldosterone or cortisol excess. Globally, these processes involve the same partners as those observed in the normal adrenal gland but frequently exhibit molecular defects that tend to reinforce the potency of the intra-adrenal paracrine mechanisms to stimulate steroid production. The main abnormal findings noticed so far in the tissues include hyperplasia of cells producing the paracrine signals, pathological expression of the paracrine factors and/or their receptors, and illicit coupling of the latter.

Sources of paracrine regulatory factors in adrenocortical neoplasms

Chromaffin cells The coexistence of two lesions, i.e., pheochromocytoma or medullary hyperplasia with adrenocortical adenoma, observed in the same adrenal gland of patients with catecholamine overproduction in combination with hyperaldosteronism (47, 48) or Cushing’s syndrome (49, 50, 51, 52), suggests a role of cortical–medullary interactions in the control of hormone secretions. This hypothesis is supported by a case of pheochromocytoma associated with focal hyperplasia of the adrenal cortex adjacent to the tumor recently reported in a patient with subclinical hypercortisolism (53). The hyperplastic adrenal cortex in the vicinity of the pheochromocytoma, contrasting with atrophy of the residual cortex, is in favor of paracrine regulation of adrenocortical hyperplasia cells by pheochromocyte secretory products. Similarly, the preferential localization of adrenocortical micronodules close to the corticomedullary junction observed in adrenal from patients with primary pigmented nodular adrenocortical disease (PPNAD) with inactivating mutation of PRKAR1A or in mouse models of PPNAD (54, 55) is consistent with cortical–medullary interactions. Intercellular communication between chromaffin and steroidogenic cells is supposed to be more intense in corticomedullary mixed tumors, as these well-circumscribed tumors are composed of an intimately admixed population of adrenocortical cells and pheochromocytes (56, 57, 58, 59). Among the 19 cases of corticomedullary mixed tumors reported in the literature, seven patients presented catecholamine overproduction that was associated with hypercortisolism in three cases (57, 59, 60). In very much the same way, our group has described the occurrence of clusters of chromogranin A-positive chromaffin cells disseminated in adrenocortical adenoma responsible for hyperaldosteronism (61) or subclinical Cushing’s syndrome (62).

Such close contact of intermingled chromaffin and steroidogenic cells constitutes an anatomical prerequisite for paracrine interactions between the two cell types in adrenal lesions (Fig. 1).

Nerve terminals Like the normal adrenal cortex, adrenocortical hyperplasias and tumors contain nerve structures that are visualized adjacent to blood vessels or close to steroidogenic cells (Fig. 2). This observation suggests that autonomic neural inputs may control the secretory activity of steroid-producing adrenal neoplasms through either an indirect action via modulation of the adrenal blood flow or a direct effect on adrenocortical cells involving local release of various neurotransmitters (5, 63). In particular, it has been speculated that they could contribute to the diurnal rhythmicity of cortisol secretion that has been noticed in patients with adrenocortical hyperplasias and tumors causing Cushing’s syndrome in spite of plasma ACTH suppression (64).

Figure 1 Intra-adrenal communication (arrows) between chromaffin cells (grey) and adrenocortical cells (yellow) in normal adrenal gland (A) and adrenal neoplasms including pheochromocytoma with adjacent cortex hyperplasia (B), corticomedullary mixed tumors (C), and adrenocortical micronodules close to the corticomedullary junction observed in primary pigmented nodular adrenocortical disease (D).
However, the density of nerve fibers seems to be lower in adrenal hyperplasias in comparison with normal adrenals and nerves appear sparse in adenomas or even absent in some carcinomas (5, 65). It therefore seems unlikely that adrenal innervation could play a significant role in the development of adrenocortical neoplasms.

**Cells of the immune system** Several case reports have described infiltration of adrenocortical tumor tissues by macrophages and lymphocytes (66, 67). Infiltrates of mononuclear cells have also been noticed in diffuse and nodular adrenal hyperplasias associated with Cushing’s syndrome (Fig. 2; 68, 69). Aiba et al. (70) have reported marked mast cell infiltration in a case of deoxycorticosterone-producing tumor. High density of mast cells has also been observed in a series of aldosterone-producing adenomas, both in tumor and peritumoral tissues (35, 70, 71). By contrast, cortisol-producing adenomas do not contain any mast cells (70). Therefore, infiltration by mast cells has been considered as a characteristic feature of Conn’s adenoma. The absence of mast cell in tumors associated with Cushing’s syndrome can be ascribed to the anti-inflammatory effect of glucocorticoid. However, the presence of numerous mast cells in close contact with adrenocortical cells has been detected by histological and immunohistochemical approaches in a carcinoma co-secretting cortisol and renin (Fig. 2; 72). Because the different cell types of the immune system can release diverse cytokines and factors that are able to activate steroid production, it is tempting to speculate that their presence in the adrenal neoplastic tissues may not only reflect an inflammatory reaction to tumor development but could also represent a histological basis for cell-to-cell interactions potentially involved in the tumorigenesis process. In this regard, mast cells are now considered as important partners of tumor cells for neoplasm expansion (73).

**Vascularization** It is generally accepted that tumor progression is dependent on growth of blood vessels under control of angiogenic factors. Vascularization of adrenocortical lesions has been examined using biomarkers of endothelial cells (CD31 and CD34; Fig. 2). Sasano et al. (12) have observed that vascular density is quite similar in carcinomas, adenomas, and normal tissues. By contrast, Bernini et al. (74) have found that vascular density is lower in carcinomas than in normal adrenal and non-malignant tumors. In spite of these conflicting data on vascular density, there is good agreement that blood vessel area increases with development of adrenocortical neoplasms. In particular, microvessel area is higher in malignant tumors than in benign lesions, i.e., hyperplasia and functional adenomas (12, 75). In addition, vasculature is disorganized in carcinomas with large vessels and irregular networks of microcapillaries (76), which could affect communication between endothelial and steroidogenic cells. In adenomas and macronodular hyperplasias, vascular architecture is equivalent to that of normal adrenal (76, 77) and could allow endothelial cells to control the steroidogenic activity of tumor cells via a paracrine mechanism, as in normal tissue. In support of this hypothesis, it has been reported that CD34 immunoreactivity in Conn’s adenoma is positively correlated with plasma aldosterone levels (74). By contrast, vascularization, which is reduced in peritumoral tissues of aldosterone-producing adenomas, is not correlated with expression of CYP11B2, the gene encoding aldosterone synthase, the key enzyme of aldosterone biosynthesis (76, 77).

