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Human type 3 5α-reductase is expressed in peripheral tissues at higher levels than types 1 and 2 and its activity is potently inhibited by finasteride and dutasteride

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Abstract

5α-Reductases are crucial enzymes involved in the biosynthesis of dihydrotestosterone, the most potent natural androgen. To date, three types of 5α-reductases, chronologically named types 1, 2 and 3 5α-reductases (SRD5a-1, 2 and 3) have been described. In the present paper, we characterized the activity and compared the mRNA expression levels of SRD5a-3 with those of SRD5a-1 and 2 in various human tissues, and determined its sensitivity to finasteride and dutasteride. We have established HEK-293 cell line that stably expressed SRD5a-3 for studying its activity and the inhibitory effect of finasteride, using [^{14}C]labeled steroids. mRNA expression levels were quantified using real-time PCR in many male and female human tissues including the prostate, adipose tissue, mammary gland, as well as breast and prostate cancer cell lines. Incubation of HEK-SRD5a-3 cells with [^{14}C]4-androstenedione and [^{14}C]testosterone allowed us to show that SRD5a-3 can catalyze very efficiently both substrates 4-androstenedione and testosterone into 5α-androstanedione and dihydrotestosterone, respectively. We observed that the affinity of the enzyme for 4-androstenedione is higher than for testosterone. The activity of SRD5a-3 and SRD5a-2 are similarly sensitive to finasteride, whereas dutasteride is a much more potent inhibitor of SRD5a-3 than SRD5a-2. Tissue distribution analysis shows that SRD5a-3 mRNA expression levels are higher than those of SRD5a-1 and SRD5a-2 in 20 analyzed tissues. In particular, it is highly expressed in the skin, brain, mammary gland and breast cancer cell lines, thus suggesting that SRD5a-3 could play an important role in the production of androgens in these and other peripheral tissues.

Keywords: androgen; dihydrotestosterone; dutasteride; finasteride; steroidogenesis; SRD5a-3; type 3 5α-reductase.

Introduction

Androgens play a crucial role in the development, growth and function of the prostate, as well as other androgen-sensitive peripheral tissues (1). In the prostate gland, androgens are involved in two common diseases, namely benign prostatic hyperplasia (BPH) which affects the majority of aging men and prostate cancer, which is diagnosed in one out of eight men during their lifetime. Androgens are also involved in skin disorders such as acne, seborrhea, androgenic alopecia and hirsutism. One objective for the treatment of these diseases is, therefore, to reduce androgen levels through inhibition of the enzymes involved in their biosynthesis, especially the 5α-reductases.

Steroid 5α-reductases are enzymes that are able to catalyze the reduction of 4-androstenedione (4-dione), testosterone (T) and progesterone (P), as well as other 4-ene-3-ketosteroids into their corresponding 5α-dihydro-3-ketosteroids. The best known 5α-reduced steroid is dihydrotestosterone (DHT), the most potent natural androgen responsible for the differentiation of male organs, including the prostate, as well as virilization of boys at puberty (1). Traditional literature indicates that DHT is produced by 5α-reduction of T. Recent data obtained by our group (2, 3), however, indicate that DHT biosynthesis does not require T as intermediate in prostate cancer cells (DU-145) and sebaceous gland cells (SZ-95).

Eighteen years ago, cDNAs (4, 5) and genes (6, 7) encoding types 1 and 2 of 5α-reductases (SRD5a-1 and SRD5a-2) were cloned and characterized. SRD5a-1 and SRD5a-2 share 48.4% amino acid sequence identity and possess similar substrate specificity. However, these two enzymes show different pH optima and sensitivity to inhibitors. SRD5a-1 possesses a broad pH optimum (pH 6–8.5), whereas SRD5a-2 shows a narrow acidic pH optimum centered around 5 (4, 6). In addition, SRD5a-1 is approximately 10-fold less sensitive to finasteride (Proscar) than SRD5a-2 (4), but it is more sensitive to cations (8). It has also been shown that SRD5a-1 catalyzes the transformation of 4-dione more efficiently than T (4, 8), thus suggesting that this enzyme could

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be involved in a pathway of DHT biosynthesis independent from the production of T (9).

