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Specific estradiol biosynthetic pathway in choriocarcinoma (JEG-3) cell line

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ABSTRACT

Estradiol (E2) plays a crucial role in all reproduction processes. In the placenta, it is well recognized that E2 is synthesized from fetal dehydroepiandrosterone sulfate (DHEAS). However, there is some controversy about the biosynthetic pathway involved, some authors suggest that E2 is produced by aromatization of testosterone (T), while others suggest that E2 is produced by the conversion of estrone (E1) into E2 by type 1 17β-HSD, subsequent to the aromatization of 4-androstenedione (4-dione) into E1. In the present report, using the precursor [^{14}C]DHEA, inhibitors of steroidogenic enzymes (chemical inhibitors and siRNA) and a choriocarcinoma (JEG-3) cell line that expresses all the enzymes necessary to transform DHEA into E2, we could determine the sequential steps and the specific steroidogenic enzymes involved in the transformation of DHEA into E2. Quantification of mRNA expression levels using real-time PCR, strongly suggests that type 1 3β-hydroxysteroid dehydrogenase (3β-HSD1), aromatase and type 1 17β-HSD (17β-HSD1) that are highly expressed in JEG-3 cells are the enzymes responsible for the transformation of DHEA into E2. Analysis of the intermediates produced in the absence and presence of 3β-HSD, aromatase and 17β-HSD1 inhibitors permits to determine the following sequential steps: DHEA is transformed into 4-dione by 3β-HSD1, then 4-dione is aromatized into E1 by aromatase and E1 is finally transformed into E2 by 17β-HSD1. Our data are clearly in favor of the pathway in which the step of aromatization precedes the step of reduction by 17β-HSD.

1. Introduction

Estradiol (E2) plays an important role in placental growth, implantation and embryo development [1]. It is synthesized in large amount in the feto-placental unit where it has been shown to increase the synthesis of the progesterone receptor (PR) [2] and several proteins in human placenta [3]. These effects of E2 are not only restricted to a normal cell, but also affect choriocarcinoma cells [4].

Human placental cells are not able to synthesize E2 *de novo* from cholesterol like ovarian cells, because of the absence of P450c17, an enzyme catalyzing the conversion of pregnenolone into DHEA. To produce E2, these cells use dehydroepiandrosterone sulfate (DHEAS) produced by the foetus [5]. DHEAS is then desulfonated by steroid sulfatase, an enzyme expressed in a large amount in the placenta [6], to produce DHEA that is subsequently taken in charge by 3β-hydroxysteroid dehydrogenase (3β-HSD), aromatase and estrogenic 17β-hydroxysteroid dehydrogenase to form E2. The human placenta has been, for many years, a major source material for purification and cloning of steroidogenic enzymes such as sulfatase, 3β-HSD1 and 17β-HSD1 [7–12].

Estrogens also play a crucial role in the development of breast and uterine normal and cancerous cells. Data from studies in aromatase knock-out mice [13–15] and in men with aromatase deficiency [16–18] reveal the importance of local and tissue-specific biosynthesis of E2. The role of local E2 synthesis is increasingly recognized and prevails over the general belief that E2 is exclusively synthesized in the gonads and delivered to peripheral tissues through the blood circulation. Although the biosynthetic pathway of E2 has been proposed a few decades ago (Fig. 1), there is still some controversy about the sequential intervention of aromatase and 17β-HSD. In fact, traditional literature and textbooks indicate that E2 is produced by the transformation of 4-androstenedione (4-dione) into testosterone (T) by 17β-HSD and then by aromatization of T into E2 (4-dione $\overset{17\beta-HSD}{\longrightarrow}$ T $\overset{Arom}{\longrightarrow}$ E2). However, it is known that aromatase possesses higher affinity for 4-dione than T. Indeed, 4- and 3.5-fold lower Km for 4-dione had been found using microsomes [19] and purified enzyme [20], respectively. Together with the cloning of many tissue- and estrogen-specific 17β-HSDs [12,21–24]. Recent data strongly suggest that 4-dione is the substrate of aromatase and accordingly, the step of aromatization precedes the step catalyzed by 17β-HSDs (4-dione $\overset{Arom}{\longrightarrow}$ E1 $\overset{17\beta-HSD}{\longrightarrow}$ E2).