**Adipocytes** Adrenal myelolipomas are neoplasms composed of mature adipocytes and scattered hematopoietic elements. These rare tumors, which represent 4% of adrenal incidentalomas, are usually non-functional and benign (78). Myelolipomas may be combined with adrenocortical nodular hyperplasia (Fig. 2), adenomas, and carcinomas (79, 80, 81, 82, 83). Some of them are associated with hormonal disorders such as Cushing’s syndrome, primary hyperaldosteronism,
or hyperandrogenism (79, 83, 84, 85, 86, 87, 88). It has been proposed that intra-adrenal adipocytes might play a role in the pathogenesis of these diseases. In this respect, a case of idiopathic hyperaldosteronism has been reported to be clinically cured after unilateral resection of the adrenal gland containing a myelolipoma. This observation suggests that intra-adrenal adipocytes released some factors that stimulated mineralocorticoid secretion by paracrine and endocrine mechanisms in the ipsilateral and contralateral glands respectively (81). Indeed, it is well established that adipocytes produce leptin, adiponectin, cytokines, and all components of the RAS that are known to modulate steroidogenesis (13). Thus, secretion of bioactive factors by intra-adrenal adipocytes seems to be involved in the paracrine control of adrenocortical cells.

**Steroidogenic cells** Steroidogenic cells influence their own secretory activity through release of steroids and ultra-short regulatory loops. Another mode of communication between steroidogenic cells involves gap junctions that allow the passage of signaling molecules from one cell to the other, a process that seems essential for the regulation of adrenocortical cell proliferation and steroidogenesis (89).

**Factors**

**Catecholamines** The main products secreted by chromaffin cells are the catecholamines adrenaline and noradrenaline. Neri et al. (90) have reported that in vitro noradrenaline does not affect cortisol secretion but stimulates aldosterone production by normal adrenocortical cells. To our knowledge, the effect of adrenaline or noradrenaline on aldosterone production by adrenocortonaoma cells has not been investigated in vitro. Mulatero et al. (91) have examined the adrenergic control of aldosterone production in patients with primary aldosteronism. They have observed that β-blockers weakly reduce plasma aldosterone concentration. However, lowering of circulating aldosterone level partially results from a concomitant decrease in plasma renin activity, giving rise to an increase in the aldosterone–renin ratio during β-blocker treatment, i.e., atenolol (91, 92). In addition, this trial could not distinguish the relative contributions of the respective blockades of circulating and intra-adrenal catecholamine effects in atenolol-induced plasma aldosterone decrease. Thus, the role of catecholamines in the paracrine control of aldosterone secretion is not clearly established in primary hyperaldosteronism. By contrast, involvement of adrenaline and noradrenaline in glucocorticoid overproduction has been documented for some patients with Cushing’s syndrome due to unilateral adenoma (93) and bilateral macronodular hyperplasia (94, 95, 96, 97). Abnormal sensitivity to catecholamines has been suspected in patients who displayed plasma cortisol responses to tests inducing elevation of endogenous catecholamines, such as upright posture or insulin-induced hypoglycemia (96). Control of hypercortisolism by administration of propranolol, a β-blocker, as well as increase in plasma cortisol levels in response to infusion of isoproterenol, a β-adrenergic receptor agonist, have revealed the implication of β-adrenergic receptors in the control of steroidogenesis (96, 98, 99, 100). Aberrant expression of β-adrenergic receptors in adenoma and macronodular hyperplasia tissues has been demonstrated in vitro by PCR and binding experiments (93, 96, 97). In accordance with these observations, functional experiments have revealed a hyper-responsiveness to the β2-receptor agonists, salbutamol and isoproterenol, by cultured cells derived from two patients with macronodular hyperplasia (95, 97). In addition, molecular and cellular biological studies have recently shown that α2-adrenergic receptors can be abnormally expressed in macronodular hyperplasia tissues (94). Our group has observed that clonidine, a α2-receptor agonist, was able to stimulate in vivo and in vitro cortisol production in one patient with macronodular hyperplasia and Cushing’s syndrome (94). Thus, the presence of chromaffin cells in the vicinity of cortisol-secreting cells that express illegitimate β- or α2-adrenergic receptors may lead to formation of an aberrant positive regulatory loop involved in the maintenance of cortisol hypersecretion in spite of suppression of circulating ACTH.

**Adrenocorticotrophin** Beside catecholamines, chromaffin cells release different peptides that may influence steroid production in adrenal lesions. In particular, intense ACTH release by pheochromocytomas leading to elevation of plasma ACTH concentration represents a rare but classical cause of ectopic ACTH Cushing’s syndrome (101, 102, 103). Therefore, it can be hypothesized that modest intra-adrenal ACTH secretion by chromaffin cells present in corticomedullary mixed tumors or adrenocortical adenoma could exert a paracrine control on the proliferative and secretory activities of adrenocortical cells. In addition, ACTH immunoreactivity has been detected in adrenocortical adenoma extracts derived from patients with primary aldosteronism and Cushing’s syndrome (104). In macronodular hyperplasia, ACTH is produced not only by sparse chromaffin cells but also by CGA-negative cells (105, 106). Adrenal hyperplastic tissues express the gene encoding the ACTH precursor POMC and produce bioactive ACTH(1–39) (97, 105). We have shown in one case that the macronodular hyperplasia tissue does not express pituitary-specific transcription factors, such as Pit1 (Pou1f1), Ptx1 (Pitx1), and Prop1, thereby excluding a pituitary phenotype of adrenal POMC-expressing cells (105). By contrast, ACTH was found to be synthesized by a subpopulation of cells exhibiting cytoplasmic lipid droplets and 17-hydroxylase immunoactivity, an enzyme involved in cortisol synthesis.
ACTH-positive cells also contain relaxin-like factor, a marker of Leydig cells, the steroidogenic cells in testis (105). Leydig cells are known to produce POMC mRNA and peptides derived from POMC (107, 108, 109, 110, 111). As steroidogenic cells of adrenal and gonad derive from a common primordium that divides into separate tissues during embryogenesis, the presence of ACTH- and relaxin-like factor-positive steroidogenic cells in adrenal lesions suggests that some gonadal stem cells have not migrated to the gonads but have been included in the adrenals during embryo development. These gonadal-like cells present in macronodular adrenal hyperplasia tissues may be regarded as the mirror image of testicular adrenal rest detected in testis tumors in patients with congenital adrenal hyperplasia associated with CYP21 (21-hydroxylase) deficiency (112). Such an abnormal embryonic development process of adreno-genital primordium may explain the intermingling of normal adrenocortical cells with clusters of ACTH-positive steroidogenic cells in macronodular adrenal hyperplasia tissues. Finally, release of ACTH by macronodular adrenal hyperplasia tissues has been detected in vitro (97, 105), suggesting that abnormal intra-adrenal ACTH production in macronodular adrenal hyperplasia tissues may be involved in the pathogenesis of primary adrenal Cushing’s syndrome. The term ‘ACTH-independent’ used to designate primary adrenal Cushing’s syndrome may therefore be inappropriate in bilateral macronodular adrenal hyperplasia with hypercortisolism and undetectable plasma ACTH levels.

