Testosterone inhibits human wild-type and chimeric aldosterone synthase activity in vitro

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Abstract

Background

Familial hyperaldosteronism type I is caused by the generation of a chimeric aldosterone synthase enzyme (ASCE) which is regulated by ACTH instead of angiotensin II. We have reported that in vitro, the wild-type (ASWT) and chimeric aldosterone synthase (ASCE) enzymes are inhibited by progesterone and estradiol did not affect.

Aim

To explore the direct action of testosterone on ASWT and ASCE enzymes.

Methods

HEK-293 cells were transiently transfected with vectors containing the full ASWT or ASCE cDNAs. The effect of testosterone on AS enzyme activities were evaluated incubating HEK-cells transfected with enzymes vectors and adding deoxycorticosterone (DOC) alone or DOC plus increasing doses of testosterone. Aldosterone production was measured by HPLC-MS/MS. Docking of testosterone within the active sites of both enzymes was performed.

Results

In this system, testosterone inhibited ASWT (90% inhibition at five μM, IC50=1.690 μM) with higher efficacy and potency than ASCE (80% inhibition at five μM, IC50=3.176 μM). Molecular modelling studies showed different orientation of testosterone in ASWT and ASCE crystal structures.

Conclusions

The inhibitory effect of testosterone on ASWT or ASCE enzymes is a novel non-genomic testosterone action, suggesting that further clinical studies are needed to assess the role of testosterone in the screening and diagnosis of primary aldosteronism.

Background

Primary aldosteronism (PA) is a known cause of hypertension. Individuals with this condition represent nearly 10% of the hypertensive population, and the prevalence of PA increases with the severity of the hypertensive disease [1]. The high prevalence of PA can be detected using the serum aldosterone/plasma renin activity ratio (ARR) for screening and aldosterone suppression as a confirmatory test (saline infusion, fludrocortisone suppression or captopril). The most frequent subtypes of the disease are idiopathic aldosteronism and aldosterone-producing adenoma. Other less frequent causes of PA are familial variants. Four forms of familial hyperaldosteronism (FH-1 to FH-IV) together with Primary

Aldosteronism, Seizures, Neurological Abnormalities (PASNA) syndrome [2] [3] [4]. FH-I, also called glucocorticoid-remediable aldosteronism, accounts for only 0.5 to 1% of PA [5] .

A hallmark of familial hyperaldosteronism type I is the presence of a chimeric aldosterone synthase enzyme, which is formed by unequal crossing-over of the genes that encode 11β-hydroxylase (CYP11B1), and aldosterone synthase (CYP11B2) enzymes. These genes are 95% identical in nucleotide sequence [5–8]. The 11β-hydroxylase enzyme (CYP11B1 gene) is normally expressed in both: human adrenal fasciculate and glomerulose zones, catalyzes the biosynthesis of cortisol and aldosterone, respectively. In the fasciculate, this gene is regulated by adrenocorticotropic hormone (ACTH). The aldosterone synthase (CYP11B2 gene) is typically expressed only in the adrenal glomerulose, and its product catalyzes the final two steps of aldosterone biosynthesis, and it is regulated by angiotensin II. The generation of the chimeric enzyme (CYP11B1/CYP11B2 gene) results in ectopic expression of aldosterone synthase in the fasciculate zone which is regulated by ACTH instead of angiotensin II, causing severe hypertension, variable hyperaldosteronism, low plasma renin activity, and normal or decreased potassium.

In recent years, some evidence has indicated that female sex steroids may modify aldosterone levels and the serum aldosterone/plasma renin activity ratio (ARR) used in screening for primary aldosteronism (PA). In women in the luteal phase, aldosterone concentrations increase, which could give a false positive in the screening and confirmatory tests for PA [9]. Moreover, our previous study in a pregnant woman carrying familial hyperaldosteronism type I demonstrated an improvement in blood pressure, concomitant with the normalization of ARR. Following childbirth, progesterone and estradiol decreased, aldosterone increased, plasma renin activity was suppressed, and ARR was very high [10]. These observations support our previously reported in vitro study in which both the wild-type and chimeric aldosterone synthase enzyme activities were inhibited by progesterone, but estradiol demonstrated no effect [11].

