Steroid hormone production in patients with aldosterone producing adenomas

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Abstract

Primary aldosteronism encompasses two major underlying causes: (1) aldosterone producing adenoma and (2) bilateral adrenal hyperplasia. In addition to the aldosterone excess, increased production of other compounds of the steroidogenic pathways may be involved. Until recently, most studies examined the production of steroids other than aldosterone in tumor tissue, urine or peripheral plasma samples but several new studies have also addressed steroid levels in adrenal venous blood samples using liquid chromatography tandem mass spectrometry. Plasma and tissue levels of several precursors of aldosterone with mineralocorticoid activity are higher in patients with aldosterone producing adenomas than in those with bilateral hyperplasia. These include corticosterone, deoxycorticosterone and their 18-hydroxylated metabolites. Similarly, urinary, peripheral and adrenal venous concentrations of the hybrid steroids 18-oxocortisol and 18-hydroxycortisol are higher in patients with aldosterone producing adenomas than in bilateral hyperplasia.

Differences in the pathophysiology and in clinical and biochemical phenotypes caused by aldosterone producing adenomas and bilateral adrenal hyperplasia may be related to the differential expression of steroidogenic enzymes, and associated to specific underlying somatic mutations. Correct appreciation of differences in steroid profiling between aldosterone producing adenomas and bilateral adrenal hyperplasia may not only contribute to a better understanding of the pathogenesis of primary aldosteronism but may also be helpful for future subtyping of primary aldosteronism.

Keywords: aldosteronism, steroids, adenomas
Introduction

Steroid hormones produced in the adrenal cortex play a pivotal role in human physiology and in pathophysiology of adrenocortical disease. Histologically, the adrenal cortex can be divided into three layers, the zona glomerulosa (ZG), the zona fasciculata (ZF) and the zona reticularis (ZR), producing mineralocorticoids, glucocorticoids and androgens [1]. Aldosterone as a major mineralocorticoid, is synthesized in the ZG, and plays a crucial role in the regulation of electrolyte and fluid homeostasis [2, 3].

$11\beta$-hydroxylase (CYP11B1) and aldosterone synthase (CYP11B2) determine the essential steps in the synthesis of cortisol and aldosterone (Fig. 1). It was previously thought that the expression of these steroidogenic enzymes was linked to functional zonation of the adrenal cortex. However, recent studies using monoclonal antibodies against CYP11B1 and CYP11B2 showed that the distribution of both enzymes is complex and variable [4]. In the normal adrenal cortex, CYP11B1 immunopositive cells, although predominantly present in the ZF, appear to be localized in all cortical layers while CYP11B2 immunopositive cells are scattered or clustered as aldosterone producing cells in the subcapsular ZG [4]. These CYP11B2 positive cells, called aldosterone producing cells, may harbor somatic mutations that are known to be involved in aldosterone secretion [5].

An additional important steroidogenic enzyme for the synthesis of aldosterone and cortisol is the upstream $3\beta$-hydroxysteroid dehydrogenase (HSD3B), which is expressed in both the ZG and ZF of the normal adrenal cortex and converts pregnenolone and 17OH-pregnenolone to progesterone and 17OH-progesterone respectively, thereby providing substrates for the synthesis of aldosterone and cortisol. HSD3B has two isoforms, HSD3B type 1 (HSD3B1) and HSD3B type 2 (HSD3B2) which are expressed in the ZG and ZF, respectively [6].

Dysfunctional mechanisms controlling aldosterone secretion may lead to a syndrome of excess aldosterone production called primary aldosteronism (PA). This syndrome is mainly due to two
subtypes: 1. aldosterone producing adenoma (APA) and 2. bilateral adrenal hyperplasia (BAH) [7, 8]. The clinical features in patients with APAs are usually more severe than those with BAH because aldosterone production by APAs is usually more pronounced than in BAH. Except for aldosterone, tumoral production of other adrenal steroids may occur in both APAs and BAH as outlined below.

Despite our current knowledge of the pathophysiology of PA, the biochemical differentiation between both subtypes of PA remains challenging. Therefore, a better understanding of the pathogenic mechanisms underlying these subtypes and a better identification of novel biomarkers such as specific steroids or steroid patterns may be helpful in the diagnosis and future treatment of PA. This paper reviews the specific patterns of steroid production in APAs but first summarizes the enzymes controlling steroid production in patients with APA, taking into account the recently discovered underlying mutational changes in these tumors.

