cAMP signaling in cortisol-producing adrenal adenoma

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Abstract

The cAMP signaling pathway is one of the major players in the regulation of growth and hormonal secretion in adrenocortical cells. Although its role in the pathogenesis of adrenocortical hyperplasia associated with Cushing’s syndrome has been clarified, a clear involvement of the cAMP signaling pathway and of one of its major downstream effectors, the protein kinase A (PKA), in sporadic adrenocortical adenomas remained elusive until recently. During the last year, a report by our group and three additional independent groups showed that somatic mutations of PRKACA, the gene coding for the catalytic subunit α of PKA, are a common genetic alteration in patients with Cushing’s syndrome due to adrenal adenomas, occurring in 35–65% of the patients. In vitro studies revealed that those mutations are able to disrupt the association between catalytic and regulatory subunits of PKA, leading to a cAMP-independent activity of the enzyme. Despite somatic PRKACA mutations being a common finding in patients with clinically manifest Cushing’s syndrome, the pathogenesis of adrenocortical adenomas associated with subclinical hypercortisolism seems to rely on a different molecular background. In this review, the role of cAMP/PKA signaling in the regulation of adrenocortical cell function and its alterations in cortisol-producing adrenocortical adenomas will be summarized, with particular focus on recent developments.

Introduction

The cAMP signaling pathway plays a fundamental role in regulation of metabolism, cell proliferation, differentiation, and apoptosis in endocrine tissues (1). A major effector of cAMP is protein kinase A (PKA), which phosphorylates a number of substrates, including cytosolic and nuclear targets, most notably transcription factors of the CREB/ATF family (2). Activation of the cAMP pathway causes both increased function and replication of several endocrine cells, including those of the adrenal cortex (2). Thus, alterations of this pathway have been found in different endocrine diseases. Loss-of-function mutations are responsible for syndromes of

Invited Author's profile

Davide Calebiro is an endocrinologist and a receptor scientist. Since 2009, he is Group Leader at the Bio-Imaging Center/Rudolf Virchow Center and Institute of Pharmacology of the University of Würzburg, Germany. His group utilizes optical methods such as fluorescence resonance energy transfer and single-molecule microscopy to investigate the spatiotemporal dynamics of receptor signaling in living cells. His major recent contributions include the discovery of GPCR signaling in the endosomal compartment and the identification of mutations in the catalytic subunit of protein kinase A (PKA) as a cause of adrenal Cushing’s syndrome.
hormone resistance like pseudohyoparathyroidism (3) or thyrotropin (TSH) resistance (4). Conversely, mutations leading to constitutive activation of this pathway lead to tumor development and hormone excess, as in the case of thyroid adenomas or growth hormone (GH)-secreting pituitary adenomas (5, 6, 7).

Under normal conditions, adrenocortical cells are under the tight control of the adrenocorticotropic hormone (ACTH), which signals via the melanocortin 2 receptor (MC2R), a G protein-coupled receptor (GPCR) expressed in high abundance on the surface of adrenocortical cells. Activation of the MC2R, which is coupled to the stimulatory Gs protein, leads to an increase of the intracellular concentration of cAMP and activation of PKA, ultimately stimulating glucocorticoid production. Whereas the role of ACTH in stimulating the proliferation of adrenocortical cells remains a matter of investigation, in vitro data, animal models, and, most importantly, a number of human genetic defects clearly demonstrate the central role of the cAMP/PKA pathway in the pathophysiology of adrenal hyperplasia and hypercortisolism. In contrast to familial forms of adrenal Cushing’s syndrome, the pathogenesis of the much more frequent cases of sporadic, unilateral cortisol-secreting adrenal adenomas had remained largely obscure. Recently, we and three additional independent groups have identified a high frequency (35–65%) of somatic mutations affecting the catalytic alpha subunit of PKA (PRKACA) in cortisol-secreting adrenal adenomas (8, 9, 10, 11). In this review, we provide an overview on the role of cAMP/PKA in the control of adrenocortical cell function and its alterations in Cushing’s syndrome, with a particular focus on recent developments.