Recently, a third type of 5α-reductase (SRD5a-3) has been described (10). The enzyme efficiently catalyzes the transformation of T into DHT and has been reported to be highly expressed in the pancreas and refractory prostate cancers, as well as in prostate cancer cell lines (10). Genomic sequence analysis (GenBank accession numbers: NM_024596 and NT_022853) indicates that the human SRD5a-3 gene contains five exons separated by four introns. The position of the introns is essentially identical to that of the SRD5a-1 and SRD5a-2 genes, thus suggesting that these three genes originate from primordial gene duplication. It is worth noting that most amino acids responsible for 5α-reductase activity deficiency found in SRD5a-2 are conserved in SRD5a-3 (Figure 1).

In the present paper, we further characterize the enzymatic properties of SRD5a-3 and its tissue distribution. We found that SRD5a-3 is highly sensitive to finasteride and dutasteride and shows similar sensitivity as SRD5a-2 for finasteride, while it is much more sensitive to dutasteride. The enzyme is highly expressed in the human skin, brain, mammary gland and breast cancer cells. Our data strongly suggest that SRD5a-3 could be the main enzyme responsible for 5α-reductase activity, previously believed to be associated with SRD5a-1, in many tissues and cell lines.

Materials and methods

Construction of HEK-293 cells stably expressing human SRD5a-1, 2 and 3

Using the prostate cDNA library and commercially available prostate RNA (Ambion Inc., Austin, TX, USA), we amplified cDNA fragments of the coding region of SRD5a-1, 2 and 3 with the following oligonucleotide primer pairs, namely (5′-cga-att-cca-cca-tgg-caa-cgg-cga-cgg-gggt-3′) and (5′-cgg-aat-tcg-cac-tta-aaa-caa-aaa-tgg-aat-3′); (5′-gga-att-cgg-cgc-gat-gca-ggt-tca-gtg-cca-g-3′) and (5′-ggg-gtc-gac-cat-ggc-tcc-ctg-ggc-gga-ggc-cga-g-3′) and (5′-ggg-tct-aga-tta-aaa-caa-aaa-tgg-tag-gaa-agc-tt-3′) and (5′-aat-ccc-cag-gcc-agc-tgg-cag-3′), respectively. Oligonucleotide sequences were derived from GenBank database sequences with accession numbers NM_001047, NM_000348 and NM_024592, respectively. The resulting amplified cDNA fragments were then subcloned into a pCMVneo vector and the resulting plasmids transfected into HEK-293 cells using Exgen 500 (MBI Fermentas, NY, USA). HEK-293 cells stably expressing SRD5a-1, 2 and 3 were selected among positive clones which are resistant to G418 as previously described (11).

Assay of enzymatic activity

The enzymatic activity was determined using intact cells in culture, as previously described (12). Briefly, 0.1 μM of the [^{14}C]labeled 4-dione and T (specific activity 56 mCi/mmol) (American Radiolabeled Chemicals Inc., St. Louis, MO, USA) was added to 12-well

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SRD5a-3 MAPWAE*AEHS ALNPLRAVWL TLTAAFLLTL LLQLLPPGLL PGCAIFQDLI        50
SRD5a-2 -QVQCQQ**** ********** ********** ********** **********        7
SRD5a-1 -ATATGV--ER L-******** ********** ********** **********        12

                                                  +
SRD5a-3 RYGKTKCGEP SRPAACRAFD VPKRYFSHFY IISVLWNGFL LWCLTQSLFL        100
SRD5a-2 ********** -PVL-GS-TL -ALGALALYV AKPS***-YG KHTESLKPA*        43
SRD5a-1 ********** LAAL-YLQCA -GCAV-ARNR QTNS***VYG RHA**LPSH*        46

                    +                                         +
SRD5a-3 GAPFPSWLHG LLRILGAAQF QGGELALSAF LVLVFLWLHS **LRRLFECLY        148
SRD5a-2 ATRL-ARAAW F-QE-PS**- AVPAGI-ARQ PLSL-GPPGT *V-LG--CLH-        91
SRD5a-1 RLRV-ARAAW VVQE-PS**- ALPLYQYASE SAPRLRSAPN CI-LAM-LVH-        95


SRD5a-3 VSVFSNVMIH VV**Q*YCFG LVYYVLVGLT VLSQVPMDGR NAYITGKNLLM        197
SRD5a-2 FHRTFVYSLL NR*GRPYPAI -ILRG*TAFC TGNG-LQGYY LI-CAEYPDGW        140
SRD5a-1 GHRCLIYPFL MRGGKPMPLL ACTMA*IMFC TCNGYLQSRY LSHCAVYADDW        145