In the present report, to determine the specific pathway of E2 biosynthesis, we incubate choriocarcinoma (JEG-3) cells that possess all the enzymes necessary to transform DHEA into E2, with [^{14}C]DHEA and determined the sequential steps of the E2

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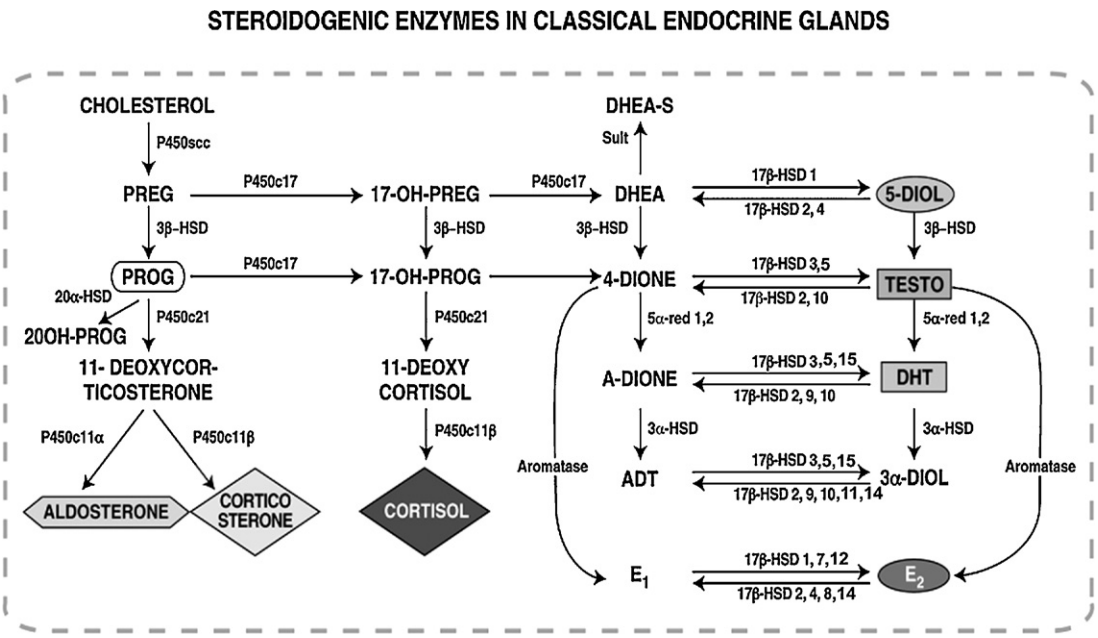

Fig. 1. General steroidogenic pathway. Active hormones are boxed and colored.

biosynthetic pathway by analyzing the metabolites produced in the presence and absence of specific inhibitors and siRNAs, specific types of enzymes involved in the pathway were determined using real-time PCR.

2. Materials and methods

2.1. Cell culture

The placental choriocarcinoma cells JEG-3 obtained from ATCC (HTB-36) were maintained in minimum essential medium (MEM) (Invitrogen Life Technologies, Inc.) supplemented with 10% (v/v) FBS (Wisent Inc.) at 37 °C under a 95% air:5% CO_2 humidified atmosphere.

2.2. Chemical inhibitors

1 μM of inhibitors was added 10 min before the addition of steroids, directly in cell culture media. The inhibitors used were epostane [25] for *HSD3B1* inhibition, formestane [26] for *CYP19A1* inhibition, and CC156 (3-(3,17β-dihydroxy-estra-1,3,5(10)-trien-16β-ylmethyl)-benzamide) [27], graciously provided by Dr. Donald Poirier, for *HSD17B1* inhibition.