**Clinical spectrum of adrenal Cushing’s syndrome**

Endogenous hypercortisolism associated with unilateral adrenocortical adenomas is the most common form of ACTH-independent Cushing’s syndrome, occurring in almost one-third of patients with overt Cushing’s syndrome (12). The clinical picture of patients with Cushing’s syndrome is characterized by severe comorbidities and adverse events, due to the effect of excessive cortisol production. Given that the glucocorticoid receptor is widely expressed in almost all tissues, the effects of cortisol hypersecretion inevitably involve many organ systems. The classic clinical picture of a patient affected by Cushing’s syndrome is characterized by easy bruising, facial plethora, proximal muscle weakness, striae rubrae, and hirsutism (Table 1) (13). In addition, hypertension is a very common feature of the syndrome, occurring in 80% of the patients, and is characterized by drug resistance in up to 17% of cases, as well as by loss of nocturnal dipping (14, 15, 16). Alterations of glucose and lipid metabolism are also common findings in patients with Cushing’s syndrome. All those comorbidities, together with the typical hypercoagulable state due to alterations of clotting factors (17), severely impair the cardiovascular profile of patients with Cushing’s syndrome, leading to an increased incidence of cardiovascular events and mortality, if left untreated (18, 19). Considering that the cardio-metabolic impairment in those patients is often associated with an increased rate of severe infectious complications (20), an increased incidence of osteoporotic fractures, and psychiatric disorders (21), it is clear that patients with Cushing’s syndrome are characterized by a life-threatening disease that requires a precise causative diagnosis and a rapid therapeutic strategy. A comprehensive review on the complications of Cushing’s syndrome and their treatment has been recently published elsewhere (19) and is beyond the scope of this review.

The term ‘subclinical hypercortisolism’ has been commonly used in the last years to define a condition of hypercortisolism, detected by hormonal alterations of the HPA axis, in patients without a clear phenotype recalling that of Cushing’s syndrome. By definition, subclinical hypercortisolism is diagnosed in patients with incidentally

<table>
<thead>
<tr>
<th>Cushing’s syndrome</th>
<th>Specific signs</th>
<th>Easy bruising</th>
<th>Facial plethora</th>
<th>Proximal myopathy</th>
<th>Striae (red-purple, &gt;1 cm wide)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Symptoms</td>
<td>Weight gain, changes in appetite</td>
<td>Depression, mood changes</td>
<td>Reduced concentration and memory</td>
<td>Insomnia, fatigue</td>
<td>Decreased libido</td>
</tr>
<tr>
<td>Less discriminatory signs and symptoms</td>
<td>Facial fullness, central obesity</td>
<td>Buffalo hump, supraclavicular fullness, acne, and hirsutism</td>
<td>Thin skin, poor wound healing</td>
<td>Peripheral edema</td>
<td></td>
</tr>
<tr>
<td>Biochemical evidence of mild cortisol excess without specific signs of Cushing’s syndrome</td>
<td></td>
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discovered adrenal tumors. Therefore, in the last years, subclinical hypercortisolism has been used for patients who do not fulfill the criteria either for Cushing’s syndrome or for non-secreting tumors. In the last decades, the study of subclinical hypercortisolism has gained more interest because of the increasing number of cases reported in a relevant fraction of patients with adrenal incidentalomas (up to 30%) (22), which are discovered in a relatively large number of subjects (up to 4% in radiological series) (23).

The clinical picture of patients with subclinical hypercortisolism is characterized by several comorbidities such as hypertension, type 2 diabetes, and dyslipidemia (Table 1), but also by clinically relevant outcomes, mainly such as hypertension, type 2 diabetes, and dyslipidemia. Hypercortisolism is characterized by several comorbidities when compared to non-secreting adrenocortical adenomas (24, 25, 26, 27, 28, 29). Moreover, three recent retrospective studies showed a role for mild cortisol hypersecretion in the development of cardiovascular diseases in patients with subclinical hypercortisolism (24, 26, 27, 28, 29). The clinical spectrum of patients with subclinical hypercortisolism (Table 1), but also by clinically relevant outcomes, mainly osteoporotic vertebral fractures, cardiovascular diseases, and related mortality. Several cross-sectional studies have highlighted that glucocorticoid-induced osteoporosis (GIO) and its related complications indeed occur also in patients with subclinical hypercortisolism (24, 25, 26, 27, 28, 29). Moreover, three recent retrospective studies showed a role for mild cortisol hypersecretion in the development of cardiovascular diseases in patients with subclinical hypercortisolism (30, 31). Indeed, the incidence of cardiovascular diseases was higher in those patients with respect to their non-secreting counterpart, and the increase in cortisol levels over time was independently associated with the risk of cardiovascular events. Similarly, the survival rate was lower in patients with subclinical hypercortisolism, when compared to non-secreting adenocortical adenomas (31) and to the general population (32), due to an increased cardiovascular mortality and infectious complications.