Arginine vasopressin (AVP) AVP produced by chromaffin cells in normal adrenal glands may exert a positive control on aldosterone and cortisol secretion via activation of type 1a receptors (V1a) positively coupled to phospholipase C (113, 114). Intra-adrenal AVP secretion by pheochromocytomas has been evidenced by hormone measurement in preoperative adrenal vein sampling (115, 116) and confirmed by in vitro experiments (117). A case of Cushing’s syndrome caused by a pheochromocytoma secreting AVP, in combination with corticotropin-releasing hormone and ACTH, has been reported (118). In addition, clusters of AVP producing cells have been observed in adrenocortical lesions, including Conn’s adenomas (61) and macronodular hyperplasias (95). These observations indicate that intra-adrenal AVP secretion may exert a positive tone on steroid production by adrenocortical cells. The role of AVP in glucocorticoid overproduction has been well documented in patients with overt and subclinical Cushing’s syndrome due to unilateral adenoma and bilateral macronodular hyperplasia (95, 119, 120, 121). Abnormal adrenal sensitivity to AVP has been shown in patients who displayed high plasma cortisol responses to injection of AVP or physiological tests modifying plasma AVP levels (water or hypertonic saline absorption) (122, 123, 124), and exaggerated cortisol responses of adrenocortical cells to AVP have been observed in vitro (95, 97, 119, 120, 123). The increased sensitivity of adrenocortical cells to AVP has been ascribed to overexpression of the eutopic V1a receptor subtype (120, 125, 126, 127) and/or expression of ectopic V1b and V2 receptors (127, 128, 129). Similarly, mRNAs encoding the V1a and V2 receptors have been detected at variable levels in aldosterone-producing adenomas (61, 130, 131). In vitro pharmacological experiments have revealed that the aldosterone response to AVP is mediated by activation of V1a receptors (61). Only a few clinical studies have evaluated the effect of vasopressin receptor antagonists in patients with primary adrenal steroid excess. A clinical trial has demonstrated that administration of the V1a receptor antagonist SR49059 to patients with Conn’s adenoma did not affect plasma aldosterone concentration but surprisingly triggered plasma aldosterone response to upright stimulation test (61). Conversely, a non-peptidic V1a antagonist administered orally was found to significantly decrease urinary cortisol level in a patient with an AVP-sensitive AIMAH associated with hypercortisolism (123). Concomitant intra-adrenal AVP production and aberrant expression of functional V1a/V2 receptors in adrenocortical neoplasms strongly suggest that AVP is involved in the physiopathology of hypercorticism.

Adrenomedullin Adrenomedullin was initially isolated from human pheochromocytoma tissue as a potent hypotensive peptide (132). A related peptide, named adrenomedullin 2 or intermedin, was subsequently discovered in 2004 (133). Adrenomedullin and adrenomedullin 2, which are produced by proteolytic cleavage from large precursors, share structural similarities with the regulatory peptides of the calcitonin family, including calcitonin, calcitonin gene-related peptide (CGRP), and amylin. Adrenomedullin acts through two subtypes of receptor complexes formed by dimerization of calcitonin-like receptor and one receptor activity-modifying protein (RAMP) type 2 or 3 but does not bind calcitonin-like receptor associated with RAMP1. Adrenomedullin 2 seems to be able to activate the three receptor subtypes formed by calcitonin-like receptor and RAMP1–3 (134). Gene expression of a complete adrenomedullin system, i.e., mRNAs encoding adrenomedullin precursor, the enzyme converting immature adrenomedullin, calcitonin-like receptor, and RAMP1–3, has been detected in zona glomerulosa but not zona fasciculata–reticularis in the normal adrenal gland (23). Genes encoding each different component of the adrenomedullin system are translated into proteins. In particular, the presence of adrenomedullin and its receptors has been well documented in adrenal zona glomerulosa (135, 136, 137). Functional in vitro studies have revealed that adrenomedullin stimulates cell proliferation by inhibiting apoptosis and reduces aldosterone secretion induced by ANG II or potassium in cultured zona glomerulosa cells.
(23). These data indicate that, in normal adrenal, adrenomedullin is a negative paracrine/autocrine regulator of aldosterone secretion. Adrenomedullin receptors are positively coupled to nitric oxide synthase and guanylyl cyclase in rat glomerulosa cells (138). Coupling of adrenomedullin receptors to similar transduction pathways could explain the inhibitory effect of adrenomedullin on mineralocorticoid production in human glomerulosa cells, as both nitric oxide and guanylyl cyclase have been reported to reduce aldosterone synthesis in H295 cells (139, 140).

The occurrence of an adrenomedullin system in adrenocortical tumors has also been investigated. Molecular, biochemical, and immunohistochemical studies have revealed that adrenomedullin is weakly or not produced in cortisol-producing adenomas, non-functioning adenomas, and adrenocortical carcinomas (ACC), whereas these approaches have clearly given evidence for intratumoral production of the peptide in aldosterone-producing adenomas (137, 141, 142, 143). On the other hand, adrenomedullin 2 has been detected in all types of adrenocortical neoplasms (142). The ability of Conn’s adenomas to secrete adrenomedullin has also been assessed in vivo by peptide quantification in plasma of patients with hyperaldosteronism (144). This clinical study has revealed i) higher levels of plasma adrenomedullin in patients than in normal subjects, ii) a decrease in plasma peptide concentration after adrenalectomy, and iii) positive correlation between circulating adrenomedullin and tumor size (144). These data thus indicate that Conn’s adenomas produce adrenomedullin together with aldosterone. Expression of calcitonin-like receptor and RAMP1–3 has been reported in adrenal tumors, including aldosteronomas (141, 142). In addition, adrenomedullin binding sites have been detected in aldosterone-producing adenomas (135). Adrenomedullin proved to be a more potent inhibitor on basal and ANG II-induced aldosterone production in cultured aldosteronomas than in normal zona glomerulosa cells (135). The increased sensitivity of adrenocortical cells to adrenomedullin could be due to overexpression of calcitonin-like receptors and subsequent activation of signal transduction pathways. In addition, a recent clinical study has shown that adrenomedullin infusion reduces plasma aldosterone levels in patients with primary hyperaldosteronism (145). These observations indicate that the endogenous adrenomedullin system exerts a potential negative control on aldosterone secretion through an autocrine/paracrine mechanism in Conn’s adenomas that may limit aldosterone hypersecretion. Conversely, the proliferative and anti-apoptotic actions of adrenomedullin on zona glomerulosa cells (23) suggest that the peptide could contribute to tumor growth. This hypothesis is reinforced by the positive correlation between plasma adrenomedullin in the patient and size of the aldosterone-producing adenoma (144).

