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ANDROGENIC 17β-HYDROXYSTEROID DEHYDROGENASE ACTIVITY OF EXPRESSED RAT TYPE I 3β-HYDROXYSTEROID DEHYDROGENASE/Δ5-Δ4 ISOMERASE

Yvan de Launoit, Jacques Simard, Francine Durocher and Fernand Labrie
Medical Research Council Group in Molecular Endocrinology, CHUL Research Center and Laval University, Quebec GIV 4G2, Canada.

ABSTRACT - Transient expression in nonsteroidogenic mammalian cells of the rat wild type I and type II 3β-hydroxysteroid dehydrogenase/Δ^5-Δ^4-isomerase (3β-HSD) cDNAs shows that the encoded proteins, in addition to being able to catalyze the oxidation and isomerization of Δ^5-3β-hydroxysteroid precursors into the corresponding Δ^4-3-ketosteroids, interconvert 5α-dihydrotestosterone (DHT) and 5α-androstane-3β,17β-diol (3β-diol). When homogenate from cells transfected with a plasmid vector containing type I 3β-HSD is incubated in the presence of DHT using NAD^+ as cofactor, a somewhat unexpected metabolite is formed, namely 5α-androstanedione (A-dione), thus indicating an intrinsic androgenic 17β-hydroxysteroid dehydrogenase (17β-HSD) activity of this 3β-HSD isoform. Although the relative Vmax of 17β-HSD activity is 14.9-fold lower than that of 3β-HSD activity, the Km value for the 17β-HSD activity of type I 3β-HSD is 7.97 μM, a value which is in the same range as the conversion of DHT into 3β-diol which shows a Km value of 4.02 μM. Interestingly, this 17β-HSD activity is highly predominant in unbroken cells in culture, thus supporting the physiological relevance of this "secondary" activity. Such 17β-HSD activity is inhibited by the classical substrates of 3β-HSD, namely pregnenolone (PREG), dehydroepiandrosterone (DHEA), Δ^5-androstene-3β,17β-diol (Δ^5-diol), 5α-androstane-3β,17β-diol (3β-diol) and DHT, with IC_{50} values of 2.7, 1.0, 3.2, 6.2, and 6.3 μM, respectively. Although dual enzymatic activities have been previously reported for purified preparations of other steroidogenic enzymes, the present data demonstrate the multifunctional enzymatic activities associated with a recombinant oxidoreductase enzyme. In addition to its well known 3β-HSD activity, this enzyme possesses the ability to catalyze DHT into A-dione thus potentially controlling the level of the active androgen DHT in classical steroidogenic as well as peripheral intracrine tissues.

INTRODUCTION

The conversion of 3β-hydroxy-Δ^5 steroids into the corresponding Δ^4-3-ketosteroids by membrane-bound 3β-hydroxysteroid dehydrogenase/Δ^5-Δ^4-isomerase, hereafter called 3β-HSD, is a crucial step in the biosynthesis of all classes of hormonal steroids (1). Three β-HSD, in addition to being found in classical steroidogenic tissues, is also present in several peripheral tissues including the mammary gland, prostate, skin, adipose tissue, liver, kidney and epididymis (1-6), where it contributes to the intracrine formation of sex steroids acting locally (7). We have recently characterized multiple types of rat cDNAs encoding 3β-HSD proteins of 372 amino acids (2,3). Transient expression of rat type I and type II 3β-HSD cDNAs in mammalian nonsteroidogenic cells reveals that 3β-ol dehydrogenase and Δ^5-Δ^4 isomerase activities reside within a single protein (3,8). Moreover, these two isoenzymes which share 94% homology also catalyze the interconversion of 3β-hydroxy- and 3-keto-5α-androstane steroids (3). Further characterization of the structure-activity relationships of these two isoenzymes by site-directed mutagenesis revealed that the lower activity of the type II compared to the type I protein is due, at least in part, to a change of four amino acid residues potentially involved in a putative membrane-spanning domain (MSD) located between residues 75 to 91 (8).

