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Biotransformation of the mineralocorticoid receptor antagonists spironolactone and canrenone by human CYP11B1 and CYP11B2: Characterization of the products and their influence on mineralocorticoid receptor transactivation

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ABSTRACT

Spironolactone and its major metabolite canrenone are potent mineralocorticoid receptor antagonists and are, therefore, applied as drugs for the treatment of primary aldosteronism and essential hypertension. We report that both compounds can be converted by the purified adrenocortical cytochromes P450 CYP11B1 and CYP11B2, while no conversion of the selective mineralocorticoid receptor antagonist eplerenone was observed. As their natural function, CYP11B1 and CYP11B2 carry out the final steps in the biosynthesis of gluco- and mineralocorticoids. Dissociation constants for the new exogenous substrates were determined by a spectroscopic binding assay and demonstrated to be comparable to those of the natural substrates, 11-deoxycortisol and 11-deoxycorticosterone. Metabolites were produced at preparative scale with a CYP11B2-dependent *Escherichia coli* whole-cell system and purified by HPLC. Using NMR spectroscopy, the metabolites of spironolactone were identified as 11β-OH-spironolactone, 18-OH-spironolactone and 19-OH-spironolactone. Canrenone was converted to 11β-OH-canrenone, 18-OH-canrenone as well as to the CYP11B2-specific product 11β,18-diOH-canrenone. Therefore, a contribution of CYP11B1 and CYP11B2 to the biotransformation of drugs should be taken into account and the metabolites should be tested for their potential toxic and pharmacological effects. A mineralocorticoid receptor transactivation assay in antagonist mode revealed 11β-OH-spironolactone as pharmaceutically active metabolite, whereas all other hydroxylation products negate the antagonist properties of spironolactone and canrenone. Thus, human CYP11B1 and CYP11B2 turned out to metabolize steroid-based drugs additionally to the liver-dependent biotransformation of drugs. Compared with the action of the parental drug, changed properties of the metabolites at the target site have been observed.

1. Introduction

Aldosterone represents the main human mineralocorticoid. It regulates renal water and sodium retention as well as potassium secretion via the mineralocorticoid receptor (MR) and thereby controls water and electrolyte homeostasis, which directly projects onto blood pressure [1–4]. The MR is a ligand-activated transcription factor inducing genomic effects upon mineralocorticoid binding, subsequent translocation into the nucleus and regulation of transcription [2,5]. In humans, aldosterone is synthesized from 11-deoxycorticosterone (DOC) in the *Zona glomerulosa* of the adrenal cortex by a member of the cytochrome P450 superfamily (CYP, P450), CYP11B2, that catalyzes a reaction sequence consisting of 11β- and 18-hydroxylations followed by an 18-oxidation [6–10]. The other member of the human CYP11B subfamily, CYP11B1, which shares 93% sequence identity with CYP11B2 on protein level, synthesizes the major human glucocorticoid cortisol in the adrenal *Zona fasciculata* by 11β-hydroxylation of 11-deoxycortisol (RSS) [6–8,11,12]. Both CYP11B isoforms are

Abbreviations: AdR, adrenodoxin reductase; Adx, adrenodoxin; DOC, 11-deoxycorticosterone; *E. coli*, *Escherichia coli*; MR, mineralocorticoid receptor; MRA, mineralocorticoid receptor antagonist; NMR, nuclear magnetic resonance; RSS, 11-deoxycortisol (Reichstein's substance S).

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located in the inner mitochondrial membrane and require NADPH as electron donor for their catalysis as well as an electron transfer system consisting of the NADPH-dependent adrenodoxin reductase (AdR) and adrenodoxin (Adx), which transfers electrons to the P450 for the activation of molecular oxygen [13].

Aldosterone induced activation of the MR is a key step in the development of hypertension and resultant cardiac and renal abnormalties [14]. In addition, MR activation in cardiac tissues also seems to play a direct role in cardiac fibrosis [3]. Consecutively, the MR represents an attractive drug target for blood pressure reducing therapies and mineralocorticoid receptor antagonists (MRAs) have been used successfully for decades in the treatment of primary aldosteronism and essential hypertension as well as congestive heart failure [15–20]. Spironolactone, which has been clinically applied since the early 1960s, is still a widely used MRA and has been shown to reduce the risks of morbidity and mortality among patients with severe heart failure [17]. Upon administration, it is rapidly dethioacetylated to its principal pharmacologically active metabolite canrenone [21–23]. However, the use of spironolactone is limited by its non-selective receptor binding properties, which result in significant adverse effects, most of them being sex-related due to binding to the androgen receptor [24,25]. As a consequence, the development of more selective MRAs, such as eplerenone, is of great interest to reduce steroid receptor cross-reactivity [18,19,26].