**Vasoactive intestinal peptide** The presence of nerve fibers immunopositive for VIP has been reported in both benign and malignant adrenocortical tumors including steroid-producing and non-functioning lesions (5). As VIP has been shown to stimulate corticosteroid secretion from adrenocortical cells (146, 147), it is possible that VIP may control corticosteroidogenesis in some adrenal tumors responsible for steroid excess. In agreement with this hypothesis, the human ACC cell line H295R has been found to express type II pituitary adenylyl cyclase-activating polypeptide/VIP receptors (148).

**Cytokines** It has long been postulated that lymphocytic infiltration of steroid-secreting adrenocortical neoplasms may play a role in the physiopathology of tumor development and hypersecretion through local release of various cytokines (149, 150). In agreement with this hypothesis, lymphocytic infiltrate in a cortisol-secreting adenoma was found to be associated with abnormal expression of IL1 receptors by adenoma cells (66). In addition, IL1, which was also detected in the tumor tissue, was able to stimulate cortisol secretion from cultured adenoma cells. It was postulated that tumor formation may have resulted from high local concentrations of IL1 associated with illicit expression of IL1 receptors by some adrenocortical cells, leading to clonal expansion of the latter (66). However, this mechanism has not been confirmed in other neoplasms. Changes in the levels of expression of IL6 and its receptor have also been investigated in adrenal adenomas. Cortisol-producing adenomas have been reported to express both IL6 and IL6 receptor mRNAs (151). Nevertheless, in vitro experiments have revealed that adenomatous adrenal cells derived from patients with Cushing’s syndrome and the H295 cell line displayed lower sensitivity to IL6 than normal adrenal gland (151). In addition, high production of IL6 has been observed in an adrenocortical oncocyteoma in one patient without hypercortisolism (152). These data rather suggest that IL6 does not play a role in the pathogenesis of hypercortisolism.

**Serotonin (5-HT)** In the normal adrenal gland, 5-HT, produced by perivascular mast cells, stimulates aldosterone and cortisol secretion through a paracrine mechanism involving activation of type 4 5-HT receptors (5-HT4) positively coupled to adenylyl cyclase and calcium influx (153). After its release by mast cells, 5-HT can also be metabolized through oxidative deamination by monoamine oxidase type A expressed in chromaffin cells (7). The 5-HT4 receptor is primarily expressed in zona glomerulosa cells and, as a result, 5-HT is more efficient to stimulate in vitro aldosterone than cortisol secretion (153). In accordance with these observations, administration of 5-HT reuptake inhibitors and 5-HT4 receptor agonists (metoclopramide, zacopride, and cisapride) to healthy volunteers...

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is followed by an increase in plasma aldosterone levels whereas cortisol concentration remains unaffected (34, 154, 155). The physiological role of adrenal 5-HT is currently not known but the development of new 5-HT4 receptor antagonists for clinical use will provide valuable tools to determine the contribution of 5-HT in the global regulation of aldosterone secretion.

Recent studies indicate that 5-HT may also play a role in the physiopathology of adrenocortical hyperplasias and tumors. First, several observations suggest a reinforcement of the serotonergic intra-adrenal stimulatory tone in aldosterone-producing adenomas and cortisol-secreting hyperplasias and tumors. In particular, it has been noticed that the density of mast cells in aldosterone-producing adenomas is significantly higher than in normal adrenals (35). 5-HT is also abnormally detected in the sub-populations of steroidogenic cells in aldosteronoma (71), and macronodular hyperplasias (95) and carcinomas (72) responsible for hypercortisolism, providing a histological basis for autocrine regulation of steroid production in addition to paracrine mast cell–steroidogenic cell interaction. Besides the increase in potential intra-adrenal sources of 5-HT, other defects favoring the stimulatory action of 5-HT on steroidogenic cells have been described in adrenocortical neoplasms. In particular, patients with aldosteronoma exhibit exaggerated plasma aldosterone responses to the 5-HT4 receptor agonists metoclopramide (131, 156) and tegaserod (157). This increased responsiveness of aldosterone-producing adenomas to serotonergic agents can be ascribed to overexpression of 5-HT4 receptor mRNAs, which has been well documented in aldosteronoma tissues in comparison with normal adrenals (71, 131, 158, 159). Interestingly, the expression pattern of 5-HT4 receptor isoforms was found to be different in aldosterone-producing adenomas when compared with that of normal adrenals, suggesting that the increase in 5-HT4 receptor agonist-induced aldosterone secretion by aldosteronomas may also result from abnormal splicing of 5-HT4 receptor primary transcripts in adenoma cells (71). Specific 5-HT4 receptor antagonists, like GR113808, strongly inhibit 5-HT- and cisapride-induced aldosterone secretion from aldosterone-producing adenomas in vitro (160). However, none of these drugs is currently available for in vivo treatments and non-specific serotonergic antagonists, such as cyproheptadine and ketanserin, only weakly decrease plasma aldosterone levels in patients with primary aldosteronism (161, 162). Clinical studies have also shown that the 5-HT4 receptor antagonists metoclopramide, cisapride, mosapride, and tegaserod abnormally increase cortisol levels in some patients with Cushing’s syndrome related to macronodular hyperplasias and adenomas (95, 122, 163, 164, 165, 166, 167). Consistent with these data, 5-HT activates more potently and/or more efficiently cortisol production in vitro from hyperplasias and tumors than in the normal adrenal gland (95, 119). The enhanced sensitivity of adrenocortical hyperplasias and tumors to the effect of 5-HT and 5-HT4 agonists could be explained by an overexpression of the eutopic 5-HT4 receptor and/or expression of 5-HT7 receptors (163, 168). 5-HT7 receptors, which are not physiologically present in normal adrenocortical cells, must therefore be considered as adrenal ectopic membrane receptors together with receptors for glucose-dependent insulino-tropic peptide and luteinizing hormone (1, 121, 122, 169). Abnormal expression of 5-HT7 receptor has also been observed in a case of ACC co-secreting cortisol and renin (72). More rarely, 5-HT may paradoxically inhibit cortisol secretion in some benign adrenocortical neoplasms (170). The molecular mechanism involved in this paradoxical effect is not known. It may potentially implicate ectopic expression of 5-HT receptors negatively coupled to adenyl cyclase, like the 5-HT1 receptors, or abnormal coupling of eutopic 5-HT4 receptors. Finally, transcriptomic analyses of various types of adrenal tissues have revealed an overexpression of the 5-HT2B receptor in macronodular hyperplasia and ACCs (171, 172). However, the sites of expression of the receptor in the neoplastic adrenal tissues have not yet been identified so that the physiopathological significance of this finding remains unknown. As 5-HT3 receptors are known to be widely expressed in blood vessels, it is conceivable that overexpression of the 5-HT2B receptor in adrenal neoplasms may only reflect abnormal vascularization of the tissues (173). The main defects affecting the serotonergic control of steroid production in adrenocortical neoplasms are summarized in Fig. 3.