Interestingly, it has been reported that some purified preparations of oxidoreductase enzymes show dual steroidogenic activities (9-11). Since the previous data on dual enzymatic activities could be due to the lack of purity of the preparations, transient expression of a recombinant enzyme in nonsteroidogenic mammalian cells should permit one to characterize without ambiguity the presence of such multiple enzymatic activities. The present study was designed to investigate the possibility that rat 3β-HSD isoenzymes possess secondary steroidogenic activities.

MATERIALS AND METHODS

The full-length cDNA inserts corresponding to the rat type I (ro3β-HSD56) and type II (ro3β-HSD112) 3β-HSD clones were used (3). We also constructed, by site-directed mutagenesis, two chimeric cDNAs in which the four codons for Ala^{83}, Ile^{85}, Val^{87} and His^{89} potentially involved in a membrane-spanning domain (MSD) predicted between residues 75 to 91 in the type I 3β-HSD protein were substituted by those for Ser^{83}, Met^{85}, Phe^{87} and Arg^{89} present in the type II 3β-HSD protein and *vice versa*, thus leading to cDNA inserts encoding a type I 3β-HSD protein without the potential MSD (I-MSD) or a type II 3β-HSD protein containing type I MSD (II+MSD) as described (8). These full-length type I, type II, I-MSD and II+MSD clones were then cloned into the unique EcoRI site of the pCMV vector, downstream from the cytomegalovirus (CMV) promoter, to produce the recombinant plasmids pCMV-type I 3β-HSD, pCMV-type II 3β-HSD, pCMV-I-MSD and pCMV-II+MSD, respectively (3,8). Expression of the plasmids was performed in the HeLa human cervical carcinoma cells or in the JEG-3 human choriocarcinoma cells by the calcium phosphate transfection method as described (3-5,8).

In order to determine enzymatic activity, cells were incubated for the indicated time intervals at 37 C in the presence of [1,2-^{3}H]5α-dihydrotestosterone (DHT; 47.3 Ci/mmol, NEN) or [4-^{14}C]DHT (58.3 mCi/mmol, NEN) in 50 mM Tris buffer (pH 7.5) containing 1 mM of the appropriate cofactor, namely NAD^+ or NADH, in the presence or absence of the unlabeled competitor steroids PREG, DHEA, Δ^5-diol, 3β-diol or DHT. Transfected cells were also incubated directly in petri dishes with labeled steroids in order to determine 17β-HSD activity in non disrupted cells. The enzymatic reaction was stopped by adding 4 volumes of ether/acetone (9/1, v/v). The organic phase was then evaporated and separated either on TLC plates using a 4:1 mixture of benzene and acetone as previously described (3-5,8) or by HPLC using a System Gold unit (Beckman) consisting of a model 126 pump, a 507 automatic injector, a Radial-Pak NovaPak C_{18} column (8 mm X 10 cm) and a model Beckman 168 photodiod array detector. The mobile phase for A-dione, DHT and 3β-diol was H_2O/methanol/tetrahydrofuran/ acetonitrile (50/35/10/5, v/v/v/v) at a flow rate of 1.5 ml/min over a 30-min period. Radioactivity was monitored in the eluent using a Beckman 171 HPLC Radioactivity Monitoring System using Formula 963 (NEN) as scintillation mixture at a flow rate of 4.5 ml/min. Km values as well as Vmax values were calculated by using Enzfitter Software (Biosoft, Cambridge, UK).

RESULTS AND DISCUSSION

As demonstrated by thin layer chromatography, a 8-h incubation of protein homogenate from HeLa cells transfected with the pCMV vector containing the rat type I 3β-HSD or type II 3β-HSD insert in the presence of NADH as cofactor led to the conversion of DHT into its metabolite 3β-diol (Fig. 1). It can also be seen in this figure that type I 3β-HSD is much more active than the type II isoform, in agreement with our previous observation for the classical substrates of 3β-HSD, namely PREG and DHEA as well as for DHT and 3β-diol (3,8).

