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KINETIC ANALYSIS OF 3β-HYDROXYSTEROID DEHYDROGENASE ACTIVITY IN MICROSOMES FROM COMPLETE HYDATIDIFORM MOLE

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Summary—Microsomes isolated from complete hydatidiform moles (CHM) were able to convert [^{3}H]pregnenolone to [^{3}H]progesterone which indicates the presence of 3β-hydroxysteroid dehydrogenase/isomerase (3β-HSD) activity. The kinetic parameters found ($K_m = 0.63\ \mu M$ and $V_{max} = 1$–3.05 nmol/min/mg of protein) were like those observed in microsomes from normal early placenta (NEP) of similar gestational age (herein) and term placenta [1] suggesting that the enzymes from the three sources are kinetically similar. Testosterone, progesterone and estradiol in a dose range of 0.05–5 μmol/l inhibited differently the *in vitro* conversion of [^{3}H]pregnenolone to [^{3}H]progesterone in a dose-dependent manner. The steroid concentrations necessary to inhibit the conversion of pregnenolone to progesterone by 50% (ID_{50}) in CHM were 0.1 μM for testosterone, 0.6 μM for progesterone and 3 μM for estradiol, whereas in NEP they were 2.5, 1 and 5 μM respectively. The K_i values calculated from these ID_{50} in CHM together with the reported levels of endogenous steroids indicate that the accumulation of testosterone and progesterone inside the molar vesicle could physiologically regulate the rate of further conversion of pregnenolone to progesterone. The present findings could provide an explanation for the low level of progesterone in patients with CHM in the second trimester of pregnancy which in turn may directly or indirectly affect the spontaneous expulsion of this aberrant tissue.

INTRODUCTION

The placental 3β-hydroxysteroid dehydrogenase/isomerase enzyme (3β-HSD) plays a key role in the production of progesterone during pregnancy. This complex enzyme which converts pregnenolone into progesterone is associated both to the mitochondria [2–4] as well as microsomal preparations [1, 2, 5–7]. Their kinetic properties and some regulation effectors have been described only in normal term placenta [1–8]. However, nothing is known about the kinetic parameters of this enzyme in complete hydatidiform moles (CHM), which is characterized by the presence of aberrant placenta with hyperplasia of cyto and syncytiotrophoblast and by absence of maternal genetic information in the nucleus [9, 10]. Studies on molar tissue incubating C_{19} or C_{21} steroid precursors indicate the presence of 3β-HSD [11, 12]. In the present study we investigated the enzyme properties of 3β-HSD activity in microsomes from CHM and the effects of testosterone, progesterone and estradiol on its activity. For comparative purposes similar experiments were performed in microsomes from normal early placenta of the same gestational age (NEP). In addition, we present evidence indicating that the *in vivo* regulation of 3β-HSD activity may be affected by the testosterone and progesterone levels inside the molar vesicle.

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EXPERIMENTAL

Chemicals

[7-^{3}H(N)]Pregnenolone (sp. act. 24.2 Ci/mmol) was purchased from New England Nuclear (Boston, Mass). Its purity was checked by TLC. All other nonradioactive steroids and NAD^+ Grade IV were obtained from Sigma Chemical Co. (St Louis, Mo.). The solvents were analytical grade from Carlo Erba (Italy).

Tissue preparation

The present study was carried out on 7 CHM and 2 NEP obtained after therapeutic abortion from patients with an amenorrheic age of 64–117 days' duration. The CHM were identified on the basis of gross morphology and histopathology. The tissue was repeatedly washed with cold saline and cut into small pieces. Aliquots of 1 g minced tissue were homogenized in 2 ml of cold isolation buffer (0.01 M potassium phosphate pH 7.4, 0.25 M sucrose, 0.1 mM EDTA, 0.1 mM DTT, 0.1 mM bacitracin)

with a Teflon glass motor driven homogenizer for 2 min at 2000 rev/min and 20 strokes. The homogenate was then centrifuged at 12,000 *g* for 20 min. Microsomes were obtained by centrifuging the 12,000 *g* supernatant at 100,000 *g* for 1 h. The precipitate was washed with 0.15 M KCl, 10 mM potassium phosphate (pH 7.5), 1 mM EDTA. The microsomal pellet was then resuspended in an adequate volume of suspension buffer (0.1 M potassium phosphate pH 7.4, 0.1 mM EDTA, 0.1 mM DTT and 20% v/v glycerol) to obtain the indicated microsomal concentrations. All the steps were carried out at 4°C. Protein concentration was measured according to Lowry *et al.*, using BSA as standard [13].