**Steroidogenic enzyme expression in APAs**

Most APAs consist of tumor cells that resemble the mineralocorticoid producing cells of the ZG while BAH is characterized by bilateral hyperplasia of the adrenal ZG. However, many adrenals with an APA may have a hyperplastic zone while many adrenals with BAH may have nodular hyperplasia with nodules dispersed throughout the ZF [9, 10]. These APAs produce aldosterone as a major compound but also other steroids with mineralocorticoid effects. In addition, the disrupted functional zonation of the APAs may result in cross-utilization of substrates, thus contributing to an increased production of the hybrid steroids 18-oxocortisol (18-oxoF) and 18-hydroxycortisol (18-OHF) [11, 12] (Fig 1).

In patients with APAs, *CYP11B2* mRNA but not *CYP11B1* mRNA levels were higher than in non-functional adenomas [13]. Localization studies using monoclonal antibodies against CYP11B1 and CYP11B2 showed that CYP11B1 immunoreactivity was diffusely dispersed with
CYP11B2 heterogeneously spotted throughout the adenomas and barely detectable in the adjacent non-adenomatous tissue [14]. Semiquantitative assessment of CYP11B2 immunostaining of APAs using different scoring systems resulted in variable results [15]. Taking into account the size of the adenoma, several studies established a positive relationship between the CYP11B2 staining score and plasma or urinary aldosterone levels [10, 16, 17]. In contrast, Nakamura et al. could not establish a stronger immunoreactivity of CYP11B2 than in normal adrenal glands although it was higher in the APA than in the adjacent hyperplastic tissue [14]. In addition to these findings, microarray based gene expression analysis showed that the expression of CYP11B2 in APAs was considerably higher than the ZG of adjacent adrenal tissue [18].

Localization studies of HSD3B1 and HSD3B2 in APAs showed that HSD3B2 mRNA was more abundantly present in APAs than HSD3B1 mRNA and yet it was HSD3B1 mRNA that was correlated with CYP11B2 mRNA [19]. Immunohistochemical studies also documented the presence of HSD3B2 immunopositive cells but again it was HSD3B1 expression that was correlated with that of CYP11B2. Both HSD3B1 and CYP11B2 are co-localized in the same APA cells, thus suggesting that HSD3B1 may also be involved in the production of aldosterone in APAs.

The hyperplastic ZG of BAH demonstrates that HSD3B1 immunoreactivity is markedly increased and this may provide an explanation for the increased aldosterone production by the hyperplastic tissue in patients with BAH. Doi et al. have shown that BAH expresses HSD3B1 but not HSD3B2 suggesting that in contrast to APAs, where HSD3B2 is also associated with increased aldosterone production, HSD3B1 is responsible for the autonomous production of aldosterone in BAH [20, 21].

Sakuma et al. have proposed to take diagnostic advantage of the high HSD3B2 in APAs by using a low CYP17/HSD3B2 ratio combined with a high expression of CYP11B2 as a biomarker.
for the diagnosis of APA [13]. However, additional research is required to establish the benefits of this marker for diagnostic purposes.

All these data suggest that based on their expression and function in steroidogenesis, CYP11B1, CYP11B2 and HSD3B may not only play an important role in the overproduction of aldosterone in APA but also of other steroids. An additional question is if and how the expression of steroidogenic enzymes in APAs is related to the different underlying somatic mutations in the genes that have been identified over the last few years in patients with APAs including, KCNJ5, ATP1A1, ATP2B3, CACNA1D [22]. These genes encode for the G-protein-activated inward rectifier potassium channel 4 (GIRK4), sodium-potassium transporting ATPase subunit α1 (ATP1A1), plasma membrane calcium ATPase, type 3 (PMCA3) and the calcium channel, voltage-dependent, L-type alpha 1d subunit (Ca,1.3), respectively. Mutations in KCNJ5 have been demonstrated to be most prevalent in APA (38%), followed by CACNA1D (9.3%), and ATP1A1 (5.3%) and ATP2B3 (1.7%) [23-26]. Irrespective of the gene affected, all somatic APA mutations lead to an activation of CYP11B2 gene transcription and enhanced aldosterone synthesis.

APAs containing a mutation in KCNJ5 consist mainly of ZF-like cells, whereas adenomas with a mutation in ATP1A1 and CACNA1D have been demonstrated to consist of ZG-like cells [27, 28]. Williams et al. demonstrated that APAs with ATP1A1 and ATP2B3 mutations display increased CYP11B2 expression compared to those with KCNJ5 or no known mutations [26]. Monticone et al. demonstrated that ZG-like adenomas with ATP1A1, ATP2B3 and CACNA1D mutations were characterized by an increased expression of CYP11B2, while ZF-like APAs with KCNJ5 mutations mainly expressed CYP11B1 [16]. This may account for differences in distinctive steroid profiles between APA patients harboring different mutations [29]. Dekkers et al. showed that KCNJ5 mutations were also associated with high CYP11B2 expression, present in both single adenomas, and nodular hyperplasia, while mutations in ATP2B3, and ATP1A1 and CACNA1D were solely found in single adenomas, and nodular hyperplasia, respectively [30].
Steroidogenesis in APA