                                          +                + +
SRD5a-3 QARWFHILGM MMFIWSSAHQ YKCHVILGNL RKNKAGVVIH CNHRIPFGDWFEY      250
SRD5a-2 YTDIRFSL-V FL--LGMGIN IHSDY--RQ- --P**-EISY ***---Q-GL-T-      188
SRD5a-1 VTDPRFLI-F GLWLTGMLIN IHSDH--RN- --P**-DTGY ***K--R-GL-E-      193

           +   +                                          +  +
SRD5a-3 VSSPNYLAEL MIYVSMAVTF GFHNLTWWLV VTNVFFNQAL SAFLSHQFYK         300
SRD5a-2 ---A-F-G-I IEWIGY-LAT WSLPALAFAF FSLC-LG**- R--HH-R--L        236
SRD5a-1 -TAA-YFG-I -EWCGY-LAS WSVQGAAFAF F-FC-LS**G R-KEH-EW-L        241

                 +
SRD5a-3 SKFVSYPKHR KAFLPFLF                                           318
SRD5a-2 KM-ED---S- --LI--I-                                           254
SRD5a-1 RK-EE---F- -III--L-                                           259
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Figure 1 Alignment of amino acid sequences of the human steroid 5α-reductases SRD5a-1, SRD5a-2 and SRD5a-3.
(–) Indicates identical amino acid, whereas (*) indicates missing amino acid. (+) Indicates conserved amino acids that are affected by mutations in patients having a mutated SRD5a-2 gene. Amino acids are numbered on the right.

culture plates containing 1 mL of culture medium per well. After 24 h of incubation, the medium was removed, steroids were extracted twice with 1 mL ethyl-ether and the metabolites analyzed by thin layer chromatography (TLC). The organic phases were pooled and evaporated to dryness. Steroids were solubilized in 100 μL methylenechloride and separated on Silica gel 60 TLC plates (Merck, Darmstadt, Germany), using the toluene/acetone (4:1) solvent system. Substrates and metabolites were identified by comparison with reference steroids and quantified by a PhosphoImager Storm 860 system (Molecular Dynamics Inc., Sunnyvale, CA, USA).

RNA quantification by real-time PCR

Total RNA was obtained from Ambion, Inc. or extracted from 1×10^6 cultured cells using a RNeasy mini kit (Qiagen, Mississauga, Canada) according to the manufacturer's protocol. Then, 5 μg 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 dithiothreitol and 0.5 mM dNTPs. cDNA was purified with a QIAquick PCR purification kit (Qiagen). Quantification of mRNA levels was performed by a real-time PCR method on the LightCycler Real-Time PCR apparatus (Hoffman-La Roche Inc., Nutley, NJ, USA) using SYBR Green and second derivative detection of crossing point and double correction as previously described (13). In brief, a cDNA amount corresponding to 30 ng of initial total RNA was used to perform fluorescent-based real-time PCR quantification. Reagents obtained from the same supplier were used as described by the manufacturer. The conditions for the PCR reactions were: denaturation at 94°C for 15 s, annealing at 55°C for 10 s and elongation at 72°C for 35 s. The data were normalized using the mRNA expression levels of a housekeeping gene, namely ATP5o (subunit O of ATPase) as internal standard. The mRNA expression levels are expressed as numbers of copies/μg total RNA using a standard curve of Cp vs. logarithm of the quantity. The standard curve is established using known cDNA amounts of 0, 10^2, 10^3, 10^4, 10^5 and 10^6 copies of ATP5o and a LightCycler 3.5 program provided by the manufacturer (Roche Inc., Nutley, NJ, USA).

Results

Amino acids involved in SRD5a-2 deficiency are conserved in SRD5a-3

Mutations of many amino acids in the SRD5a-2 sequence have been found to cause male pseudohermaphroditism due to 5α-reductase activity deficiency (5). These amino acids are thus likely to be crucial for the activity of SRD5a-2. Interestingly, as shown in Figure 1, these amino acids are conversed in the SDR5a-3 sequence, suggesting that the two enzymes possess similar specificity of activity and substrates. It is also observed that SRD5a-3 possesses ~50 additional amino acids in the NH2-terminal compared with SRD5a-1 and SRD5a-2, while it shares an overall homology of 20% identity in the NH2-terminal region. In addition, SRD5a-3 shares 50% and 54% identity, respectively, with SRD5a-1 and SRD5a-2 in the C-terminal that contains the cofactor binding site.