Endothelin Endothelin (ET-1) is a 21 amino acid peptide (ET-1(1–21)) released by endothelial cells. This peptide is generated by proteolytic cleavage of its precursor, big ET-1, by the endothelin-converting enzyme (ECE). Big ET-1 may also be cleaved by chymase, a mast cell enzyme, to produce a larger peptide, ET-1(1–31). Endothelin peptides act through two membrane receptor subtypes, named ETA and ETB. A complete local endothelin signaling system exists in normal adrenal cortex, including ECE, prepro-ET-1, ET-1, and ETA and ETB (36, 174, 175, 176). It has been demonstrated that both ET-1(1–21) and ET-1(1–31) stimulate in vitro aldosterone and cortisol productions by dispersed normal adrenocortical cells (36). The occurrence and role of endothelin system in the control of steroidogenesis have been extensively investigated in adrenal lesions associated with hyperaldosteronism. Expression of genes encoding prepro-ET-1, ECE-1, and ETA and ETB receptors has been detected in aldosterone-producing adenomas by PCR (177). Gene expression of the complete endothelin system was confirmed by microarray analysis, at levels equivalent to those of normal adrenal (178). In situ hybridization experiments have localized prepro-ET-1, ECE, and ETA and ETB receptor mRNAs in vascular
structures (177). They have also revealed the presence of ECE and ETA receptor transcripts in steroidogenic adenoma cells (177). In addition, it is now clear that genes encoding the different components of the endothelin system are translated into proteins. In particular, the presence of ET-1 in Conn’s adenomas has been demonstrated by immunohistochemistry (179). Higher amounts of ET-1(1–21) in aldosterone-producing adenomas than in normal adrenal have been found by RIA (179). These data argue in favor of an increased ET-1 secretion in aldosterone-producing adenomas. However, Morello et al. (178) have detected similar ET-1 levels in inferior vena cava and left and right adrenal vein blood in patients with hyperaldosteronism due to bilateral adrenal hyperplasia or aldosteronoma. ET-1 acts via high-affinity binding sites present in aldosterone-producing adenoma and idiopathic hyperaldosteronism tissues (179). Binding experiments have revealed lower maximal binding capacity of ET-1 receptors in unilateral tumors than in normal adrenal and idiopathic hyperaldosteronism, suggesting downregulation of ET-1 receptors in Conn’s adenoma. The occurrence of ET-1 and its receptors in aldosterone-producing adenoma is highly suggestive for the existence of an endothelin-mediated control of endothelial cell on aldosterone secretion by adenoma cells through a paracrine mechanism. In this respect, Rossi et al. (176) have demonstrated that ET-1 is as efficient as ANG II to stimulate in vitro aldosterone secretion by dispersed cells derived from Conn’s adenomas. Pharmacological experiments have revealed that the aldosterone response to ET-1 is mediated by activation of both ETA and ETB receptors and stimulation of the protein kinase C and cyclooxygenase pathways (176). A clinical

Figure 3 Alterations of the paracrine serotonergic control of corticosteroidogenesis in adrenocortical neoplasms compared with normal adrenal. Aldosterone-producing adenomas harbor mast cell proliferation and overexpression of the serotonergic receptor type 4 (5-HT4). In cortisol-producing adenomas (CPA), a subpopulation of steroidogenic cells abnormally synthesize serotonin (5-HT) which, together with 5-HT released by mast cells, stimulates cortisol secretion through activation of overexpressed 5-HT4 receptors and ectopic receptors (5-HTx). In some CPA tissues, 5-HT exerts a paradoxical inhibitory effect on cortisol production via a non-characterized subtype of receptor (5-HTy). Bilateral macronodular adrenal hyperplasias are associated with illicit synthesis of 5-HT in some steroidogenic cells and occurrence of illegitimate receptors including overexpression of the eutopic 5-HT4 receptor and expression of ectopic 5-HT7 receptors.
trial has demonstrated that combined administration of ETA and ETB receptor antagonists (BQ-123 and BQ-788) to patients with primary aldosteronism transiently reduced plasma aldosterone concentration without changing plasma ACTH and cortisol levels or renin activity (37). Altogether, these data support a role of the intra-adrenal endothelin system in the control of aldosterone secretion in aldosterone-producing adenoma, which is at least as potent as in normal adrenal gland. However, endothelin does not seem to play a crucial role in the pathogenesis of primary hyperaldosteronism, but the peptide could contribute to the maintenance of aldosterone production at excessive levels in spite of suppression of the RAS.

Steroids Most of the physiological responses to aldosterone are mediated by the mineralocorticoid nuclear receptor. Molecular studies have revealed expression of mineralocorticoid receptor mRNA in normal adrenal gland and aldosterone-producing adenomas (180, 181). Nevertheless, the lack of effect of the mineralocorticoid receptor antagonists, canrenone and eplerenone, on aldosterone production by adrenocortical cells derived from normal gland and Conn’s adenoma (181), indicates that aldosterone does not exert a control on its own release. It has been demonstrated that, in mice, glucocorticoids exert an intra-adrenal negative feedback loop on corticosterone production via activation of glucocorticoid receptor and nuclear hormone receptor Dax-1 (182, 183). The existence of an ultra-short intra-adrenal regulatory loop involving cortisol has also been investigated in humans. The presence of glucocorticoid receptor mRNAs has been evidenced in normal cortex, as well as in cortisol-producing and non-functioning adrenocortical adenomas by northern blot, RT-PCR, and microarray experiments (181, 184, 185, 186). However, other studies failed to detect the glucocorticoid receptor by binding experiments (187) or immunohistochemistry in normal cortex and benign adrenocortical tumors (186, 188). In addition, a recent western blot analysis has revealed that the glucocorticoid receptor D isoform is present in adrenocortical cells in normal cortex and tumor tissues but it is known that this molecular form displays weak transcriptional activity (189). In agreement with these biochemical findings, in vivo studies conducted in patients with hypopituitarism have shown that ACTH-induced cortisol secretion was not affected by dexamethasone administration (187). Similarly, dexamethasone administration during Liddle’s test is known not to affect plasma or urinary cortisol levels in patients with macronodular adrenal hyperplasia (190). In addition, in vitro experiments have revealed that dexamethasone has no effect on cortisol production by adrenocortical cells derived from macronodular adrenal hyperplasia (188). These data indicate that intra-adrenal glucocorticoid receptors are not involved in control of steroidogenesis in normal tissue and macronodular adrenal hyperplasia.