In summary, while the clinical spectrum of adrenal-dependent hypersecretion of cortisol is wide, it has become quite clear that in most instances adrenal hypercortisolism represents a relevant clinical problem.

**Role of cAMP/PKA signaling in adrenocortical cells**

The second messenger cAMP and its effector PKA are key regulators of virtually all cellular functions, such as growth and differentiation, and mediate the effects of several hormones and neurotransmitters that signal via GPCRs. The discovery of this pathway represented fundamental milestones in cell biology, which culminated with the assignment of three Nobel prizes: the first in 1971 to Earl Sutherland for the discovery of cAMP, the second in 1992 to Edmond Fischer and Edwin Krebs for the discovery of PKA, and the third in 1994 to Alfred Gilman and Martin Rodbell for the discovery of G proteins (33).

PKA is a prototypical serine/threonine kinase and a primary example of allosteric regulation (34). The PKA holoenzyme in its inactive form is a tetramer consisting of a dimer of two regulatory (R) and two catalytic (C) subunits (34). There are three known isoforms of C subunits (Cα, Cβ, Cγ) and four of R subunits (RIα, RIβ, RIIα, RIIβ), each coded by a separate gene (34). In addition to the C subunits, the human protein kinase X also can associate with the R subunits of PKA (35). The regulatory subunits form homo- or heterodimers via the docking and dimerization (D/D) domain (34, 36). This domain is also responsible for the binding of A kinase-anchoring proteins, which tether different PKA isoforms to specific subcellular structures (34).

In the inactive holoenzyme an inhibitory sequence derived from each R subunit occupies the active site cleft of the corresponding C subunit, behaving as a tethered substrate or pseudo-substrate and thus precluding the access of PKA substrates (34). Upon binding of two cAMP molecules to each R subunit, the C subunits are released from the holoenzyme and can phosphorylate their targets localized in the cytosol as well as in the nucleus (37). In adrenocortical cells, this leads to an acute stimulation of glucocorticoid synthesis as well as to a later, transcriptional induction of steroidogenic enzymes and genes involved in cell replication (38, 39) (Fig. 1).

**Genetic defect associated with adrenal Cushing’s syndrome**

A number of genetic defects in the cAMP/PKA pathway have been associated with adrenal diseases (40). The first defect was identified in patients affected by the McCune–Albright syndrome, characterized by bone fibrous dysplasia, café au lait skin spots, and hypersecretion from different endocrine glands. These patients were found to carry mosaic, activating mutations in the gene coding for the Gαs protein (GNAS) (41). A minor fraction of McCune-Albright patients develop Cushing’s syndrome due to adrenal hyperplasia (42). A second genetic defect involves the gene coding for the RIIα subunit of PKA (PRKARIA). PRKARIA mutations are responsible for Carney complex, another multiple endocrine neoplasia syndrome, characterized by the presence of primary pigmented nodular adrenocortical disease, cutaneous and neuronal tumors, cardiac myxomas, as well as characteristic pigmented lesions of the skin and mucosae (43). These mutations cause a reduced expression of the RIIα subunits or impair its association with C subunits, thus leading to constitutive PKA activation (44). More recently,
inactivating mutations in the genes coding for two phosphodiesterases (PDE8 and PDE11A), which are responsible for the degradation of cAMP, have been shown to predispose to the development of adrenal hyperplasia (45, 46, 47, 48).

Considering the importance of impaired cAMP/PKA signaling in the development of adrenal hyperplasia associated with cortisol hypersecretion, the study of those alterations has been extended to sporadic adrenocortical tumors as well. Indeed, somatic mutations of GNAS (9, 10, 11), PRKAR1A (49), and PDE8B (47) have been found also in sporadic adrenocortical adenomas associated with cortisol hypersecretion. However, those mutations were able to explain only a small number of cases, and the molecular pathogenesis of these tumors, which represent a relevant cause of Cushing’s syndrome, remained until recently largely obscure.