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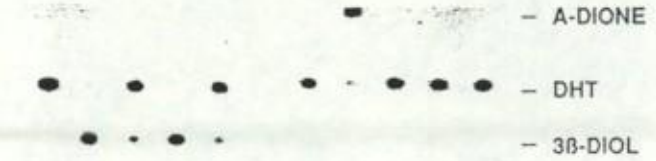

CONTROL	TYPE I	TYPE II	II + MSD	I - MSD	CONTROL	TYPE I	TYPE II	II + MSD	I - MSD
1mM NADH					1mM NAD^+				

Figure 1. Enzymatic conversion of DHT by wild type I and type II 3β-HSD as well as by chimeric II+MSD and I-MSD 3β-HSD proteins. Sixty μg of protein from homogenates of HeLa cells transfected with pCMV alone (CONTROL), pCMV-type I 3β-HSD (TYPE I) or pCMV-type II 3β-HSD (TYPE II) plasmid, as well as the pCMV plasmid containing the recombinant fragments of type I 3β-HSD devoid of the predicted membrane-spanning domain (MSD) (I-MSD) between residues 75 to 91 or type II having gained this MSD (II+MSD) were incubated with 300 nM [^{14}C]-DHT for 8 h in the presence of 1 mM NADH or 1mM NAD^+. DHT and its metabolites 3β-diol and A-dione were separated by TLC and identified by autoradiography.

Somewhat unexpectedly, we then observed that homogenate obtained from cells transfected with the pCMV-type I 3β-HSD in the presence of the oxidative form of the cofactor, i.e. NAD^+, converts DHT into a highly polar metabolite (Fig. 1). This metabolite has been shown by HPLC to correspond to the 17β-oxidative form of DHT, namely A-dione (Fig.2), thus demonstrating that the expressed enzyme possesses 17β-HSD activity. In fact, as illustrated in Fig 2, endogenous 17β-HSD activity in HeLa cells transfected with pCMV alone is very low (Fig. 2B) whereas, after a 12-h incubation in the presence of 1 mM NAD^+, homogenate from cells transfected with pCMV-type I 3β-HSD converts about 65% of DHT into A-dione (Fig. 2C).

As illustrated in Fig. 1, homogenates from cells transfected with pCMV type I-3β-HSD and pCMV-II+MSD transform DHT into 3β-diol to the same extent in the presence of 1mM NADH. On the other hand, type II and I-MSD possess similar but considerably lower activity than the two other proteins. As mentioned earlier, the change of residues 83, 85, 87 and 89 in the wild-type rat type II 3β-HSD protein prevents the formation of a potential MSD present in the rat type I 3β-HSD enzyme between residues 75 and 91. From the data presented above, it is likely that the absence of this putative MSD in type II 3β-HSD explains its much lower activity (8). In contrast to the type I 3β-HSD protein, it can be seen that type II 3β-HSD, as well as chimeric I-MSD and II+MSD proteins, do not possess the androgenic 17β-HSD activity (Fig. 1). Finding that the chimeric II+MSD 3β-HSD protein is devoid of 17β-HSD activity strongly suggests that the lack of such 17β-HSD enzymatic activity for the type II 3β-HSD isoenzymes is not due to the absence of a MSD between residues 75 to 91, but rather to another structural difference resulting from one or several of the 19 other amino acid changes observed between the type I and type II isoforms.