By contrast, glucocorticoid receptors seem to play a significant role in the control of steroidogenesis in patients with primary hypercortisolism due to ACC or PPNAD. In fact, it has been clearly demonstrated that NR3C1, the gene encoding the glucocorticoid receptor, is overexpressed in ACCs associated with Cushing’s syndrome (186). In addition, 3H-dexamethasone binding sites have been detected (186, 187) and glucocorticoid receptors have been visualized by immunohistochemistry in the nucleus of carcinoma cells (186). Nuclear localization of glucocorticoid receptor is in favor of the existence of functional receptors in neoplastic cells. In support of these observations, dexamethasone has been reported to increase gene transcription of STAR and the steroidogenic enzyme 3βHSD in the adrenocortical cell line H295R (191). Dexamethasone also stimulates cortisol production by H295R cells (191). However, there is no evidence that cortisol exerts an intra-adrenal positive regulatory loop that might contribute to glucocorticoid overproduction in patients with ACCs.

A positive role of glucocorticoid receptors in the control of cortisol synthesis in patients with PPNAD has been clearly established on the basis of the following observations: i) the gene encoding the glucocorticoid receptor is overexpressed in nodules of PPNAD tissues (188); ii) immunohistochemical studies have revealed the presence of glucocorticoid receptors in the cytoplasm of adrenocortical cells in pigmented nodules (188); iii) dexamethasone administration during Liddle’s test provokes a paradoxical increase in plasma cortisol levels in patients with PPNAD (192); and iv) dexamethasone has been reported to stimulate in vitro cortisol production by cultured cells derived from PPNAD tissues (183, 188, 193). These data demonstrate that glucocorticoid exerts an intra-adrenal positive regulatory loop that contributes to cortisol overproduction in patients with PPNAD. Occurrence of bilateral micronodular adrenal hyperplasia is frequently associated with germline or somatic PRKAR1A inactivating mutations (194, 195), suggesting that these mutations might confer abnormal glucocorticoid sensitivity to adrenocortical cells. In support of this hypothesis, Bertherat et al. (196) have reported paradoxical cortisol responses to dexamethasone in some patients with cortisol-producing adenomas associated with somatic PRKAR1A inactivating mutations. Our group has demonstrated that the positive effect of dexamethasone on cortisol secretion in micronodular adrenocortical cells is reduced by the glucocorticoid receptor antagonist mifepristone or the protein kinase A inhibitor H89 (183). These observations indicate that the stimulatory action of dexamethasone on cortisol synthesis is mediated by cytoplasmic glucocorticoid receptors interacting with protein kinase A catalytic subunits.
The adrenal RAS The adrenal cortex has been shown to synthesize all of the components of the RAS, including prorenin, renin, angiotensinogen, and angiotensin-converting enzyme. Adrenal renin synthesis appears to be subtly regulated. In rats, the adrenal concentration of renin, which has been found in dense bodies of glomerulosa cells, is increased by nephrectomy, ACTH, potassium, and low-sodium diet whereas high-sodium diet decreases adrenal renin content and renin mRNA level (46). Interestingly, ANG II, the active final compound of the system, is detected in zona glomerulosa cells, indicating that the intra-adrenal RAS likely controls aldosterone secretion. Because ANG II is able to stimulate renin secretion by adrenocortical cells in primary culture, the adrenal RAS may constitute an amplificatory system of the action of circulating ANG II. Locally produced ANG II may also control adrenal blood flow, as it does in other organs, and adrenal growth through its stimulatory action on the mitogenic activity of adrenocortical cells. Intriguing results have described the presence of a non-secretory renin isoform in adrenal cell mitochondria but the physiological function of this intracellular protein remains unclear.

Little is known about the physiopathological role of the adrenal RAS in steroid-producing neoplasms. Aldosterone-producing adenomas express type 1 and type 2 ANG II receptors (AT1R and AT2R respectively) as well as renin, angiotensin, and angiotensin-converting enzyme mRNAs (197, 198). However, most of them are unresponsive to the action of ANG II, suggesting the occurrence of post-ANG II receptor defects in aldosterone-producing adenomas. The fact that AT2R mRNAs are overexpressed in the subgroup of ANG II-responsive in comparison with ANG II-unresponsive aldosterone-producing adenomas indicates that AT2R may be involved in aldosterone hypersecretion in some adenomas (197). It cannot also be excluded that ANG II may play a role in aldosterone-oma formation through its mitogenic properties.

Expression of renin has been observed in adrenocortical cortisol-secreting and non-secreting adenomas and carcinomas (72, 199), sometimes associated with the occurrence of angiotensinogen (72, 199). In one case, renin secretion by cultured tumor cells was found to be inhibited by 5-HT in a paracrine fashion revealing complex regulations within the tumor tissue (72). The pathophysiological relevance of these findings is not clear but the recent identification of a membrane receptor for renin, which mediates angiogenic and mitogenic actions of the protein through interaction with the Wnt/β-catenin pathway (200, 201), could suggest that renin may be involved in adrenocortical tumorigenesis (202, 203, 204).

Insulin-like growth factor 2 IGF2, which is known to play a pivotal role in the development of the adrenal cortex, acts as a mitogen autocrine factor to promote cell cycle progression and proliferation through activation of the type 1 IGF receptor (IGF1R). In the Beckwith-Wiedeman syndrome, increased expression of IGF2 results in an elevated rate of ACCs (205). In addition, several recent transcriptomic studies have demonstrated strong up-regulation of IGF2 in the great majority of sporadic ACCs in contrast to adenomas and normal adrenal tissues (206), a finding that had previously been reported by Gicquel et al. as early as 1994 (207). This important observation suggests that IGF2 may be a major actor of the development of malignant adrenocortical tumors, an hypothesis that is also supported by the fact that IGF2 has been shown to stimulate cell proliferation in the human ACC cell line H295R (208). However, recent data obtained from transgenic mice overexpressing IGF2 in the adrenal cortex show that IGF2 has no tumor-initiating potential in this model (209). Nevertheless, several investigators have initiated preclinical studies with pharmacological agents targeted to the IGF2 pathway, aimed at developing new treatments of ACC. This approach has already produced promising results showing that IGF1R antagonists induce a significant inhibition of growth in ACC cell lines (210).