Assay of 3β-HSD activity

The enzyme activity was determined by measuring the conversion of [^{3}H]pregnenolone to [^{3}H]progesterone in microsomes from CHM and NEP. Incubation was carried out for 8 min at 37°C. Protein concentrations were adjusted so that the rate of [^{3}H]progesterone formation was linear for 8 min. Aliquots of microsomal solution containing 60 μg of proteins were incubated with [^{3}H]pregnenolone (200,000 cpm, 5 μM) in 2 ml of suspension buffer containing 1 mM NAD^+. For testing the effects of testosterone, progesterone and estradiol on the 3β-HSD activity present in the microsomal fractions, varying amounts of them were added to the tubes containing [^{3}H]pregnenolone and propylene glycol. At the end of incubation the steroids were extracted 3 times with 5 vol of ethyl-ether. Then 100 μg of unlabelled progesterone and pregnenolone were added as carriers, submitted to TLC on silica gel GF 254 and developed using benzene–ethanol (95:5 v/v) as solvent system. The identification of [^{3}H]pregnenolone and [^{3}H]progesterone which were isolated from the chromatogram was done by crystallization to constant specific activity. After appropriate correction for recovery, the mass of product was calculated from the known specific activity of the substrate and the reaction velocity was expressed as nmol of product/min/mg of protein. Values of K_m and V_{max} were estimated graphically from plots of 1/V vs 1/S. The results of the experiments performed to examine the effects of other steroid compounds on 3β-HSD are expressed as percentage taking the conversion of pregnenolone to progesterone in the absence of other compounds as 100%.

RESULTS

A first-order enzyme kinetic for CHM (initial velocity) can be achieved within an incubation time of 8 min using a protein concentration of 40 μg/ml (Fig. 1A). A linear relationship between the 3β-HSD activity and the protein concentration of microsomes is observed within a protein concentration of 40 μg/ml (Fig. 1B). Similar results were obtained with microsomes from NEP.

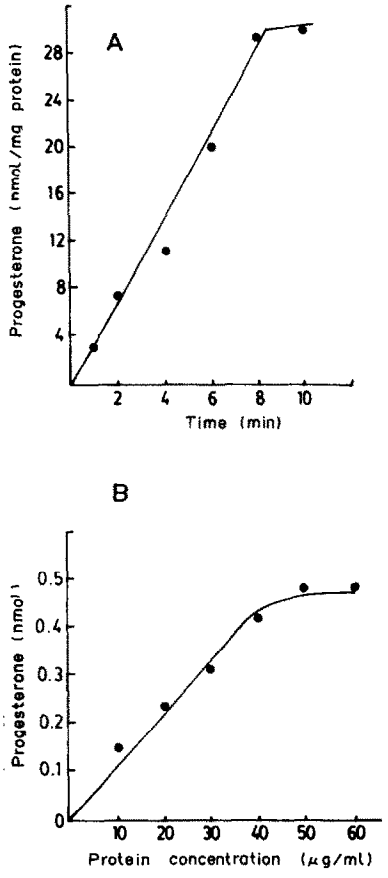

Fig. 1. (A) Time-related linearity of microsomal 3β-HSD from complete hydatidiform mole containing 40 μg/ml of protein; (B) 3β-HSD activity as a function of protein concentrations (incubation time of 4 min). Each point represents one experiment in duplicate.

