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INHIBITORY EFFECT OF SYNTHETIC PROGESTINS, 4-MA AND CYANOKETONE ON HUMAN PLACENTAL 3β-HYDROXYSTEROID DEHYDROGENASE/5→4-ENE-ISOMERASE ACTIVITY

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Summary—Human placental 3β-hydroxysteroid dehydrogenase/5→4-ene isomerase (3β-HSD) purified from human placenta transforms C-21 (pregnenolone and 17α-hydroxy pregnenolone) as well as C-19 (dehydroepiandrosterone and androst-5-ene-3β, 17β-diol) steroids into the corresponding 3-keto-4-ene-steroids and is thus involved in the biosynthesis of all classes of hormonal steroids. Trilostane, epostane and cyanoketone are potent inhibitors of 3β-HSD with K_i values of approximately 50 nM. 4-MA, a well known 5α-reductase inhibitor, is also a potent inhibitor of 3β-HSD with a K_i value of 56 nM. Synthetic progestin compounds such as promegestone and RU2323 show relatively strong inhibitory effects with K_i values of 110 and 190 nM, respectively. Cyproterone acetate, a progestin used in the treatment of hirsutism, acne and prostate cancer as well as norgestrel and norethindrone that are widely used as oral contraceptives also inhibit 3β-HSD activity at K_i values of 1.5, 1.7 and 2.5 μM, respectively.

INTRODUCTION

In mammalian steroid-producing tissues, the conversion of 3β-hydroxy-5-ene-steroids to 3-oxo-4-ene-steroids is catalyzed by the membrane-bound enzyme complex: 3β-hydroxysteroid dehydrogenase (EC 1.1.1.145) and 5→4-ene-isomerase (EC 5.3.3.1), hereafter called 3β-HSD [1]. This enzymatic complex is the rate-limiting step in the conversion of pregnenolone to progesterone, 17α-hydroxypregnenolone to 17α-hydroxyprogesterone, dehydroepiandrosterone (DHEA) to androstenedione and 5-androstene-3β,17β-diol to testosterone.

In human placenta, 3β-HSD has been found in both microsomes and mitochondria where it catalyzes the production of progesterone from maternal pregnenolone and androstenedione from fetal and maternal dehydroepiandrosterone sulfate [2]. Androstenedione is further converted into estrone and estradiol via aromatase and 17β-hydroxysteroid dehydrogenase [3]. Inhibition of the placental enzyme by various steroids has been suggested as playing an important role in the control of estrogen and progesterone synthesis in the placenta [4, 5].

The enzymatic activity of 3β-HSD, in addition to being required for the synthesis of all classes of steroids, could well be involved in the preferential transformation of precursor steroids into mineralocorticoids, progestins, glucocorticoids or sex steroids. Moreover, there is still debate about the presence of one or more 3β-HSD(s) and clinical data suggest the existence of more than one 3β-HSD in humans [6]. The existence of three different substrate-specific 5→4-ene-isomerases has been suggested in bovine adrenals [7]. In order to have a better knowledge about 3β-HSD, we have purified the enzyme from human placenta and performed detailed inhibition studies using

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Abbreviations: Cyanoketone, 2α-cyano-4,4,17α-trimethyl-17β-hydroxy-5-androstene-3-one; 4-MA, *N,N*-diethyl-4-methyl-3-oxo-4-aza-5α-androstan-17β-carboxamide; trilostane, 2α-cyano-4α,5α-epoxy-17β-hydroxy-androstane-3-one; epostane, 2α-cyano-4α,5α-epoxy-17β-hydroxy-4,17-dimethyl-androstane-3-one; promegestone, 17,21 dimethyl 19 nor 4,9 pregnadiene 3,20 dione; RU2323, 13-ethyl-17β-hydroxy-18,19(17α)-dinor-4,9,11-pregnadiene-20-yn-3-one; Norgestrel, 13-ethyl-17β-hydroxy-19(17α)-nor-4-pregnene-20-yn-3-one; norethindrone, 17β-hydroxy-19(17α)-nor-4-pregnene-20-yn-3-one; cyproterone acetate, 6-chloro-1α,2α-methylene-17α-hydroxy-4,6-pregnadiene-3,20-dione.

the purified enzyme and two substrates, namely pregnenolone and dehydroepiandrosterone. Human placenta was used to purify 3β-HSD since it contained large amount of 3β-HSD activities and was a readily available source of human tissues.