Qualitative measurements of the enzymatic pathways involved in the production of aldosterone and other steroids in APAs is nowadays addressed by modern immunohistochemical techniques applied to representative tissue slices of adenomas or hyperplastic tissue. Quantitative production of steroids, including aldosterone by adenomas or hyperplasia has been assessed at multiple levels: in adenoma or hyperplastic tissue itself, in peripheral venous blood samples, in urine, and in adrenal venous blood samples. Theoretically, it would be expected that with very sensitive, specific and reliable techniques, measurements of steroids in the tissue itself together with immunohistochemical studies on steroidogenic enzyme expression would be the best way to define the steroid profiles in APAs and BAH. Based on the relative expression levels of \textit{CYP11B1} and \textit{CYP11B2} in the context of the disordered functional zonation of APAs, the direct precursors of aldosterone could possibly be affected in patients with these tumors.

Adenoma tissue

The first studies that reported the aldosterone and steroid content of APAs date back over 50 years. To determine the steroid content and profile in APAs, surgically removed adenomas from patients with typical characteristics of PA, including hypertension and hypokalemia, were investigated. Once dissected, aldosterone and other steroids were extracted from adrenocortical tumors and normal adjacent adrenal gland tissue and separated by chromatography.

In an early study, Biglieri et al. showed that, although the cortisol content of APA tissue was normal, these tumors had a nearly 3-fold elevated content of corticosterone (B) and deoxycorticosterone (DOC) while 11-deoxycortisol was minimally elevated compared to normal adrenal glands [31]. In some patients the B secretion rate was also elevated. Similar findings were described by Louis and Conn [32] and later by Kaplan et al. who demonstrated that APAs not only displayed higher aldosterone levels but also higher B levels compared to normal adrenal glands.
and to adenomas in normotensive and primary hypertensive patients [33]. *In vitro* data on steroid production, obtained after incubating tissue slices from APAs showed that basal levels of B, DOC, and 18-hydroxcorticosterone (18-OHB), were at least two-fold higher in supernatants from APA than from adrenals of control patients [34]. These compounds have marked mineralocorticoid and blood pressure elevating activities. Finally, previous *in vitro* studies reported that both APAs and hyperplastic tissues were capable of secreting other compounds with mineralocorticoid and hypertensive properties in supernatants such as 19-nor-aldosterone (19-nor-aldo), 18-hydroxy-19-nor-corticosterone (18-OH-19-nor-B) and 18,19-dihydroxycorticosterone (18,19(OH)₂-B) [35]. The biological significance of these compounds in patients with PA is not well known but is probably limited.

**Peripheral venous plasma**

Previous *in vitro* data were confirmed by elevations in B, DOC, 18-OHB and 18-OHDOC in peripheral plasma vein samples from PA patients as compared to normal adrenal glands [34]. Most *in vivo* studies have examined steroid concentrations in peripheral venous plasma to investigate steroid production by APAs while some studies used urine assays to analyze steroidogenesis in PA patients [36-40]. Earlier studies have mainly used immunoassays to quantify steroids while more recently liquid chromatography tandem mass spectrometry methods have been used. The latter have a better accuracy for steroid measurements than immunoassays [41-43].

Ulick et al. were the first to recognize the unique urinary steroidogenic profile in patients with PA [11, 40]. The urinary excretion of the hybrid steroids 18-oxoF and 18-OHF were elevated in patients with APAs in contrast to those with BAH. This was later confirmed by Mulatero et al. who also found significantly higher plasma and urinary levels of these hybrid steroids in patients with APAs than with BAH [39]. The same applied for serum 18-OHB. Mosso et al. could confirm that plasma 18-OHF levels were not increased in patients with BAH, thus this is also in line with
previous studies [44]. A very recent study of Satoh et al. also demonstrated that plasma 18-oxoF and 18-OHF were nearly 10-fold and 2-fold elevated respectively in patients with APAs compared to BAH [45]. Of particular clinical relevance was their finding that peripheral plasma 18-oxoF was useful to discriminate APA from BAH patients.

*Adrenal venous plasma*

Adrenal venous sampling is nowadays considered the reference method to determine whether excess aldosterone production in PA patients is unilateral (usually APA) or bilateral (usually BAH). The plasma concentrations of aldosterone and other steroids in adrenal venous blood are considered to be a better reflection of their production by the adrenal glands than the peripheral vein plasma concentrations. Most recent reports on steroid quantification in adrenal venous plasma of patients with PA have used mass spectrometry [43, 46-48].