On the other hand, little information on the role of male hormones in aldosterone synthase activity is available, although some authors have reported that males have a higher blood pressure than women [12]. Few studies have analyzed the effect of testosterone on aldosterone production, and the majority of these studies were performed using animal or experimental models. During our study with a male index case carrying FH-I and his pedigree consisting of 4 generations, we observed that aldosterone and ARR decreased with age [10]. Based on this family observation, we postulated that changes in male gonadal hormones observed during the transition from childhood to adulthood might also alter aldosterone levels, which in turn might explain the normalization of ARR in adulthood.

We had previously assessed the direct action of progesterone and estradiol on wild-type and chimeric aldosterone synthase activities using HEK-293 cells transiently transfected with wild-type aldosterone synthase or CYP11B1/CYP11B2 chimeric enzymes. Aldosterone production was determined using deoxycorticosterone (DOC) as a substrate. In this system, we demonstrated that progesterone inhibited wild-type aldosterone synthase with similar efficacy and higher potency than the chimeric enzyme, while estradiol had no effect on any of the enzymes [11]. Using these models, in this work, we explore the direct

action of testosterone on wild-type aldosterone synthase (ASWT) and chimeric aldosterone synthase (ASCE).

Methods

Reagents and cells

An in vitro assay using HEK-293 cells transiently transfected with a vector containing the promoter for cytomegalovirus (PCMV) and either ASCE or ASWT cDNA for the aldosterone synthase enzyme was developed as previously described9. In brief, the chimeric CYP11B1/B2 gene used in this assay consisted of a fusion of exons 1 to 3 of CYP11B1 (1-573 bp) and exons 4 to 9 of CYP11B2 (574–1512 bp). Transfection efficiencies were analyzed by counting cells that express the green fluorescent protein (pZsGreen1-n1, Clontech, California, USA) used as a marker of transfection efficiency as we described in a previous study (11). The transfected efficiency was comparable between the different constructs.

The mRNA expression levels of ASCE and ASwt in transfected HEK-293 cells were similar as we described in a previous study (Fig. 1B,) (11). In brief, it was evaluated by qRT-PCR using Maxima SYBR (Thermo Scientific, California, USA) (11). The mRNA expression was quantified by ΔΔCt method relative to that of GAPDH (13).

The activity of both enzymes was evaluated incubating, HEK-293 cells transfected with PCMV-CYP11B1, PCMV-CYP11B1/B2, and PCMV-CYP11B2 with increasing concentrations of deoxycorticosterone as the substrate (DOC, Steraloids Inc., Andover, MA, USA). Aldosterone production was quantified by HPLC-MS/MS (Agilent 1200, ABI Sciex API 4000 Qtrap). The apparent kinetic parameters obtained were Km = 1.163 μM and Vmax = 36.98 μM/24 h for ASWT and Km = 1.191 μM and Vmax = 27.08 μM/24 h for the ASCE (11). In this system, we analyzed the effect of testosterone (Steraloids Inc., Andover, MA, USA) on the ASWT and ASCE enzymes.

Molecular modeling of CYP11B2 and CYP11B1/B2 chimeric enzymes and steroid docking. To examine the potential binding mode of testosterone within the active site of ASWT and ASCE enzymes, we first used the crystal structure of human ASWT in complex with DOC (PDB id 4DVQ) to generate an ASCE model (14). Comparative modelling was performed using the MODELLER program implemented in the Build Homology Models protocol in Discovery Studio v2.1 (Accelrys Inc., San Diego, USA). MarvinSketch (ChemAxon, Budapest, Hungary) was used to draw and generate a 3D model of testosterone (https://chemaxon.com/products/marvin)(13). The docking of testosterone within the active sites of both enzymes was performed using FRED v3.2.0.2 (OpenEye Scientific Software, Santa Fe, NM), and the solutions were ranked according to the ChemGauss 4 scoring function (https://www.eyesopen.com/) (15).

Data analysis. Data are expressed as mean with SEM, Differences between means were analyzed by repeated-measures ANOVA and Tuckey as post hoc test. Statistical analysis was performed using Prism v5.03 program (GraphPad Software, Inc.) Differences were considered significant at $p < 0.05$.