PRKACA mutations

With the aim of identifying the underlying genetic alterations, our groups recently performed whole exome sequencing in sporadic cortisol-secreting adrenocortical adenomas (8). We found two mutations in the gene coding for the Cα subunit of PKA (PRKACA) in about 30% of the tumors (8). All mutated adenomas were associated with overt Cushing’s syndrome (8). The more frequent of the two mutations (p.Leu206Arg) resulted in the substitution of a leucine residue at position 206 with arginine; the second mutation (Leu199_Cys200insTrp) caused insertion of a tryptophan residue between the amino acid 199 and 200 (8). Functional experiments revealed that both mutations caused constitutive PKA activation (i.e., PKA activation in the absence of cAMP), thus providing a molecular explanation for the development of cortisol-secreting adrenocortical adenomas (8). These findings were confirmed by three independent studies by other groups, as summarized in Table 2 (9, 10, 11). In a subsequent targeted analysis of the PRKACA gene, we have identified two novel mutations (p.Cys200_Gly201insVal in three adenomas and p.Ser213Arg_Cp.Leu212_Lys214 inslle-Ile-Leu-Arg in one) (50).

All the mutations found in cortisol-secreting adrenocortical adenomas involve amino acids that reside on the surface of the C subunit and are located at the interface with the R subunit (8, 50, 51). Indeed, Leu206 is part of the active site cleft of the Cα subunit and participates in forming a hydrophobic pocket (8, 51). This hydrophobic pocket is responsible for substrate binding and recognition as well as for the interaction with the inhibitory sequence of the R subunit. Therefore, we predicted that the substitution of Leu206 with a bulky and positively charged amino acid such as arginine would lead to steric hindrance between the side chain of this amino acid and residues Val115 and Tyr228 of the regulatory subunit (8, 51) (Fig. 2). This prediction has been well confirmed by a recent X-ray structure of the Leu206Arg mutant (52).

Similarly, residues Leu199 and Cys200 are located next to Thr198, which is part of the so-called ‘activation loop’ and is phosphorylated during the synthesis of the Cα subunit (8, 34, 51). This region of the C subunit is oriented parallel to the inhibitory sequence of the R subunit.

Table 2  Summary of PRKACA somatic mutations identified by next generation sequencing in patients with hypercortisolism.

<table>
<thead>
<tr>
<th>Clinical phenotype (n(%)</th>
<th>Reporting studies</th>
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<tbody>
<tr>
<td></td>
<td>(8)</td>
</tr>
<tr>
<td>Cushing’s syndrome</td>
<td>22 (37%)</td>
</tr>
<tr>
<td>Subclinical hypercortisol</td>
<td>0 (0%)</td>
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www.eje-online.org
Importantly, residues Gly201 and Leu199 are involved in main-chain hydrogen bonding interactions with residues Val115 and Ala117 of the R subunit. Thus, we predicted that an insertion of an additional amino acid at this position might also interfere with the interaction between the R and C subunits (8, 51).

Finally, although the two subsequently identified mutations do not affect amino acids that directly interact with the R subunit, it is likely that they might also indirectly interfere with the formation of a stable PKA holoenzyme. The first of these mutations (p.Leu206Arg) involves a region that is oriented in parallel to the inhibitory loop of the R subunit. The substitution of Leu206 with a bulky and positively charged Arg residue is predicted to result in steric hindrance with the inhibitory sequence (∗) of the R subunit and thus to interfere with the formation of a stable holoenzyme.

![Figure 2](PRKACA mutations identified in cortisol-secreting adrenocortical adenomas interfere with the association between the C and R subunits of PKA. Shown are zoomed-in views of the interface between the R and C subunits based on the structure of the mouse full-length tetrameric RII(2):Ca(2) holoenzyme (Protein Data Bank entry 3TNP). Left, WT. Right, predicted structure of the Leu206Arg mutant. Substitution of Leu206 with a bulky and positively charged Arg residue is predicted to result in steric hindrance with the inhibitory sequence (∗) of the R subunit and thus to interfere with the formation of a stable holoenzyme.)

