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Urinary cysteinyl progestogens: Occurrence and origin

Josep Marcos [a,b], Marta Pol [a], Andreu Fabregat [a], Rosa Ventura [a,b], Nuria Renau [a], Felicia A. Hanzu [c], Gregori Casals [d], Santi Marfà [d], Bernardí Barceló [e], Antonia Barceló [e], Juan Robles [e], Jordi Segura [a,b], Oscar J. Pozo [a,*]

[a] Bioanalysis Research Group, IMIM, Hospital del Mar, Doctor Aiguader 88, 08003 Barcelona, Spain
[b] Department of Experimental and Health Sciences, Universitat Pompeu Fabra, Doctor Aiguader 88, 08003 Barcelona, Spain
[c] Department of Endocrinology and Nutrition, Hospital Clinic, Laboratory of Endocrine Disorders, IDIBAPS, University of Barcelona, Barcelona, Spain
[d] Biochemistry and Molecular Genetics Department, Hospital Clínic, IDIBAPS, CIBERehd, Barcelona, Spain
[e] Servei d'Anàlisis Cliniques Hospital Universitari Son Espases, Palma de Mallorca, Spain

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ABSTRACT

The presence of two cysteinyl progestogens, 16-cysteinyl-progesterone (16-Cys-Prog) and 16-cysteinyl-pregnenolone (16-Cys-Preg), in human urine is described for the first time. Their occurrence was unequivocally confirmed by comparison with synthesized material by using mass spectrometric detectors. Several experiments were performed in order to clarify their origin. The adrenal origin of both 16-Cys-Prog and 16-Cys-Preg can be inferred from the increase in their concentrations after ACTH stimulatory test, together with their circadian variation similar to the one observed for cortisol.

Moreover, the notable increase in excretions of 16-Cys-Prog during the luteal phase of the menstrual cycle points towards an ovarian production for this progestogen. However, the analysis of samples during the course of two pregnancies revealed that, in spite of the large amounts of progesterone produced during gestation, the human placenta lacks the capacity to make 16-Cys-Prog. The adrenal and ovarian origin has been further indicated by the absence of both metabolites in samples collected from a subject with bilateral adrenalectomy and hypogonadotrophyic hypogonadism.

Regarding liver action, *in vitro* studies with hepatocytes and progesterone indicate that, although the liver is able to metabolize progesterone to 6-dehydroprogesterone, it has not the enzymatic machinery for the generation of 16-dehydroprogesterone. Taken together, these results open the possibility for a noninvasive test for the simultaneous evaluation of progesterone biosynthesis in different organs.

1. Introduction

The first step in steroidogenesis is the conversion of cholesterol to pregnenolone by the cholesterol side-chain cleavage enzyme P450scc (CYP11A1) [1]. A cell is said to be steroidogenic if it expresses P450scc, which is located in the mitochondria [2]. Pregnenolone is mainly produced by the adrenal glands, but other organs like the brain and the skin are also important sources of pregnenolone [3,4].

Once pregnenolone is produced from cholesterol, it may be converted to progesterone. A microsomal enzyme, 3β-HSD (3β-hydroxysteroid dehydrogenase), catalyzes both conversion of the hydroxyl group to a keto group on C3 and the isomerization of the double bond from the B ring (Δ^5 steroids) to the A ring (Δ^4 steroids) [5,6]. In humans, two isoforms are present. The 3β-HSD type 1 catalyzes the activity in placenta, breast, liver, brain, and some other tissues [7,8]. This isoform is required for placental progesterone production during pregnancy, which may explain why a deficiency of 3β-HSD type 1 has never been described. In contrast, the type 2 enzyme (3β-HSD2) is the principal isoform in the adrenals and gonads [7,9].

Progesterone, the major progestogen in the body, is involved in the menstrual cycle, pregnancy, and embryogenesis of humans [10]. Progesterone is also a crucial metabolic intermediate in the production of other endogenous steroids and plays an important role in brain function as a neurosteroid. It is produced in high amounts in the ovaries (by the *corpus luteum*) from the onset of puberty to menopause, and is also produced in smaller amounts by the adrenal glands after the onset of adrenarche in both males and females [11]. To a lesser extent, it is also produced in the adipose tissue, and in the brain [12]. In addition, by the eighth week of pregnancy, the human placenta replaces the *corpus luteum* as the

* Corresponding author. Tel.: +34 933160472; fax: +34 933160499.
E-mail address: opozo@imim.es (O.J. Pozo).

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primary source. The levels of progesterone produced by the placenta will increase rapidly throughout the remainder of pregnancy [13].

The main phase I metabolites of progesterone are known for decades [14]. They arise from three ways; first, by means of 20α- and 20β-HSD which results in the active 20α- and 20β-reduced progestenolones. Secondly, by D4 reduction, the products being 5α- and 5β-pregnane-3,20-diones, and thirdly by reduction at C3 by means of 3α- and 3β-HSD, resulting in several isomeric pregnanolones such as 3α-hydroxy-5β-pregnan-20-one. When the reduction takes place at the three sites, several pregnanediols are formed. Among them, the metabolite excreted in higher amount is 5β-pregnane-3α,20α-diol (PD) [15]. In urine, they are mainly excreted in the form of glucuronides and sulfates, and in a lesser extend in the form of *N*-acetylglucosaminides [16].