By selecting these conditions so as to obtain conversion rates of less than 20% of total [^{3}H]pregnenolone, the K_m value was determined in one CHM in quadruplicate and one NEP in duplicate using substrate concentrations from 0.25 to 5 μM. By means of Lineweaver–Burk's plot for pregnenolone ranging from 3.9×10^{-1} to 7.9 K_m, the apparent K_m of pregnenolone was calculated to be 0.63 μM for CHM (Fig. 2) and 0.66 μM for NEP (data not shown). The maximal velocity of reaction ranged between 1–3.05 nmol/min/mg of protein in seven different CHM.

The effects of testosterone, progesterone and estradiol on the microsomal 3β-HSD activity from CHM are shown in Fig. 3A. All the three steroids with the dose range of 0.05–5 μmol/l inhibited the conversion of pregnenolone to progesterone in a dose-dependent manner in the microsomal preparations. From the dose–response curves for their inhibitory effects, it was possible to estimate graphically the dose of inhibition that reduced the conversion of [^{3}H]pregnenolone to [^{3}H]progesterone by 50% (ID_{50}). These values in CHM were 0.1 μM for

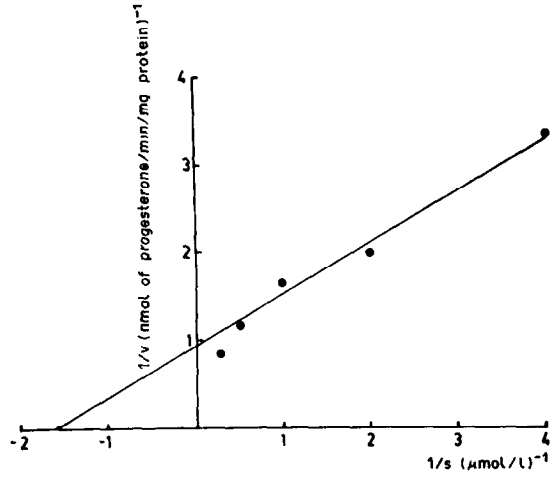

Fig. 2. Lineweaver–Burk plot for the determination of the K_m values for pregnenolone (apparent K_m 0.63×10^{-6} M) in assays of microsomal 3β-HSD of complete hydatidiform mole. Results are presented as means of one experiment in quadruplicate.

testosterone, 0.6 μM for progesterone and 3 μM for estradiol (Fig. 3A) and the corresponding results in NEP were 2.5, 1 and 5 μM (Fig. 3B). The hyperbolic dose–response curves suggest an apparent competitive inhibition by these steroids. Using the formula reported by Brandt *et al.* for competitive inhibitors [14] it was possible to calculate their K_i (Table 1). For comparison purposes, and in order to know the possible impact that a single inhibitor can have upon the *in vivo* 3β-HSD activity, the mean concentration of testosterone, progesterone and estradiol in the molar vesicle fluid and the plasma concentrations of these steroids from patients with CHM and NEP are also shown in Table 1 [15–18]. These data demonstrate that circulating testosterone and progesterone from patients with CHM reach a concentration high enough to inhibit the 3β-HSD activity, whereas only progesterone can do this in NEP. Moreover, the accumulation of these steroids inside the molar vesicle is sufficient to inhibit this activity.