Several studies have also addressed steroids other than aldosterone, such as 18-OHB and the two hybrid steroids 18-oxoF and 18-OHF [46-48]. Auchus and co-workers showed that, similar to aldosterone, adrenal vein plasma concentrations of 18-OHB were much higher in the vein draining from the diseased gland compared to that draining from the non-diseased contralateral gland [46]. However, 18-OHB was inferior to aldosterone for assessing the lateralization of aldosterone excess.

Nakamura et al. demonstrated in a small study that 18-oxoF levels were elevated in adrenal venous samples from APAs as compared to BAH [47]. Furthermore, the adrenal vein ratio of 18-oxoF/cortisol was helpful in discriminating APA from BAH. In a later study, the same authors showed that 18-oxoF concentrations were lower in the contralateral vein draining from the non-diseased gland than the vein draining from the APA, thus illustrating that 18-oxoF was suppressed as might be expected [48]. The same group of investigators also examined the adrenal vein concentrations of 9 unconjugated C_{19} steroids, estrone, and estradiol. The most pronounced elevations in APA were found for 11β-hydroxyandrostenedione (11OHA),
dehydroepiandrosterone (DHEA) and androstenedione (A4) while active androgens such as testosterone were produced to a lesser degree [49].

Conclusion

Studies using adenoma tissue, urine, peripheral and adrenal vein plasma have consistently shown that APAs in PA patients produce increased amounts of B, DOC, 18-OHB, 18-oxoF and 18-OHF in addition to the excess production of aldosterone. This specific pattern of increased steroid production by APAs may be explained by the dysregulated functional zonation of the enzymes involved in steroid synthesis (CYP11B1, CYP11B2 and HSD3B) in APAs. Underlying genetic mutations associated with different subtypes of PA may play an important role in the functional expression of these enzymes but this is still incompletely understood. It is also not fully clear whether this specific steroid profile associated with APAs will be of use for subtyping PA. With the advent of the more sensitive and specific mass spectrometry for measuring steroids, peripheral steroid biomarkers may prove to have a potential role in differentiating between BAH and APA. Prospective studies in large numbers of patients with different subtypes of PA are needed to validate the utility of steroids other than aldosterone for subtyping different forms of PA.

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Conflict of interest
The authors declare no conflict of interest

Figure 1. Mineralocorticoid and glucocorticoid synthesis in the human adrenal gland.

Cholesterol side chain cleavage enzyme (CYP11A); 3β-hydroxysteroid dehydrogenase (HSD3B2); 17α-hydroxylase (CYP17A1); 21β-hydroxylase (CYP21), 11β-hydroxylase (CYP11B1), aldosterone synthase (CYP11B2).

The hybrid steroids 18-hydroxycortisol and 18-oxocortisol are mainly produced after conversion of 11-deoxycortisol by aldosterone synthase (CYP11B2). The 18-hydroxycortisol can also be produced from cortisol by aldosterone synthase (CYP11B2) but this pathway is much less effective (indicated by the dashed line).

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Fig. 1

Cholesterol
  \( \xrightarrow{\text{Cholesterol side chain cleavage enzyme}} \)
  \( \xrightarrow{\text{þ-hydroxysteroid dehydrogenase}} \)
  \( \xrightarrow{17\alpha\text{-hydroxylase}} \)
  \( \xrightarrow{17\alpha\text{-hydroxylase}} \)
  \( \xrightarrow{21\beta\text{-hydroxylase}} \)
  \( \xrightarrow{11\beta\text{-hydroxylase}} \)
  \( \xrightarrow{18\text{-hydroxylase}} \)
  \( \xrightarrow{18\text{-methyl oxidase}} \)
  \( \xrightarrow{\text{aldosterone synthase (CYP11B2)}} \)

Pregnenolone
  \( \xrightarrow{\text{þ-hydroxysteroid dehydrogenase}} \)
  \( \xrightarrow{17\alpha\text{-hydroxylase}} \)
  \( \xrightarrow{17\alpha\text{-hydroxylase}} \)

Progesterone
  \( \xrightarrow{21\beta\text{-hydroxylase}} \)
  \( \xrightarrow{11\beta\text{-hydroxylase (CYP11B1)}} \)

11-deoxycorticosterone
  \( \xrightarrow{11\beta\text{-hydroxylase}} \)
  \( \xrightarrow{18\text{-hydroxylase}} \)

Corticosterone

18-hydroxycorticosterone

Aldosterone

17-hydroxyprogesterone

11-deoxycortisol