The identification of all these biotransformations has been possible through many analytical techniques, mass spectrometry being the most relevant for both the unequivocal elucidation of the structure of the metabolic products and their quantitation in biological samples [17,18]. The use of hyphenated techniques, first with gas chromatography [19,20], and lately with liquid chromatography [21–23] has revealed the general picture of steroid metabolism in humans. However, this complex picture is far from being completed as proved by the discoveries of many recent investigations [24–28].

As a result of a recent research, a novel form of excretion has been described for testosterone and other steroid hormones having a Δ^4-3-keto structure *e.g.* progesterone and cortisol [29]. The first step of the postulated metabolic pathway is a phase I reaction that leads to the formation of a polyunsaturated Δ^6 steroid. In a phase II reaction 7α-cysteinyl conjugates are formed by the reaction between the thiol moiety (either coming from cysteine or from glutathione residues) and the electrophilic Δ^6-testosterone and Δ^6-progesterone [30].

In the case of steroid hormones having a keto group at C20, such as pregnenolone and progesterone, the formation of Δ^{16}-steroid may generate another active electrophilic site able to be conjugated with cysteine.

The goals of the present study are to investigate the presence of 16-cysteinyl progestogens in human urine and to shed light on their biological origin. For these purposes, 16-cysteinyl progestogens have been synthesized, studied by mass spectrometry and the change in their excretions under several circumstances has been evaluated.

2. Materials and methods

2.1. Chemicals and reagents

Pregn-4,16-diene-3,20-dione (Δ^{16}-Prog), 3β-hydroxypregn-5,16-dien-20-one (Δ^{16}-Preg) and 5β-pregnan-3α,20α-diol (pregnanediol, PD) were obtained from Steraloids Inc. (Newport, USA). Cortisol and d4-cortisol were obtained from Sigma–Aldrich (St. Louis, MO, USA). Cysteine (Cys), trifluoroacetic acid (TFA) and all other chemicals were purchased from Sigma–Aldrich Química S.A. (Madrid, Spain). Unless otherwise stated, all the chemicals were used without further purification.

The β-glucuronidase preparation (from *Escherichia coli* type K12) was purchased from Roche Diagnostics (Mannheim, Germany). Analytical grade potassium carbonate, hydrochloric acid, di-sodium hydrogen phosphate, sodium hydrogen phosphate, *tert*-butyl-methyl ether, ammonium iodide, sodium hydroxide, acetonitrile and methanol (LC gradient grade), formic acid, ammonium formate (LC–MS grade), ethyl acetate, and cyclohexane were obtained from Merck (Darmstadt, Germany). *N*-Methyl-*N*-trimethylsilyl-trifluoroacetamide (MSTFA) was from Karl Bucher Chemische Fabrik GmbH (Waldstetten, Germany) and 2-mercaptoethanol was from Sigma–Aldrich (St. Louis, MO, USA). Ultrapure water was obtained using a Milli-Q purification system (Millipore Ibérica, Barcelona, Spain).

Dulbecco's modified Eagle's medium (DMEM), penicillin, streptomycin and L-glutamine were purchased from Life Technologies (Alcobendas, Madrid, Spain). Fetal bovine serum (FBS) medium and Hank's balanced salt solution (HBSS) were purchased from PAA laboratories (Houdstone Business Park, UK). HepG2 cells were obtained from the American Type Culture Collection (Manassas, VA, USA).

2.2. Synthesis of cysteinyl progestogens

The synthesis of 16-cysteinyl progestogens was based on the published procedure for other cysteinyl steroids [27]. The general scheme of the synthesis is shown in Fig. 1. Each progestogen (1 equiv.) was dissolved in 3 volumes of MeOH and the solution was

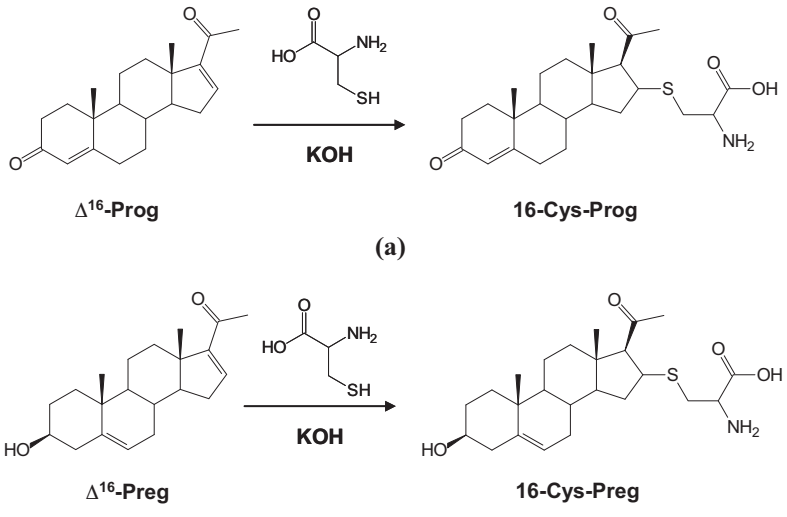

Fig. 1. Scheme for the synthesis of (a) 16-Cys-Prog and (b) 16-Cys-Preg.