MATERIALS AND METHODS

Materials

NAD^+, $NADP^+$, digitonin and norgestrel were purchased from Sigma Chemicals Co. (St Louis, Mo.). Hydroxylapatite Biogel HTP was from Bio-Rad Laboratories, Richmond, Calif. [4,7-^{3}H]pregnenolone and [1,2-^{3}H]dehydroepiandrosterone were obtained from DuPont Canada Inc. (Mississauga, Ont.). The purity of the radioactive steroids was confirmed by TLC on silica gel (Merck, Darmstadt, F.R.G.). All non-radioactive steroids were purchased from Steraloids Ltd while cyproterone acetate was obtained from Schering AG, West Germany and RU2323 and norethindrone were from Roussel–Uclaf, Paris, France. Promegestone was from DuPont Canada Inc. (Mississauga, Ont.) while trilostane was kindly provided by Sterling-Winthrop Research Institute, Rensslaer, New York and 4-MA was a gift from Merck Sharp & Dohme Research Laboratories, Rahway, N.J.

Purification of 3β-HSD

Trophoblasts from freshly delivered human placentas were separated from connective tissue by dissection. The tissue fragments were rinsed in saline and homogenized in 2 vol of 50 mM sodium phosphate, pH 7.4, 250 mM sucrose and 1 mM EDTA. Homogeneization was performed for 3 × 10 s at full speed in a cold waring blender. The homogenate was then centrifuged at 800 *g* for 30 min. The resulting supernatant was decanted and recentrifuged at 10,000 *g* for 30 min. Following centrifugation of the 10,000 *g* supernatant at 105,000 *g* for 60 min, the microsomal pellet was washed with 100 mM pyrophosphate buffer, pH 7.4, 20% glycerol and 1 mM EDTA. The resulting 105,000 *g* pellet was then resuspended in buffer A containing 50 mM sodium phosphate, pH 7.4, 20% glycerol and 1 mM EDTA. The microsomal fraction was diluted to 20 mg/ml and a 20% sodium cholate solution prepurified on a DEAE column was added to a final 1% concentration. The cholate suspension was then incubated at 4°C for 1 h, diluted to 0.6% cholate with buffer A and then centrifuged at 105,000 *g* for 60 min. The resulting supernatant was loaded on a phenyl-Sepharose 4B column preequilibrated with buffer A + 0.4% cholate and the column was washed with 4 column volumes of equilibrium buffer. Elution was performed by adding 0.3% NP-40, a non-ionic detergent. Fractions containing high specific 3β-HSD activity were pooled and dialyzed overnight against buffer A containing 0.4% cholate and 0.1% NP-40. The dialyzed fraction was applied to a 30 ml hydroxylapatite column equilibrated with the same dialyzis buffer. The column was then washed with 4 column volumes of the equilibrium buffer and eluted with a phosphate gradient (10–200 mM) in the presence of 0.1% NP-40. Fractions containing high 3β-HSD activity were pooled and dialyzed against buffer A containing 0.4% cholate and 150 mM phosphate. The dialyzed material was applied to a 10 ml hydroxylapatite column equilibrated with the dialyzis buffer. The column was then washed with 5 column volumes of the equilibrium buffer and eluted with a NP-40 gradient (0.02–0.2%). Fractions containing high activity were pooled, concentrated, dialyzed and stored in buffer A at −70°C.

Enzymatic assay

Radioactive steroid (20–100 μM, 10 × 10^4 cpm) and inhibitor (0–200 μM) dissolved in benzene were transferred to an incubation tube containing a drop of propylene glycol. Following evaporation of the solvent under a stream of nitrogen, the purified enzyme (7–17 μg protein) and 0.8 mM NAD^+ in 50 mM potassium phosphate buffer (pH 7.4) containing 20% glycerol and 1 mM EDTA were added. The final volume of the incubation mixture was adjusted to 0.5 ml, and the incubation was performed for 10 min with constant agitation at 37°C.