2.3. Assay of enzymatic activity

Enzymatic activity was determined using intact cells in culture as previously described [24]. Briefly, 0.2 μM of the [^{14}C]-labeled DHEA (Dupont Inc.) was added to 12-well culture plates containing 1 ml of culture medium and 150,000 cells per well. Where indicated, 1 μM of Epostane, a 3β-HSD inhibitor or 1 μM of formestane, an inhibitor of aromatase or 1 μM of CC156, an inhibitor of type 1 17β-HSD was added 10 min before addition of the substrate DHEA. After 0, 2, 6, 12 or 24 h of incubation, steroids were extracted twice with 1 ml ethyl-ether. The organic phases were pooled and evaporated to dryness. The steroids were then solubilized in 100 μL methylene chloride, and separated on thin layer chromatography (TLC) Silica gel 60 plates (Merck, Darmstad, Germany), using the chloroform:ethyl acetate (4:1) solvent system. Radio-labeled steroids were revealed and quantified by a PhosphoImager Storm 860 system and identified by comparison with reference steroids. In our TLC system, DHEA, 4-dione, T, E1 and E2 are very well separated.

2.4. siRNA-mediated knock-down analysis

siRNAs against aromatase (Hs_CYP19A1_1 and Hs_CYP19A1_2), and AllStars Negative Control was obtained from Qiagen Inc., Canada (Mississisauga, Ont). One day before transfection, cells were plated in 12-well falcon flasks to 150,000 cells/well. Transfections of siRNAs were performed using HiPerFect (QIAGEN) according to the manufacturer's protocol. 72 h after transfection, assay of enzymatic activity was performed and cells were harvested for total RNA extraction and Western blot analysis. Two siRNAs were tested and HS_CYP19A1_1 that showed a more potent inhibitory effect has been chosen for further experiments. Experiments have been performed in triplicate. Negative control had been done using AllStars Negative Control.

2.5. RNA extraction and quantification by real-time PCR

Total RNA was extracted from 1×10^6 cells using RNeasy mini kit (QIAGEN) according to the manufacturer's protocol. Five micrograms of total RNA was converted to cDNA by incubation at 42 °C for 2 h with 200 U SuperScript II reverse transcriptase (Invitrogen), using oligo-d(T)24 as primer in a reaction buffer containing 50 mM Tris–HCl pH 8.3, 75 mM KCl, 3 mM $MgCl_2$, 10 mM DTT and 0.5 mM dNTPs. cDNA was purified with QIAquick PCR purification kit (Qiagen). Quantification of mRNA levels of steroidogenic enzymes was performed using an improved real-time PCR method as previously described [28].

3. Results

3.1. mRNA expression levels of steroidogenic enzymes in JEG-3 cells

To identify the mRNA encoding the specific steroidogenic enzymes involved in the pathway of the transformation of DHEA into E2 in JEG-3 cells, we have quantified the mRNA expression levels of P450scc, P450c17, aromatase, sulfatase, 3β-HSDs, 17β-HSDs