As illustrated in Table I, the Km values of the expressed type I 3β-HSD protein using DHT as substrate and NADH as cofactor for the transformation of DHT into 3β-diol is 4.02 μM after a 1-h incubation. This value is in the same range as those recently obtained with the human type II 3β-HSD protein which is the almost exclusive type present in the adrenals and gonads where the Km value for DHT was measured at 2.7 μM (5) but higher

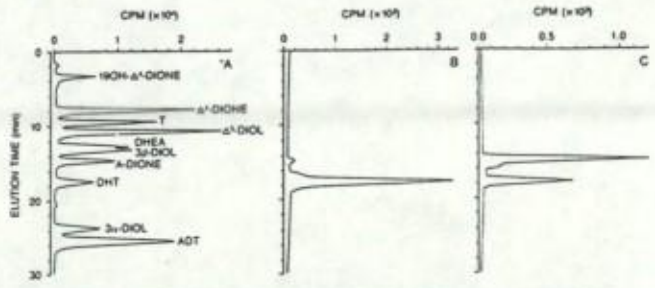

Figure 2. HPLC analysis of steroids obtained after transient transfection of pCMV alone (Panel B) or pCMV-type I 3β-HSD (Panel C) plasmid in HeLa cells in the presence of 300 nM [^{3}H]-DHT and 1 mM NAD^+. Panel A shows the elution profile of steroids used as standards in the HPLC assay.

than the Km value obtained with the human placental type I 3β-HSD (5,12). When NAD^+ is used as cofactor, the androgenic 17β-HSD activity of rat type I 3β-HSD is obtained at a Km value of 7.97 μM (Table I). As measured in HeLa cell homogenate, the Vmax of 3β-HSD activity of expressed type I 3β-HSD is much higher than that of 17β-HSD activity (24.9 ± 1.18 versus 1.67 ± 0.13 nmol/min/mg).

However, when the same enzymatic assays are performed in unbroken JEG-3 human choriocarcinoma cells transfected with the type I 3β-HSD, 17β-HSD activity is clearly predominant over 3β-HSD activity. In fact, it can be seen in Fig. 3 that cells transfected with type I 3β-HSD convert DHT exclusively into A-dione while no 3β-diol formation can be detected over basal values obtained in cells transfected with pCMV plasmid. The % of A-dione formed raised above 50% after 12 h of incubation in cells transfected with pCMV-type I 3β-HSD plasmid. The low 3β-HSD activity of type I isoenzyme observed in living cells is probably due to the low concentration of the reductive cofactor NADH compared to the oxidative cofactor NAD^+ (Fig. 3). The present data suggest that 17β-HSD activity, although "secondary" in homogenate of transfected cells in the presence of NADH, could well play a major role in the metabolism of DHT in the NAD^+-rich environment of living cells.The 17β-HSD activity of 3β-HSD seems to be specific to 5α-androstane steroids, since the other classical substrates of 17β-HSD, namely DHEA, Δ^5-diol, testosterone, Δ^4-androstenedione, 17β-estradiol or estrone, were not transformed into their respective metabolites in homogenates of HeLa cells transfected with the pCMV-type I 3β-HSD plasmid in the presence of appropriate cofactors (data not shown).

As illustrated in Fig. 4, the classical substrates of 3β-HSD, i.e. the Δ^5-3β-hydroxysteroids PREG, DHEA and Δ^5-diol as well as the 3β-hydroxy- (3β-diol) and 3-keto- (DHT) 5α-androstanes inhibited 17β-HSD activity in homogenate from transfected cells

TABLE 1

Kinetic properties of the 3β-HSD and 17β-HSD activities endowed by rat type I 3β-HSD. Twenty μg of protein from homogenate of cells transfected with the pCMV-type I 3β-HSD plasmid were incubated with increasing concentrations of tritiated DHT in the presence of 1 mM NADH or 1mM NAD^+ for 1 h. Kinetic parameters were determined using Enzfitter software analysis on duplicate samples and are expressed as means ± S.E.