Immediately after incubation, steroids were extracted twice from the incubation mixture with 2 ml of methylene dichloride. The extract was then dried with anhydrous sodium sulfate and evaporated under a stream of nitrogen. The steroid products were separated by TLC on silica gel in the system benzene–acetone (4/1; v/v), developing system. Radioactivity was measured by a liquid scintillation spectrometer (Beckman LS 3801) and were identified by comparison with authentic pregnenolone and progesterone and dehydroepiandrosterone and 4-androstenedione.

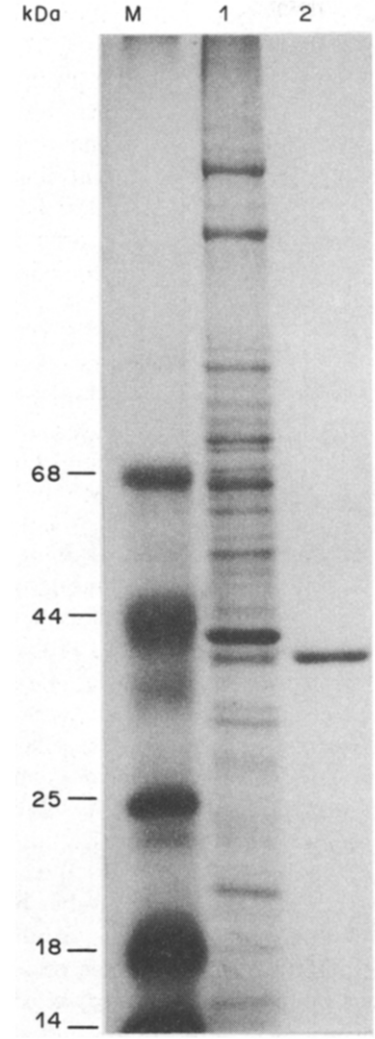

Fig. 1. Analysis of 3β-HSD by SDS–PAGE. Protein fractions were separated by SDS–PAGE and stained by Coomassie blue: microsomal fraction (1), enzyme purified from the same fraction (2) and protein markers (M). Electrophoresis was performed as described in Materials and Methods.

Electrophoresis

SDS–polyacrylamide gel electrophoresis was performed according to the method of Laemmli [8] using a gradient of 5–15% polyacrylamide as the separating gel. Protein markers of 68,000, 44,000, 25,000, 18,000 and 14,000 from Bethesda Research Laboratories were used.

Protein determination

Protein was measured by the method of Bradford [9] using bovine serum albumin (BSA) as standard.

RESULTS AND DISCUSSION

Homogeneous 3β-HSD fraction, as judged by SDS–PAGE (Fig. 1), has been obtained by purification through hydrophobic chromatography column (phenyl-Sepharose 4B) and followed by two hydroxylapatite column. It transforms efficiently DHEA and pregnenolone to 4-androstenedione and progesterone, respectively, with almost the same K_m value of 1.2 μM [10]. The purified enzyme was used to perform the following inhibition studies.

As illustrated in Fig. 2, trilostane, a well-known inhibitor of 3β-HSD [11] used in the management of Cushing's syndrome [12] is a potent inhibitor of purified human placental 3β-HSD. K_i values were measured at 61 and 40 nM by Dixon plot analysis, when DHEA and pregnenolone were used as substrates, respectively.