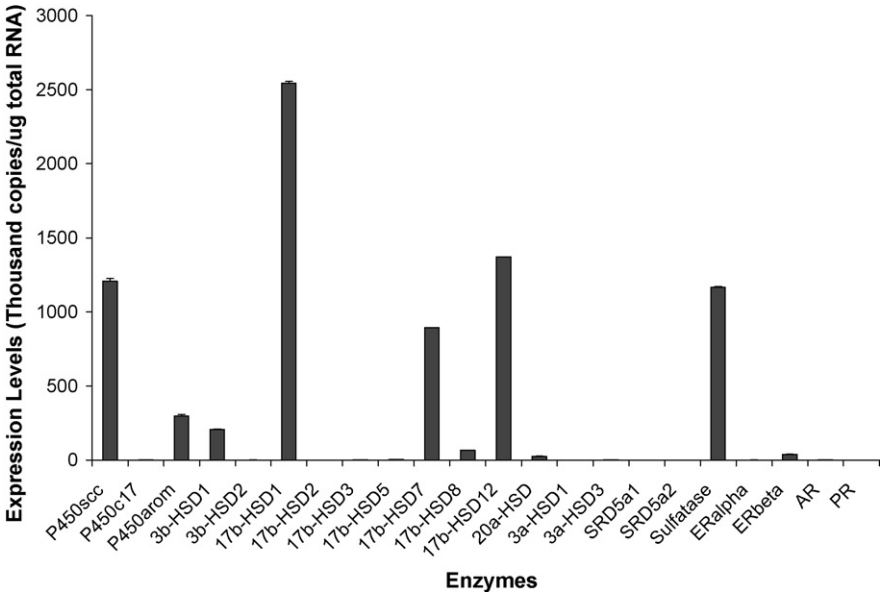

Fig. 2. mRNA expression levels of steroidogenic enzymes in JEG-3 cells. Graphs showing mRNA expression levels of steroidogenic enzymes in JEG-3 cells quantified by real-time PCR. Experimental procedures were as described in Section 2. Data are expressed as means ± S.D. of triplicate measurements.

and 5α-reductases in JEG-3 cells using real-time PCR. As shown in Fig. 2, the enzymes P450scc, aromatase, sulfatase, 3β-HSD1, 17β-HSD1, 7 and 12 are highly expressed. The high expression levels of P450scc and 3β-HSD1 and the absence of P450c17 are in agreement with the observation that the human placenta can produce progesterone from cholesterol but is unable to synthesize DHEA. On the other hand, the high levels of sulfatase, an enzyme catalyzing the transformation of DHEAS into DHEA, is also in agreement with the observation that the maternal placenta use DHEAS provided by the fetus to produce DHEA, the later is further transformed into E2. The high expression levels of 3β-HSD1, aromatase and estrogenic 17β-HSDs along with the absence of 5α-reductases confirms the main role of the placenta is to produce active estrogens not androgens using DHEA as substrate.

3.2. Profile of DHEA transformation in JEG-3 cells

As illustrated in Fig. 3, JEG-3 cells possess very high 3β-HSD activity causing a rapid decrease of the DHEA concentration. Approximately, 85% DHEA is metabolized after only 6 h of incubation while it is metabolized almost completely at 12 h. Androstenedione accumulates at 3 and 6 h of incubation and is totally metabolized after 12 h of incubation. Concomittant with the decrease of DHEA and 4-dione, there is an increased accumulation of E2 that is the end product.

3.3. Effect of 3β-HSD inhibitor

As illustrated in Fig. 4, in the presence of Epostane, a potent 3β-HSD activity inhibitor [25], there is no transformation of DHEA. The data confirm that the production of 4-dione and E2 described above requires prior transformation of DHEA into 4-dione by 3β-HSD and subsequent transformation of 4-dione into E2.

3.4. Effect of aromatase inhibitor and siRNA

Aromatase is the enzyme that catalyzes the transformation of 4-dione into E1 and T into E2. There is, however, some controversy about whether the main activity of aromatase in the pathway of E2 biosynthesis is to transform 4-dione into E1 or T into E2. To elucidate this controversy, we have incubated JEG-3 cells with [^{14}C]DHEA in the presence of 1 μM formestane, a well known aromatase inhibitor [26]. As illustrated in Fig. 5A, in the presence of formestane, the

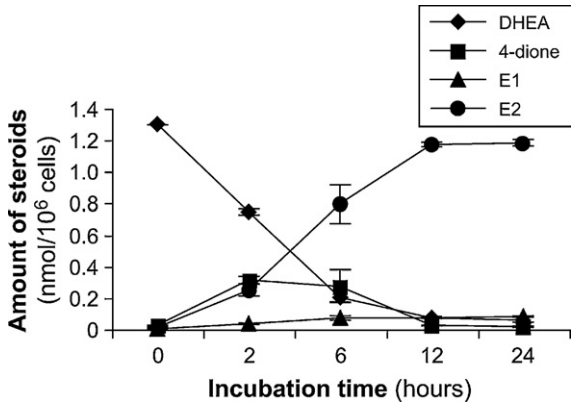

Fig. 3. Transformation of [^{14}C]DHEA in JEG-3 cells. Curves showing metabolic profiles of 0.2 μM of [^{14}C]DHEA incubated for 2, 6, 12 and 24 h with JEG-3 cells cultured in 12-well plates. Experimental procedures were as described under Section 2.