These findings provide a mechanistic explanation for the constitutive activation of PKA caused by PRKACA mutations, and thus for the development of cortisol-secreting adrenocortical adenomas in the presence of these mutations.

**Genotype–phenotype correlation and interrelation between subclinical hypercortisolism and overt Cushing’s syndrome**

Notably, based on the patient cohorts published to date, the presence of PRKACA mutations is clearly associated with a clinical phenotype of overt Cushing’s syndrome (8, 9, 10, 11). Moreover, even in the subgroup of patients with Cushing’s syndrome, carriers of PRKACA mutations were found to be affected by a more severe endocrine phenotype with higher cortisol secretion at midnight, following dexamethasone suppression or in 24 h urinary collections (8). In contrast although PRKACA mutations in adrenal adenomas associated with subclinical hypercortisolism cannot be excluded, they seem to be extremely rare.

Studies investigating the natural history of subclinical hypercortisolism published in the last 16 years provide...
Table 3  Overview of long-term follow-up studies reporting the rate of conversion from subclinical to clinical hypercortisolism.

<table>
<thead>
<tr>
<th>Reference</th>
<th>Year of publication</th>
<th>Patients with incident-alomas (n)</th>
<th>Time of follow-up (months)</th>
<th>Progression to overt Cushing’s syndrome (n(%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(53)</td>
<td>1998</td>
<td>53</td>
<td>12</td>
<td>0 (0)</td>
</tr>
<tr>
<td>(54)</td>
<td>2001</td>
<td>53</td>
<td>24</td>
<td>0 (0)</td>
</tr>
<tr>
<td>(55)</td>
<td>2002</td>
<td>130</td>
<td>23.5</td>
<td>4 (3)</td>
</tr>
<tr>
<td>(56)</td>
<td>2002</td>
<td>64</td>
<td>25.5</td>
<td>0 (0)</td>
</tr>
<tr>
<td>(57)</td>
<td>2009</td>
<td>51</td>
<td>51.6</td>
<td>3 (6)</td>
</tr>
<tr>
<td>(58)</td>
<td>2009</td>
<td>77</td>
<td>62.7</td>
<td>2 (3)</td>
</tr>
<tr>
<td>(30)</td>
<td>2014</td>
<td>206</td>
<td>82.5</td>
<td>0 (0)</td>
</tr>
<tr>
<td>(31)</td>
<td>2014</td>
<td>198</td>
<td>90</td>
<td>0 (0)</td>
</tr>
</tbody>
</table>

Evidence of a low conversion rate toward clinically evident Cushing’s syndrome. A summary of the main studies analyzing the natural history of subclinical hypercortisolism and its evolution toward overt Cushing’s syndrome is provided in Table 3. This concept raises the hypothesis that the clinical correlates observed in those patients can be mainly related to the time of exposure to mild hypercortisolism. Although those results could be biased by the variability in the clinical recognition of the features of the syndrome among different physicians, it is clear that subclinical hypercortisolism has a slow evolution in terms of cortisol hypersecretion.

Taken together, these clinical observations together with recent findings on the molecular fingerprint of adrenal adenomas support the hypothesis that subclinical hypercortisolism and overt Cushing’s syndrome associated with adrenocortical adenomas could in fact represent two distinct pathological entities. It is tempting to speculate that genetic alterations directly causing cortisol hypersecretion – such as PRKACA mutations – result in a severe phenotype, thus leading to timely clinical recognition and tumor removal. In contrast, genetic – or epigenetic – events that lead to mild hormonal excess are found only incidentally or after prolonged clinical courses and only rarely acquire secondary hits that result in overt clinical symptoms.

Conclusion
The recent application of high-throughput genetic analyses is unraveling the molecular pathogenesis of sporadic cortisol-secreting adrenocortical adenomas, which was until recently largely obscure. Recent studies identified PRKACA mutations as major genetic alterations responsible for the development of these tumors. These data confirm the key role of the cAMP/PKA signaling pathway in stimulating both the function and the proliferation of adrenocortical cells. They provide insights into the development of adrenal hormonal autonomy and may provide the basis for novel approaches to the diagnosis and therapy of adrenal Cushing’s syndrome.

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

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