The 4-azasteroid 4-MA is known as a potent inhibitor of 5α-reductase [13, 14] and this category of compounds is potentially useful in the

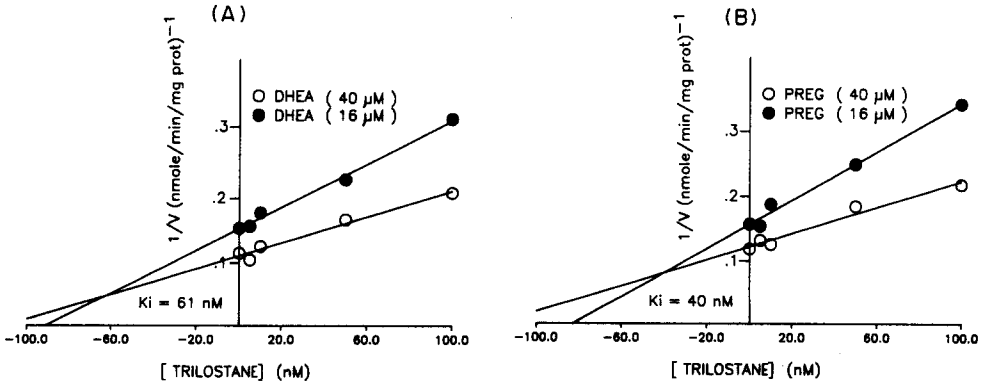

Fig. 2. Dixon plot analysis of the effect of trilostane on purified human placental microsomal 3β-HSD activity. Incubation was performed as decribed in Materials and Methods. [^{3}H]Dehydroepiandrosterone (DHEA) (A) and [^{3}H]pregnenolone (Preg) (B) were used as substrates at the concentration of 16 (●——●) or 40 μM (○——○).

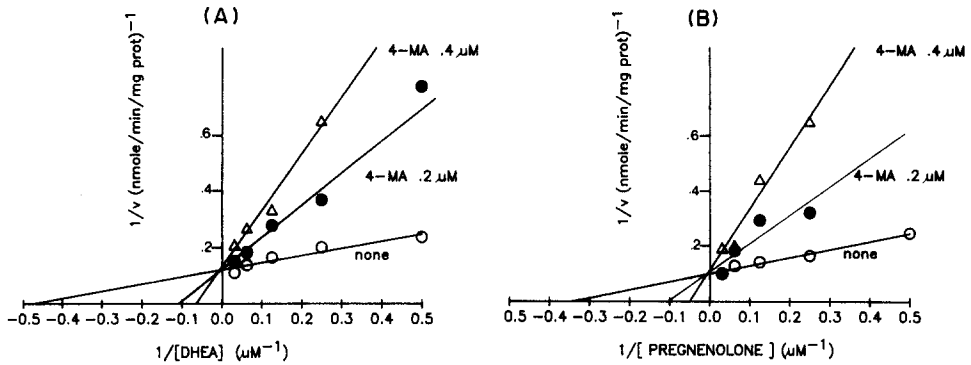

Fig. 3. Lineweaver–Burk plot analysis of the effect of 4-MA on purified human placental microsomal 3β-HSD. Incubations were performed as described in Materials and Methods. DHEA (A) and pregnenolone (B) were used as substrates at the indicated concentrations. 4-MA was added at the concentrations of zero (○——○); 0.2 μM (●——●) or 0.4 μM (△——△).

treatment of androgen-sensitive diseases, especially benign prostatic hyperplasia. As illustrated in Fig. 3, 4-MA is also a potent inhibitor of 3β-HSD activity with K_i values of 40 and 56 nM for pregnenolone and dehydroepiandrosterone, respectively. In addition to trilostane and 4-MA, epostane and cyanoketone are also potent inhibitors of purified human microsomal placental 3β-HSD (Fig. 4, Table 1), when either pregnenolone or DHEA are used as substrate, thus indicating an important role of the 2α-cyano group in the inhibition of 3β-HSD [11]. Ferre *et al.* [15] reported that progesterone preferentially inhibits the transformation of C-21 precursors while not affecting C-19 substrates in human placental microsomes. Using the purified placental enzyme, we do not observe such a dissociation in substrate specificity (Fig. 4). Since the transformation of both pregnenolone and DHEA is slightly inhibited by progesterone, whereas other progestins such as promegestone and RU2323 (Fig. 4) which possess one and two additional double bonds at position 9 and 11, respectively, exhibit more potent inhibition of 3β-HSD, the present data suggest the potentiating inhibitory effect of unsaturated groups at position 9.