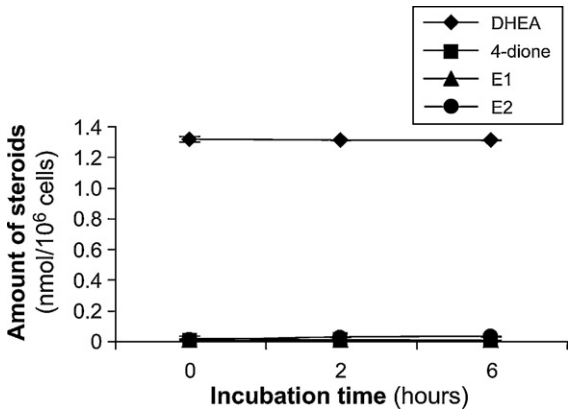

Fig. 4. Effect of the 3β-HSD inhibitor epostane (A) on the transformation of DHEA in JEG-3 cells. The experimental procedures were as described under Fig. 2, except that 1 μM of epostane, an inhibitor of 3β-HSD was added 10 min before the substrate [^{14}C]DHEA. Data are expressed as means ± S.D. of triplicate measurements.

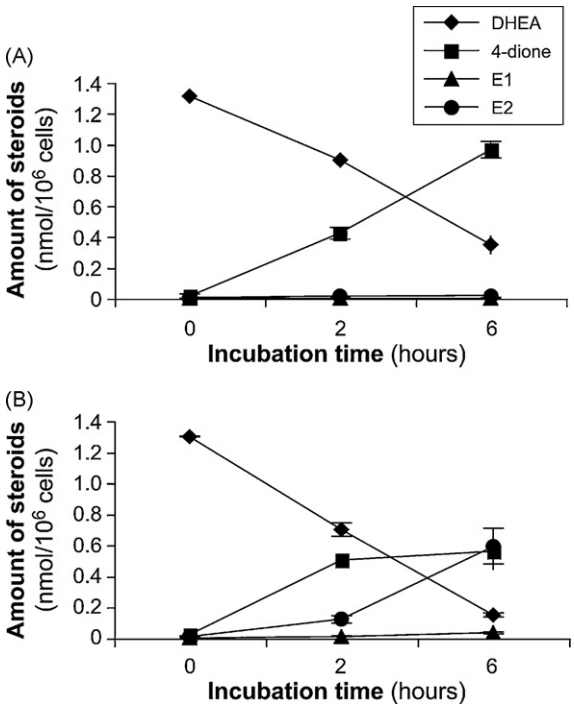

Fig. 5. Effect of aromatase inhibitor formestane (A) and siRNA (B) against aromatase on the transformation of DHEA in JEG-3 cells. Experimental procedures were as described under Fig. 2, except that 1 μM of formestane, an inhibitor of aromatase, was added 10 min before the substrate [^{14}C]DHEA. siRNA-mediated knock-down of aromatase (B) was performed as described under Section 2. Data are expressed as means ± S.D. of triplicate experiments.

production of E2 is reduced to almost zero. There is, however, an accumulation of 4-dione but not T. The data thus indicate that the substrate of aromatase is 4-dione and not T. To further confirm this observation, we have replaced formestane with siRNA against aromatase (Fig. 5B). In agreement with the data using formestane, there is accumulation of 4-dione whereas T is absent. With siRNA, there is also an accumulation of E2. This is most probably due to a weaker inhibitory effect of aromatase siRNA than formestane, a chemical inhibitor.