Inhibin/activin/TGFβ/bone mitogenic proteins Activins and inhibins are formed as combinations of inhibin α- and β-subunits. Activins are dimers of β-subunits (βA or βB) while inhibins A and B are heterodimers of the inhibin α-subunit linked to β-subunits A and B respectively. Activins exert their biological actions by means of specific receptors, i.e., the activin type I and II receptors, and the intracellular proteins SMAD. Inhibins antagonize activin signaling through binding the activin type II receptor and formation of an inactive complex with the TGFβ type III receptor β-glycan. In the normal human adrenal cortex, expression of inhibin α-subunit is mainly observed in zona reticularis while β-subunit is principally expressed in the outer zones (211, 212). In agreement with this observation, expression of inhibin α-subunit is predominantly stimulated by ACTH whereas that of inhibin β-subunit is activated by ANG II (45). The activin receptors and the inhibin co-receptor β-glycan mRNAs are also detected in the adrenal cortex raising the possibility that activins and inhibins may control corticosteroidogenesis through autocrine and paracrine mechanisms. Adrenomedullary chromaffin cells, which express inhibin β-subunits A and B (45), may also influence the adrenocortical function in a paracrine manner via the release of activins in the vicinity of adrenocortical cells. The physiological roles of activins and inhibins in the adrenal cortex are not completely understood. Activin A is able to reduce adrenocortical cell growth by favoring cell apoptosis (213) and to regulate expression of several steroidogenic enzymes leading to inhibition of corticosteroid secretion (213, 214). Conversely, no
direct action of inhibins alone on the adrenal cortex has been clearly demonstrated.

The involvement of inhibin in the physiopathology of adrenocortical tumorigenesis has been suggested by several findings obtained from rodent models. Especially, gonadectomized Inha knockout mice have been shown to develop ACCs (215). Expression of inhibin α-subunit has been investigated in various types of adrenocortical tumors. Microarray studies have shown low expression of the INHA gene in carcinomas in comparison with adenomas (216). However, only a subgroup of adrenocortical non-secreting carcinomas lack INHA expression (217), with the great majority of these tumors being positive for inhibin α-subunit at immunohistochemistry (218). In addition, ACCs express low levels of TGFβ receptor type III mRNA (216, 217). Nevertheless, the role of these findings in adrenocortical tumorigenesis remains to be elucidated. Inhibin production by adrenocortical cortisol- or estrogen-secreting tumors has been observed in vivo (219, 220, 221). The main endocrine consequence of this process is inhibition of pituitary FSH release (219, 220). It is not known whether inhibin may also affect tumor steroidogenesis through paracrine mechanisms or not.

The three isoforms of TGFβ, termed TGFβ1, TGFβ2, and TGFβ3, are widely expressed and are potent inhibitors of cell growth. They are also able to regulate hormone synthesis and secretion in various types of endocrine glands (222, 223, 224). In the human adrenal cortex, TGFβ1 immunoreactivity is primarily if not exclusively observed in zona glomerulosa and zona reticularis (225), and the three types of TGFβ receptors have been detected in human adrenocortical cells (226), suggesting that TGFβ may act as an autocrine factor to regulate corticosteroid production. In support of this hypothesis, TGFβ was found to increase levels of 3β-hydroxysteroid dehydrogenase mRNA and reduce those of 17α-hydroxylase mRNA (226). In addition, TGFβ decreases DHEAS secretion but has no effect on cortisol release (226). TGFβ also exerts an antimitotic action on fetal adrenocortical cells in vitro (227). The presence and distribution of TGFβ1 has been investigated by immunohistochemistry in various types of adrenocortical tumors (225). Among adenomas, TGFβ1 immunoreactivity was observed in steroid-secreting tumors whereas no signal could be seen in non-functioning neoplasms. Interestingly, carcinomas were negative for TGFβ whatever their secretory status, i.e., steroid-producing or not (225). On the other hand, it has been noticed that, in the human ACC SW-13 cell line, restoration of TGFβ type II receptor expression reduces the ability of malignant cells to form tumors in athymic nude mice (228). Collectively, these data suggest that decreased expression of TGFβs and TGFβ type II receptor may play an important role in the determination of malignant phenotype in human adrenocortical tumors. TGFβ may also play a role in the physiopathology of PPNA secondary to PRKAR1A mutations. In the adrenocortical cell line H295R, PRKAR1A inactivation inhibits TGFβ-induced apoptosis through a decrease in SMAD3 expression, which plays a major role in TGFβ signaling (229). This mechanism may be involved in adrenocortical hyperplasia and resistance of adrenocortical cells to apoptosis observed in PPNA (55).

Bone morphogenic proteins (BMPs) have been initially isolated on the basis of their ability to trigger bone formation (230). Subsequent studies have shown that BMPs represent a family of cytokines belonging to the TGFβ superfamily. BMPs are involved in the regulation of cell differentiation, growth, and apoptosis in a wide variety of tissues (231). They exert their biological effects through binding to specific receptors, the BMP type I and II receptors (BMPRI and BMPRII respectively), and activin receptors. Interestingly, BMPs exhibit different affinities for these diverse receptor types. For example, BMP2 principally binds to BMPR1 whereas BMP6 and BMP7 actions are mainly mediated by activin receptors. After activation by BMPs, BMPR2 transphosphorylates BMPR1, which secondarily phosphorylates SMAD proteins. Recent studies have shown that BMPs display several functions in the adrenal gland (232). BMPs (especially BMP2, BMP4, and BMP7), originating from the wall of the dorsal aorta, have been shown to play an important role in the development of adrenal medulla. BMP4 can also be released by adrenocortical cells during the formation of the adrenal gland to stimulate the development of chromaffin cells in a paracrine manner (233). In addition to their function during embryogenesis, BMPs are able to regulate catecholamine secretion from the adult adrenal medulla. This action seems to be an indirect process in which BMPs negatively modulate the stimulatory effect of glucocorticoids (234). BMPs also directly influence corticosteroidogenesis. Especially, BMP6, produced by adrenocortical cells, has been shown to enhance the stimulatory action of ANG II on aldosterone secretion through an autocrine/paracrine mode of communication (235, 236). Conversely, BMP2 and BMP5 inhibit aldosterone, cortisol, and DHEAS production from the H295R adrenocortical cell line (237). On the other hand, numerous studies have demonstrated that BMPs are involved in tumorigenesis at various sites and alteration in BMP pathways have been reported in several types of endocrine tumors including those that arise from ovary, pituitary, and thyroid. Interestingly, it has been shown that expression of BMP2 and BMP5 is downregulated in ACCs and the ACC cell lines H295R and SW13 in comparison with normal adrenals, and treatment of ACC cell lines with BMP2 and BMP5 reduced cell proliferation and viability (237). These results suggest that BMPs may act as autocrine/paracrine suppressors of tumor growth in the adrenocortical tissue.
Pathophysiological significance of paracrine factor expression in adrenocortical neoplasms