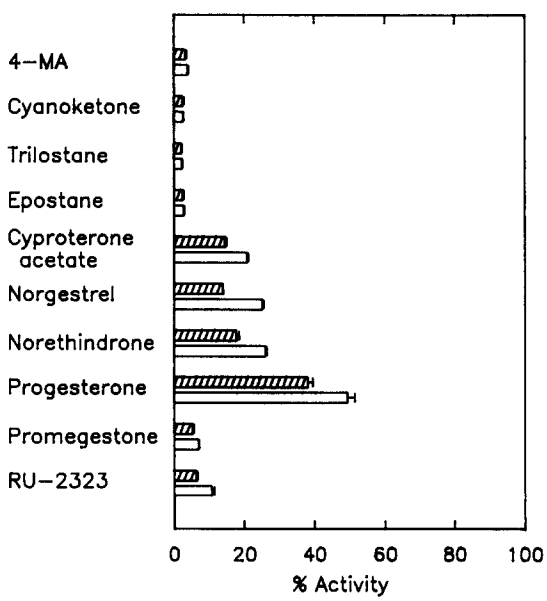

Fig. 4. Effect of 4-MA, cyanoketone and synthetic progestins on the activity of purified human placental microsomal 3β-HSD. Incubation was performed as described in Materials and Methods in the presence of 20 μM of pregnenolone (open bar) or DHEA (shaded bar) and the indicated competitors. The 100% activity corresponds to incubation in the presence of substrate alone. Each value was obtained from duplicate experiments.

Cyproterone acetate, a potent progestagen and a compound having mixed agonist and antagonist androgenic activities [16–18] used in the treatment of hirsutism, acne and prostate cancer [16] also exerts a relatively high inhibitory effects on 3β-HSD with a K_i value of 1.5 μM. Norgestrel and norethindrone, two progestagens, which are used as components of

Table 1. Potency of various steroids to inhibit activity of purified human placental 3β-HSD

Inhibitor	K_i value (μM)
4-MA	0.056
Cyanoketone	0.038
Trilostane	0.061
Epostane	0.041
Cyproterone acetate	1.5
Norgestrel	1.7
Norethindrone	2.5
Progesterone	7.1
Promegestone	0.11
RU-2323	0.19

K_i values were estimated as described in Fig. 1 using 20 μM of DHEA as substrate.

oral contraceptive pills also show relatively potent inhibition of 3β-HSD activity with K_i values of 1.7 and 2.5 μM for norgestrel and norethindrone, respectively (Table 1). An inhibition of 80% was in fact observed when these progestagens were added in a ratio of 1:1 with the substrate.

Since 3β-HSD is involved in the biosynthesis of all classes of natural steroid hormones, and the enzyme is present in adrenal, gonadal as well as in a series of peripheral tissues, knowledge about the control of its activity is of major importance. However, despite its well-recognized role, little is known about the factors or compounds able to modulate the activity of this enzyme, the available information being limited to cyanoketone [19], trilostane [11, 12], epostane [20], and more recently, 4-MA in porcine granulosa cells [21].

The availability of purified 3β-HSD permits a precise assessment of the potency of potential inhibitors. The present data clearly demonstrate that in addition to the well known effects of trilostane and epostane, the progestin derivatives cyproterone acetate, norgestrel, norethindrone, promegestone and RU2323 can also exert potent inhibitory effects on the activity of the enzyme. Moreover, the azasteroid 4-MA, a well-known inhibitor of 5α-reductase activity [13, 14], is also a relatively potent inhibitor of 3β-HSD, Thus, there is the possibility that 4-MA and its analogues as well as the synthetic progestins could, in addition to their desired therapeutical effects, inhibit 3β-HSD and the formation of mineralocorticoids, glucocorticoids, progesterone and precursors of sex steroids. However, since 3β-HSD is also present in peripheral human tissues, namely the prostate [22], skin [23] and adipose (C. Labrie *et al.*, unpublished data), such knowledge about the specificity of 3β-HSD could help the design of more efficient and better tolerated regimens for the therapy of estrogen-sensitive and estrogen-insensitive diseases.

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