Results

The effect of testosterone (0 to 10 μM) on ASWT and ASCE activities was evaluated in our bioassay using 1.5 μM DOC as the substrate (Fig. 3, panel A). Testosterone inhibited wild-type aldosterone synthase (ASWT, 90% inhibition at five μM, IC50 = 1.690 μM) with higher efficacy and potency than the chimeric enzyme (ASCE, 80% inhibition at five μM, IC50 = 3.176 μM).

To explore the putative binding mode of testosterone on the active sites of both enzymes, we performed docking simulations. Figure 1, panels B and C show the most favourable predicted binding modes obtained for testosterone within the ASWT and ASCE active sites. Our results indicate that testosterone displays a binding mode similar to that observed for DOC in the ASWT crystal structure. However, within the ASCE active site, testosterone binds in an inverse orientation with respect to DOC, with the 17-hydroxy group facing the polar pocket but failing to establish any hydrogen bond interaction.

Discussion

Our results show that testosterone inhibits the activities of the wild-type and chimeric aldosterone synthase enzymes in vitro. Testosterone showed higher efficacy for ASWT, with similar potency but lower efficacy for ASCE. These findings were similar to the results described for progesterone using the same bioassay (11). However, testosterone displayed higher potency and similar efficacy to progesterone for ASWT and higher potency and lower efficacy for ASCE.

The docking studies predicted that the binding mode of testosterone is similar to the binding mode of progesterone in ASWT. However, testosterone binds in an opposite orientation to progesterone within the ASCE active site, with its

17-hydroxy group facing the polar pocket and failing to establish any hydrogen bond interaction, thereby displaying a lower inhibitory capacity. The remaining interactions were very similar to those published for progesterone (11).

Currently, there is limited information about the effect of testosterone on aldosterone biosynthesis. Although a few studies have analyzed the effect of this hormone on aldosterone production, the majority were performed in animal or experimental models. Testosterone and the synthetic androgen methylandrostenediol have been reported to decrease the expression of cytochrome P-450 11β mRNA in the adrenal mitochondria of female rats (16). Kau et al. reported that the plasma aldosterone concentration was higher in ovariectomized (Orx) rats without testosterone replacement and demonstrated in vitro that testosterone caused a marked decrease in aldosterone secretion by zona glomerulosa cells (17). Later, Ajdžanović et al. communicated in Orx middle-age Wistar rats that the volume of zone glomerulose cells and nuclei increased significantly in Orx treated with testosterone animal by 50% and 25% ($p < 0.05$) respectively, but the serum concentration of aldosterone decreased by 60% ($p < 0.05$), all compared to the same parameters in Orx group (18). Besides, Hakki et al. used the recombinant fission yeast strain MB164, which expresses human CYP11B2, identified two testosterone

analogues as CYP11B2 inhibitors. One of these compounds (4-androstene-3,17-dione) is a testosterone precursor that displays an IC50 of 3.11 μM for human ASWT (19). Recently, More et al. reported that in six-month-old female rats prenatally exposed to testosterone, the CYP11B2 mRNA levels decreased by 40% compared to the controls (20) and Carsia et al communicate that in dispersed adrenocortical cells from ovariectomized lizard the basal rate of aldosterone production increased by 166%, respect to intact-male cells. The addition of testosterone reverted this effect (21).

Our results show an in vitro inhibitory effect of testosterone on aldosterone synthesis by ASWT or ASCE enzyme. In agreement with previously reported results in animal and cellular models, we have demonstrated that high testosterone levels reduce aldosterone synthesis. This effect is a novel regulatory mechanism of testosterone action, suggesting that further clinical studies are needed to assess the role of testosterone in the screening and diagnosis of primary aldosteronism.

Conclusion

The inhibitory effect of testosterone on ASWT or ASCE enzymes is a novel non-genomic testosterone action, suggesting that further clinical studies are needed to assess the role of testosterone in the screening and diagnosis of primary aldosteronism.