It is now well known that adrenocortical cells physiologically express neuroendocrine markers like synaptophysin, neuronal cell adhesion molecule (NCAM), neuron-specific enolase (NSE), granulophysin, protein gene product (PGP) 9.5, stanniocalcin, synaptic vesicle protein 2 (SV2), and neurofilaments (65, 238, 239, 240). Granule-like bodies have also been visualized in the cytoplasm of corticosteroidogenic cells by means of electron microscopy (241, 242). Neuroendocrine differentiation is mainly observed in zona glomerulosa cells, although zona reticularis cells seem to be able to display PGP 9.5 and SV2 immunoreactivities (65, 238, 243). As a matter of fact, aldosterone-producing adenomas have been found to express synaptophysin, NCAM, NSE, and SV2 (65, 238). Cortisol-producing and non-functioning adenomas can also contain detectable synaptophysin, NSE, PGP 9.5 immunoreactivities (244) but neuroendocrine differentiation of adrenocortical tumor tissues is mainly a feature of ACCs that are known to intensely and diffusely express neuroendocrine markers (65, 242, 243, 245). Most paracrine regulatory factors detected in adrenocortical tumor cells are neuropeptides or conventional neurotransmitters. It therefore seems reasonable to consider that their expression in corticosteroidogenic cells is an additional trait of neuroendocrine differentiation.

The occurrence of neuroendocrine differentiation in adrenocortical neoplasms is not restricted to single tumors but is also encountered in diffuse nodular hyperplasias. Indeed, expression of neuroendocrine markers has been well established by immunohistochemistry and transcriptomic approaches in PPNAD tissues (246, 247). In addition, a subpopulation of steroidogenic cells in macronodular hyperplasias has been shown to abnormally contain ACTH, AVP, and 5-HT and to contain secretory granules (95, 105). These cells seem to be different from the corticochromaffin hybrid cells that have previously been observed in the normal rat adrenal cortex (248). In that they do not express chromogranin A (105). Conversely, ACTH-positive cells in macronodular hyperplasias express Leydig cell markers, suggesting that their neuroendocrine characteristics could be regarded as a feature of gonadal-like differentiation (105). This hypothesis is also supported by the observation that AVP and 5-HT are physiologically present in the human testis (111, 249, 250, 251).

The newly demonstrated expression of stimulatory factors within steroid-secreting adrenal hyperplasias and tumors suggests that steroid hypersecretion and adrenocortical cell proliferation are driven by autocrine/paracrine loops in these neoplasms. It is also physiopathologically relevant to notice that some intra-adrenal regulatory systems exert inhibitory actions on corticosteroidogenesis that may be regarded as compensatory mechanisms aimed at counteracting the effect of the local corticotropic signals. The demonstration of the presence of adrenomedullin, which decreases steroid release, in aldosterone-producing adenomas as well as the report of paradoxical inhibitory actions of 5-HT in cortisol-secreting lesions are valuable illustrations of this concept. The global impact of intra-adrenal paracrine factors on the secretory and mitogenic activities of adrenocortical neoplasms may therefore result from a subtle balance involving stimulatory and inhibitory bioactive agents released within the adrenal tissues.

Conclusion and perspectives

Benign adrenocortical neoplasms responsible for primary adrenal hypercorticism are considered as autonomous lesions because they are independent of ANG II and ACTH, which are suppressed by steroid excess. On the other hand, the drugs currently used to control steroid hypersecretion or inhibit the peripheral actions of aldosterone and cortisol produce cumbersome or severe side effects that hamper their use in long-term treatments. For instance, the mineralocorticoid antagonist spironolactone generates impotence and gynecomastia in male patients and anti-cortisolic compounds, like mitotane, metyrapone, and ketoconazole, exhibit neurological, hepatic, metabolic, and/or hematological toxicities. Consequently, surgical ablation of the causative adrenocortical lesion is frequently necessary in order to cure steroid excess in patients with primary adrenal hypercorticism. However, the intra-adrenal paracrine stimulatory systems could be regarded as promising targets for new pharmacological treatments of hyperfunctioning adrenal disorders, which may constitute an alternative to adrenal surgery. In fact, prolonged remissions of Cushing’s syndrome with β-blocker treatments have been described in patients with macronodular hyperplasia expressing illicit adrenergic receptors (100). In addition, it is conceivable that pharmacological interventions targeted to cells of the immune system like mast cells may help control adrenocortical tumor development, as already reported in patients with neurofibromas (252, 253). Similarly, treatments aimed at inhibiting the action of intra-adrenal growth factors like IGF2 could represent an efficient strategy in the clinical management of patients with ACCs. In this regard, the main challenge for the forthcoming years will be to determine the hierarchy of the different paracrine adrenal factors with respect to their functional importance in order to identify the best therapeutic targets for future clinical studies.

There is no evidence in the literature for a possible influence of the intra-adrenal regulatory loops on the performance of the clinical biological tests employed in
the diagnosis of hyperaldosteronism and hypercortisolism. Nevertheless, it seems likely that the more the corticotropic factors are expressed in the adrenal tissues, the more steroid secretion is enhanced and insensitive to suppression tests, increasing therefore the diagnostic accuracy of the latter. On the other hand, synthetic ligands of adrenal aberrant membrane receptors of paracrine factors, such as 5-HT4 receptor agonists and vasopressin analogs, are used to detect abnormal cortisol responses in patients with primary adrenal hypercorticism. However, the systematic screening for these alterations in the adrenocortical function is not currently recommended in routine practice. In vivo imaging of receptors for hormones and neurotransmitters is now made possible by the recent development of synthetic radioligands for positron emission tomography (PET). As hyperfunctioning adrenal neoplasms aberrantly overexpress membrane receptors for paracrine regulatory factors, it is conceivable that radioligands able to bind these receptors may be of great interest for the diagnosis of the adrenal disorder. For instance, 5-HT4 receptor PET radioligands, which are now used for brain imaging, could be employed in patients with primary aldosteronism for lateralization of the source of excessive aldosterone production, which is a critical diagnostic step to guide the therapeutic management of the disease.

Finally, it is not known whether expression of paracrine factors has a significant influence on the prognosis of adrenocortical tumors or not, although it seems probable that high adrenal levels of growth factors may favor development of larger and more aggressive lesions. In this regard, it is clear that high expression of IGF2 in the adrenal tissue is an important marker for the discrimination of ACCs vs benign tumors but is a weak predictor of mortality in patients with malignant adrenocortical tumors. However, it is highly likely that the molecular research currently performed to elucidate the molecular mechanisms involved in adrenocortical tumor progression and metastasis will progressively allow identification of the main actors of these physiopathological processes, leading to improvements in the diagnostic procedures.

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

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References
17. Takahashi K, Totsune K & Murakami O. Adrenocortical peptides: autocrine or paracrine regulators for the steroid hormone


Adrenal cortex remodeling and functional zona glomerulosa hyperplasia in primary aldosteronism. *Hypertension* 2010 **56** 885–892. (doi:10.1161/HYPERTENSIONAHA.110.158543)


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