3.5. *Effect of 17β-HSD1 inhibitor*

The data described above strongly suggest that in the pathway of E2 biosynthesis, aromatase converts 4-dione into E1 that is further converted into E2 by estrogenic 17β-HSD. The very high mRNA expression levels of 17β-HSD1 in JEG-3 cells, strongly suggest that this enzyme is the major enzyme that catalyzes the transformation of E1 into E2. To further validate this hypothesis, we have incubated [^{14}C]DHEA in the presence of JEG-3 cells and 1 μM of CC156, a 17β-HSD1 inhibitor, graciously provided by Dr Donald Poirier [27]. As illustrated in Fig. 6, in the presence of CC156, E1 is accumulated, thus confirming that E1 is the substrate of 17β-HSD1.

4. Discussion

The present data, using [14C]DHEA as precursor, JEG-3 cells and inhibitors of 3β-HSD, aromatase and 17β-HSD1, clearly establish that the pathway of E2 biosynthesis involves the sequential steps catalyzed by 3β-HSD, aromatase and estrogenic 17β-HSDs, with the aromatase step preceding estrogenic 17β-HSD step (Fig. 7). Consequently, this pathway does not require T as intermediate. The step of aromatization of T to E2, as traditionally believed is thus not an essential step. The data are also in agreement with the observations

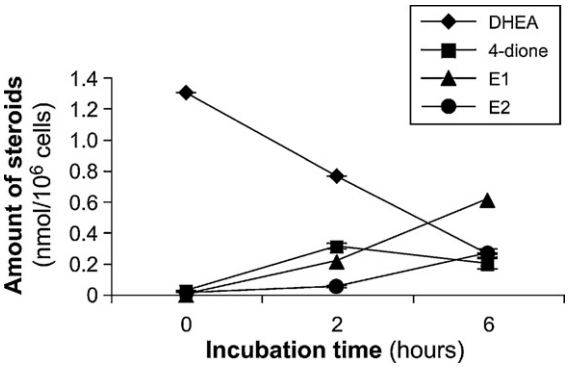

Fig. 6. Effect of type 1 17β-HSD inhibitor CC156 on the transformation of DHEA in JEG-3 cells. The experimental procedures were as described under Fig. 2, except that the 1 μM of CC156, an inhibitor of 17β-HSD1, was added 10 min before the substrate [^{14}C]DHEA. Data are expressed as means ± S.D. of triplicate measurements.

obtained using siRNA against aromatase (Fig. 5B). However, the inhibitory effect by siRNA is much less potent than with inhibitors (Fig. 5A, giving rise to reduced activities and metabolism profiles. Since this work has been performed using choriocarcinoma cells, in which membrane fluidity could be different from that in non-transformed cells and that this change could affect steroid metabolism, the pathway described in the present manuscript may not reflect the one of the normal cells. However, the higher affinity of aromatase for 4-dione than T [19,20] and the existence of estrogen-specific 17β-HSDs [12,21,23,24,29] are in favor of the existence of a pathway that does not require T as intermediate in normal cells. Although, it has been described that 17β-HSD1 could metabolize DHEA [30], this activity is relatively very weak in comparison to the metabolism of E1. The transformation of DHEA could be observed in vitro when a high amount of partially purified and purified 17β-HSD1 are used, but it is not observed in JEG-3 cells in culture where the amount of the enzyme is very low and there is competition with E1 that is produced by the cell from DHEA.