During the 1970s, several studies observed an interference of the MRAs spironolactone and canrenone with corticosteroid biosynthesis in rat adrenal tissue and mitochondrial preparations from bovine and human [27–29]. Furthermore, binding of both compounds to P450s leading to a type-I difference spectrum and the formation of hydroxylated MRA metabolites was described [28,29]. As these studies were performed with tissue-derived preparations and the availability of high and pure quantities of each of the corticosteroid synthesizing P450s was not given at that time, neither the metabolites nor the particular P450s responsible for their formation could be identified. Metabolite identification and characterization is, however, crucial for drug design and development due to potential toxicity and pharmacological diversification. The recombinant expression of the two human mitochondrial steroidogenic P450s CYP11B1 and CYP11B2 in *Escherichia coli* (*E. coli*) was finally achieved during the last years, which now enables an efficient purification and application for biotransformations in preparative scale [9,11,30]. According to the traditional P450 classification, these isozymes are counted as nondrug-metabolizing P450s and are presumed to display a narrow substrate spectrum, which is restricted to their natural function in steroid hormone biosynthesis [31,32]. Nevertheless, very recently a few exogenous substrates were described for the CYP11B subfamily [33–35] demonstrating their ability for the biotransformation of exogenous compounds. This study presents a new metabolic pathway for spironolactone and canrenone catalyzed by CYP11B1 and CYP11B2. Metabolism is demonstrated in a reconstituted *in-vitro* system and emerging metabolites were produced at preparative scale by *E. coli* based whole-cell bioconversions for structural elucidation by nuclear magnetic resonance (NMR) spectroscopy. Alterations of their bioactivity concerning the antagonist properties for the MR were studied in a transactivation assay.

2. Material and methods

2.1. Chemicals

All reagents were purchased from standard sources with the highest purity available. Steroids were purchased from Sigma-Aldrich.

2.2. Expression and purification of human CYP11B1 and CYP11B2

Human CYP11B1 and CYP11B2 were expressed in *E. coli* C43 (DE3) using constructs described [9,11]. The expression was performed in 2.8 L Fernbach flasks containing 400 mL TB medium (24 g technical yeast extract, 12 g peptone, 4 mL glycerol, 4.6 g KH_2PO_4, 25 g K_2HPO_4) supplemented with 100 μg/mL ampicillin and 50 μg/mL kanamycin. The expression culture was inoculated with an overnight culture and incubated at 37 °C and 210 rpm until an OD_{600nm} of 0.5 was reached. Protein expression was induced with 1 mM isopropyl β-D-1-thiogalactopyranoside, 4 mg/mL arabinose, 1 mM δ-aminolevulinic acid and 50 μg/mL ampicillin. Further incubation was carried out at 27.5 °C and 180 rpm for 24 h. Cells were harvested by centrifugation and sonicated in lysis buffer (50 mM potassium phosphate (pH 7.4), 20% glycerol, 500 mM sodium acetate, 1.5% sodium cholate, 1.5% Tween 20, 0.1 mM phenylmethylsulfonylflourid, 0.1 mM dithiothreitol). Cell debris was removed by ultracentrifugation (30 min, 35.000 rpm, 4 °C) and the P450 was purified from the supernatant by immobilized metal ion affinity chromatography and cation exchange according protocols established previously [9,11].

Concentrations of the purified cytochromes P450 were determined by reduced carbon-monoxide difference spectroscopy with an extinction coefficient $\varepsilon_{450nm} = 91\ mM^{-1}\ cm^{-1}$ [36].

2.3. Purification of redox partners

Human AdR [37,38] and Adx [39,40] were expressed in *E. coli* and purified as previously described. Concentrations were determined with $\varepsilon_{450nm} = 11.3\ mM^{-1}\ cm^{-1}$ mM for AdR and $\varepsilon_{414nm} = 9.8\ mM^{-1}\ cm^{-1}$ for Adx.

2.4. In-vitro *substrate conversion*

For *in-vitro* substrate conversion assays, 400 μM of the respective MRA was incubated with the P450 system that was reconstituted with the natural human redox partners, AdR and Adx. The reaction mixture (0.5 mL) consisted of 0.5 μM CYP11B1 or CYP11B2, 0.5 μM AdR and 10 μM Adx in 50 mM HEPES buffer (pH 7.4) supplemented with 20% glycerol and 100 μM 1,2-Didodecanoyl-sn-glycero-3-phosphocholine. Furthermore, a NADPH-regeneration system consisting of 5 mM glucose-6-phosphate, 4 U/mL glucose-6-phosphate dehydrogenase and 1 mM $MgCl_2$ was added. The reaction was started by adding 1 mM NADPH, incubated at 37 °C under shaking and stopped after 1 h by adding 2 reaction volumes of chloroform.

2.5. Reversed phase HPLC analysis

For the analysis of the product pattern via HPLC, samples were extracted twice with two volumes of chloroform, whereupon the chloroform phase was evaporated and the remaining steroids were suspended in acetonitrile for HPLC analysis. Steroids were separated on a Jasco reversed phase HPLC system of the LC900 series (Jasco, Groß-Umstadt, Germany) using a 4.6 mm × 125 mm NucleoDur C18 Isis Reversed Phase column (Macherey-Nagel, Düren, Germany) with an acetonitrile/water gradient (Phase A: 10% acetonitrile in water, Phase B: 100% acetonitrile; 0 min 20% B, 5 min 20% B, 13 min 40% B, 20 min 80% B, 21 min 80% B, 22 min 20% B, 30 min 20% B) at 40 °C and a flow rate of 0.8 mL/min. Steroids were detected by an UV/Vis detector (UV-2 075 Plus, Jasco) at 240 nm (for spironolactone) or 288 nm (for canrenone).

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