Cofactor	Km (μM)	Vmax (nmol/min/mg)
NADH	4.02±0.67	24.9±1.18
NAD^+	7.97±2.18	1.67±0.13

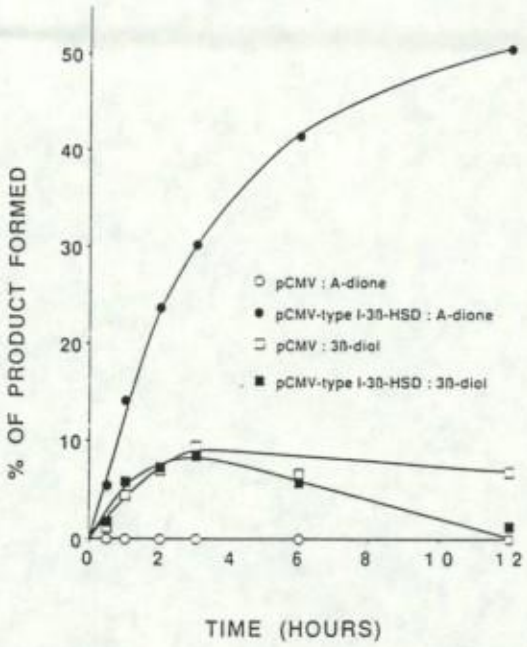

Figure 3. Time course of androgenic 17β-HSD activity of expressed rat type I 3β-HSD in JEG-3 cells. Five hundred thousand cells were plated in 60 mm well and transfected with either 5 μg pCMV or 5 μg pCMV type I 3β-HSD plasmid. Forty-eight h later, 100 nM [^{3}H]DHT was added into culture medium. Incubation was stopped at the indicated time intervals and steroids were determined by HPLC analysis.

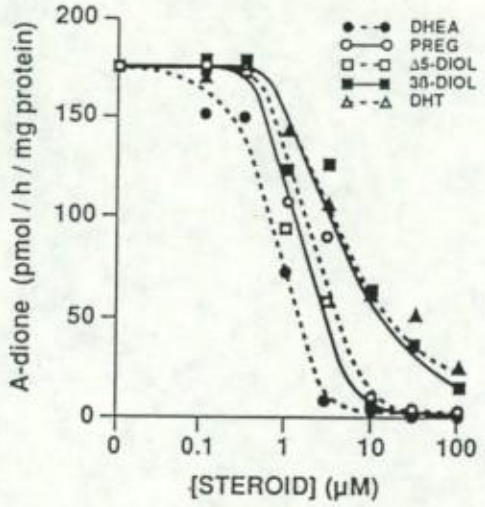

Figure 4. Competition by classical 3β-HSD substrates of the conversion of DHT into A-dione catalyzed by the expressed rat type I 3β-HSD isoenzyme. Sixty μg of protein from homogenate of cells transfected with the pCMV-type I 3β-HSD plasmid was incubated in duplicate for 8 h with 500 nM [^{14}C]DHT in the presence of 1 mM NAD^+ and the indicated concentrations of DHEA, PREG, Δ^5-DIOL, 3β-DIOL or DHT.

with the pCMV-type I 3β-HSD plasmid at IC_{50} values of 2.7, 1.0, 3.2, 6.2 and 6.3 μM, respectively, thus suggesting that the same active site is involved for both 3β-HSD and 17β-HSD activities.

Dual activity at the active site of steroid-specific enzymes is also a characteristic of preparations of purified 3β,20α-HSD from fetal lamb blood (10) and 3α,20β-HSD from fungus *Streptomyces hydrogenans* (9). The hypothesis was then advanced that some steroids can bind in opposite ways to the same enzyme active site (10,11), such an interpretation being limited by the possibility of impure enzyme preparations. In agreement with this hypothesis, the present study demonstrates such multiple catalytic activity after transient expression of a specific oxidoreductase isoenzyme. Moreover, the present data clearly demonstrate that androgenic 17β-HSD activity for 5α-androstane steroids is highly predominant in living human cells, thus strongly supporting the physiological relevance of the above-described "secondary" activity of type I 3β-HSD isoenzyme.

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