JEG-3 cells are, in fact, a very good model for steroidogenesis studies in the placenta, since they express at a high level the specific steroidogenic enzymes found in the placenta. In JEG-3 cells, 3β-HSD1 is expressed at 206 456 copies/μg of total RNA, a much

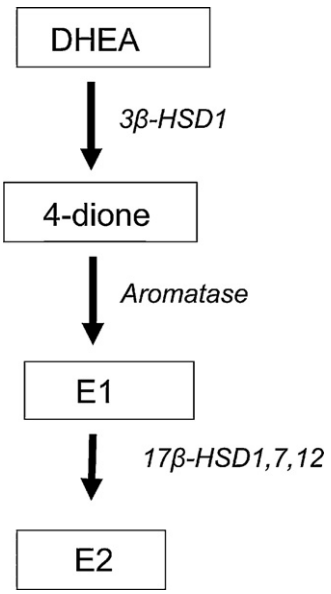

Fig. 7. Proposed E2 biosynthetic pathway that does not require the production of testosterone in JEG-3 cells.

higher level than 3β-HSD2 that is found at a non-significant level (357 copies/μg of total RNA). These data are in agreement with previous findings that 3β-HSD1 is specifically expressed in the placenta and skin [31–33], while 3β-HSD2 is expressed specifically in the adrenals and gonads [31].

Aromatase is expressed at 298 334 copies/μg of total RNA in JEG-3 cells, in agreement with the very high aromatase activity in JEG-3 cells and also in the human placenta. On the other hand, 17β-HSD1 is expressed at 2 543 963 copies/μg of total RNA, while the two other estrogenic 17β-HSDs, namely 17β-HSD7 and 12 are expressed at the lower levels of 893 012 and 1 371 739 copies/μg of total RNA, respectively. These levels are still relatively high in comparison to others steroidogenic enzymes. It is remarkable that there is usually a good correlation between mRNA expression levels and enzymatic activity [34]. The higher expression levels and efficiency of 17β-HSD1 as compared to 17β-HSD7 and 12 [24] indicate that the main estrogenic activity in JEG-3 cells is due to 17β-HSD1. Since 17β-HSD7 and 12 are multifunctional enzymes that are also involved in cholesterol biosynthesis [35] and fatty acid elongation [36], respectively, it is likely that in JEG-3 cells, they could play a role in cholesterol biosynthesis and fatty acid elongation, respectively. However, when 17β-HSD1 is inhibited, these enzymes could also exert their estrogenic 17β-HSD activity catalyzing the transformation of E1 into E2. This could explain why there is still a relative high production of E2 in the presence of a 17β-HSD1 inhibitor (Fig. 6). On the other hand, 17β-HSD1 is located in the cytoplasm [11]. Its protein levels and accessibility are much higher than those of 17β-HSD7 and 12 that are located in the endoplasmic reticulum [29,36]. These are most probably responsible for the much higher activity of 17β-HSD1, in comparison to 17β-HSD7 and 12 [24]. It is worth noting that, in contrast with unicellular organism, in human and multicellular organisms, an enzyme having higher activity does not mean that it possesses more important physiological function. For example, human type 2 3β-HSD that catalyzes the activity 10-fold less efficient than type 1 [31] is the one that is responsible for congenital adrenal hyperplasia due to 3β-HSD deficiency [37].

Estrogenic 17β-HSD activity is catalyzed by three estrogenic 17β-HSD enzymes, namely types 1, 7 and 12 17β-HSDs (17β-HSD1, 7 and 12), encoded by three distinct genes (7–11) that are expressed in a tissue-specific manner. These enzymes possess very different structure (approximately 20% amino acid identity). Thus, inhibition of estrogen production through estrogenic 17β-HSD is more tissue specific than aromatase that is encoded by a single gene, but containing multiple promoters that drive the tissue-specific expression of a unique protein. In addition, these enzymes are also involved in the E2 biosynthetic pathway using estronesulfate (E1S) as precursor. Estrogenic 17β-HSDs could thus represent interesting targets for the treatment of estradiol-sensitive diseases. Interestingly, inhibitors of 17β-HSD1, 7 and 12 have been found to inhibit estrogenic 17β-HSD activity in various cancer cell lines [38].

It is noteworthy, that the action of E2 in JEG-3 cells is most probably mediated by ER-β, since the later is significantly expressed ($38{,}000 \pm 1000$ copies/μg total RNA) while ER-α is expressed at a non-significant level (500 ± 700 copies/μg total RNA).

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