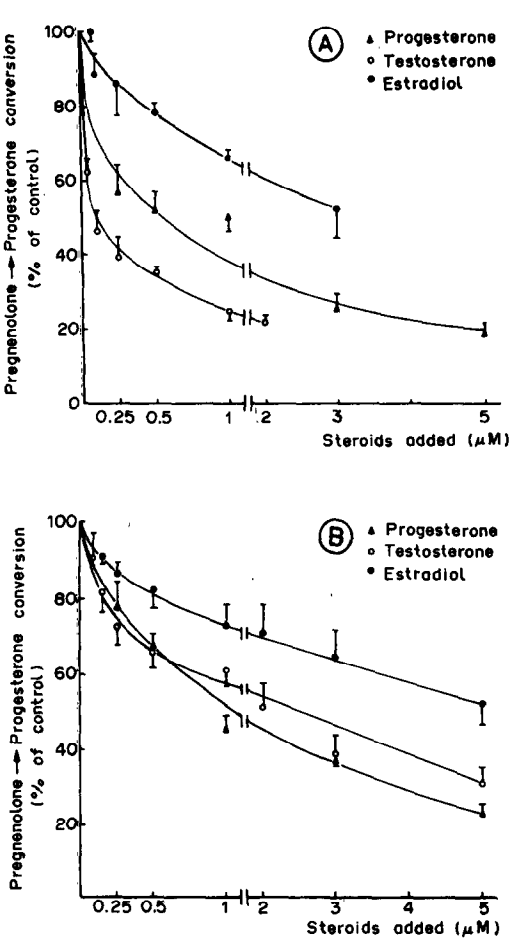

Fig. 3. Dose–response relationship of the inhibitory actions of testosterone, progesterone and estradiol on the conversion of [^{3}H]pregnenolone (5 μM) to [^{3}H]progesterone by microsomes from complete hydatidiform mole (A) and normal early placenta (B). For each dose of steroid hormone tested duplicate experiments were carried out on 3 different moles and 2 different normal early placentas. Each of the values on the graph represents the mean ± SEM and the values are given as the conversion of pregnenolone to progesterone expressed as percentage of the conversion rate in control assay 100% where no test steroid was added.

DISCUSSION

Using microsomal preparation obtained from CHM tissue, we found that pregnenolone can be converted to progesterone *in vitro* thus indicating that the 3β-HSD enzyme is present in CHM microsomes as suggested in previous conversion studies [11, 12].

Table 1. Inhibition of 3β-HSD in microsomes from complete hydatidiform mole by testosterone, progesterone and estradiol

	Complete hydatidiform mole			Normal early placenta	
Inhibitor	K_i[a]	Plasma concentration (μM)	Vesicle fluid concentration (μM)	K_i[a]	Plasma concentration (μM)
Testosterone	0.01	0.0076[b]	0.021[d]	0.34	0.0027[b]
Progesterone	0.07	0.084[c]	2.52[c]	0.14	0.113[c]
Estradiol	0.3	0.007[c]	0.039[d]	0.69	0.009[c]

[a]The K_i value was calculated from the following formula (Ref. [14])

$$K_i = \frac{I}{((S/K_m) + 1)((V_o/V_i) - 1)}.$$

[b]Reported by Dawood *et al.*[18]. [c]Reported by Ho *et al.*[17]. [d]Reported by Chew *et al.*[15]. [e]Reported by Dawood[16].

The K_m and V_{max} values for 3β-HSD obtained in microsomes from CHM were like those observed by us in microsomes from human early (herein) and term placenta [1], suggesting that the enzymes from the three sources are kinetically quite similar.

In the present study we also demonstrate that testosterone, progesterone and estradiol can decrease the conversion of pregnenolone to progesterone *in vitro* by microsomal preparations from CHM or NEP by inhibiting the 3β-HSD activity. The comparison of K_i values calculated for these steroids indicate that testosterone was 34-fold more inhibitory in CHM with respect to NEP, whereas all the other steroids are approximately in the same order to inhibition in both tissues and similar to those found by us in term placenta [1].

From these data together with reported values of testosterone, progesterone and estradiol in the molar vesicle fluid [15, 16] and the plasma concentrations of these steroids in patients with CHM [17, 18], it could be expected that some of these steroid concentrations should affect the 3β-HSD activity, particularly in absence of adaptative changes in substrate concentrations. Certainly, the K_i values calculated for testosterone and progesterone in CHM and their endogenous circulating and molar vesicle levels would suggest that the accumulation of these steroids inside the vesicle could physiologically regulate the rate of further conversion of pregnenolone to progesterone. These results could provide a theoretical explanation for the low concentration of plasma progesterone in patients with CHM in the second trimester of pregnancy [17], which in turn may directly or indirectly affect the spontaneous expulsion of this aberrant tissue [19]. Finally, phospholipids and other small membrane-bound molecules could influence the activity of the enzyme-associated membrane [20] with a consequent different order of inhibition efficiency on the 3β-HSD activity from CHM or NEP.

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