Abbreviations

ACTH: Adrenocorticotropic hormone; ARR:Aldosterone/Renin Ratio; AS_{WT}:Wild-Type Aldosterone Synthase; AS_{CE}:Chimeric Aldosterone Synthase; CYP11B1:Steroid 11β-hydroxylase; CYP11B2:Steroid 18 β-hydroxylase; FH:Familial Hyperaldosteronism; HPLC-MS/MS:High-Performance Liquid chromatography-tandem mass spectrometry; PA:Primary Aldosteronism.

Declarations

Ethics approval and consent to participate:

This article does not contain any studies with human participants or animals performed by any of the authors. N/A

Consent for publication:

The authors confirms that review and approve the final version of the paper.

Availability of data and materials:

availability to data will be through a letter request to the corresponding author

Competing interests:

The authors declare that they have no conflict of interest. The authors alone are responsible for the content and writing of this paper.

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Authors' contributions:

AV, CF, FA made substantial contributions to the conception and design of the experiments and acquisition of data. AV, CF and CAC performed the chimeric design, plasmid amplification, transfection and cellular manipulation. FA and AT participated in the measuring of aldosterone content by HPLC-MS/MS. AV and CC participated in the Western blot result. CFL performed the molecular modelling and analysed the data. AV, CFL, CC, CAC and CEF made substantial contributions to the analysis and interpretation of data. AV, CC and CEF were involved in drafting the manuscript. All authors read and approved the final manuscript.

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Figures

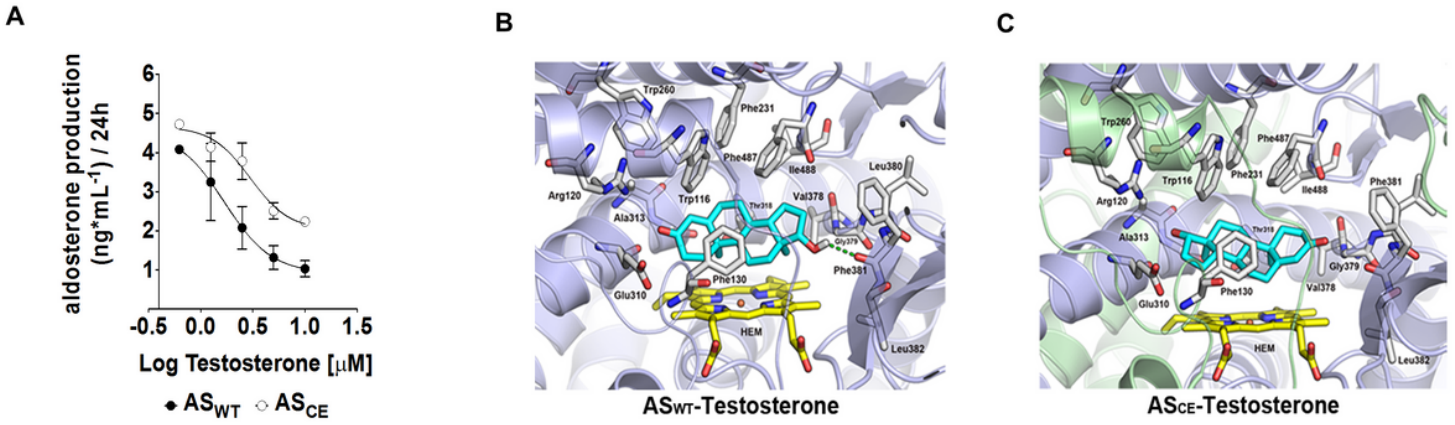

Figure 1

Inhibitory effects of testosterone on wild-type aldosterone synthase (ASWT) and chimeric aldosterone synthase (ASCE). Panel A: Effect of testosterone on aldosterone production by wild-type aldosterone synthase (ASWT, black circles) and chimeric aldosterone synthase (ASCE, white circles). The calculated

IC50 was 1.690 µmol/L for ASWT and 3.176 µmol/L for ASCE. Data are expressed as the mean ± S.E.M. of 4 independent experiments. Panel B: Binding mode of testosterone (light blue) at the active site of the wild-type aldosterone synthase enzyme. Panel C: Binding mode of testosterone (light blue) at the active site of the chimeric aldosterone synthase enzyme.