SRD5a-3 possesses higher affinity for 4-dione than T

Recently, it has been shown that the pathway of DHT biosynthesis in the prostate cancer cell line DU-145 (2) and the transformed sebaceous gland cell line SZ95 (3) does not require T as intermediate, as traditionally believed. This is in agreement with the higher affinity and activity of SRD5a-1 and SRD5a-2 for 4-dione than T (4, 8, 14). To determine whether SRD5a-3 also possesses similar relative affinity of 4-dione and T, we have determined the K_m value of SRD5a-3 using stably transfected HEK-293 cells in culture. As illustrated in Table 1, SRD5a-3 also shows an approximately 2-fold higher affinity for 4-dione than T.

SRD5a-3 is inhibited by finasteride and dutasteride

Finasteride and dutasteride are well-known inhibitors of 5α-reductase activity. Although finasteride more specifically inhibits SRD5a-2 (5) compared to dutasteride, it most strongly inhibits both SRD5a-3 and SRD5a-1 (15, 16). To determine the effect of these inhibitors on SRD5a-3 activity, we compared their inhibitory effects using HEK-293 cells stably transfected with SRD5a-1, 2 and 3 expressing vectors. As illustrated in Figure 2 and Table 2, finasteride inhibits SRD5a-3 with similar potency SRD5a-3 ($IC_{50}=17.4$ nM) and SRD5a-2 ($IC_{50}=14.3$ nM). By contrast, dutasteride is approximately 50 times more potent to inhibit SRD5a-3 ($IC_{50}=0.33$ nM) than finasteride ($IC_{50}=17.4\pm1.4$ nM). For

Table 1 K_m values of 4-dione and T for SRD5a-1, 2 and 3.

Enzyme	K_m, μM[a]	
	4-Dione	T
SRD5a-1	3.5±0.5	12.0±1.8
SRD5a-2	0.55±0.05	1.0±0.2
SRD5a-3	6.0±0.9	14.1±1.2

[a]K_m values are expressed as mean±SD of triplicate incubations. HEK-293 cells stably transfected with SRD5a-1, SRD5a-2 and SRD5a-3 in culture were used.

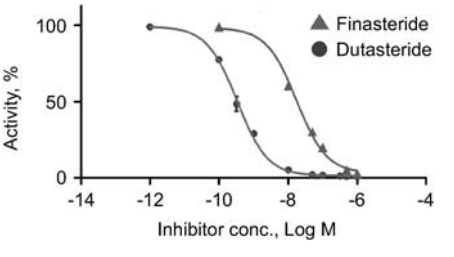

Figure 2 Graph showing inhibitory effects of finasteride and dutasteride on SRD5a-3 activity.
HEK-293 cells stably transfected with SRD5a-3 were used to determine SRD5a-3 activity using 0.1 μM [^{14}C]4-dione as substrate. Finasteride and dutasteride were added at the indicated concentrations to determine their potency. Incubation, enzymatic assay and IC_{50} determinations were performed as described in the Materials and methods section.

Table 2 IC_{50} values of finasteride and dutasteride inhibition for SRD5a-1, 2 and 3.

Enzyme	IC_{50}, μM[a]	
	Finasteride	Dutasteride
SRD5a-1	106.9 ± 17.1	8.7 ± 0.4
SRD5a-2	14.3 ± 2.7	57.0 ± 6.8
SRD5a-3	17.4 ± 1.4	0.33 ± 0.02

[a]IC_{50} values are expressed as mean ± SD of triplicate incubations. HEK-293 cells stably transfected with SRD5a-1, SRD5a-2 and SRD5a-3 in culture were used.

SRD5a-1, dutasteride is approximately 12 times more potent (IC_{50} = 8.7 nM) than finasteride (IC_{50} = 106.9 ± 17.1 nM). For type 2 5α-reductase, finasteride (IC_{50} = 14.3 ± 2.7 nM) is approximately four times more potent than dutasteride (IC_{50} = 57.0 ± 6.8 nM).

Comparison of SRD5a-3 mRNA expression levels with SRD5a-1 and SRD5a-2

To further assess the relative role of SRD5a-3 compared to SRD5a-1 and SRD5a-2, we quantified the mRNA expression levels of these enzymes in 20 human tissues (Figure 3A,B), as well as in prostate and breast cancer lines (Figure 4) using real-time PCR. As illustrated in Figure 3A,B, except for BPH and muscle, SRD5a-3 is more highly expressed than SRD5a-1 and SRD5a-2 in all tissues, including the normal prostate, mammary gland, brain, skin and adipose tissue. In addition, SRD5a-3 is also more highly expressed in human prostate and breast cancer cell lines.

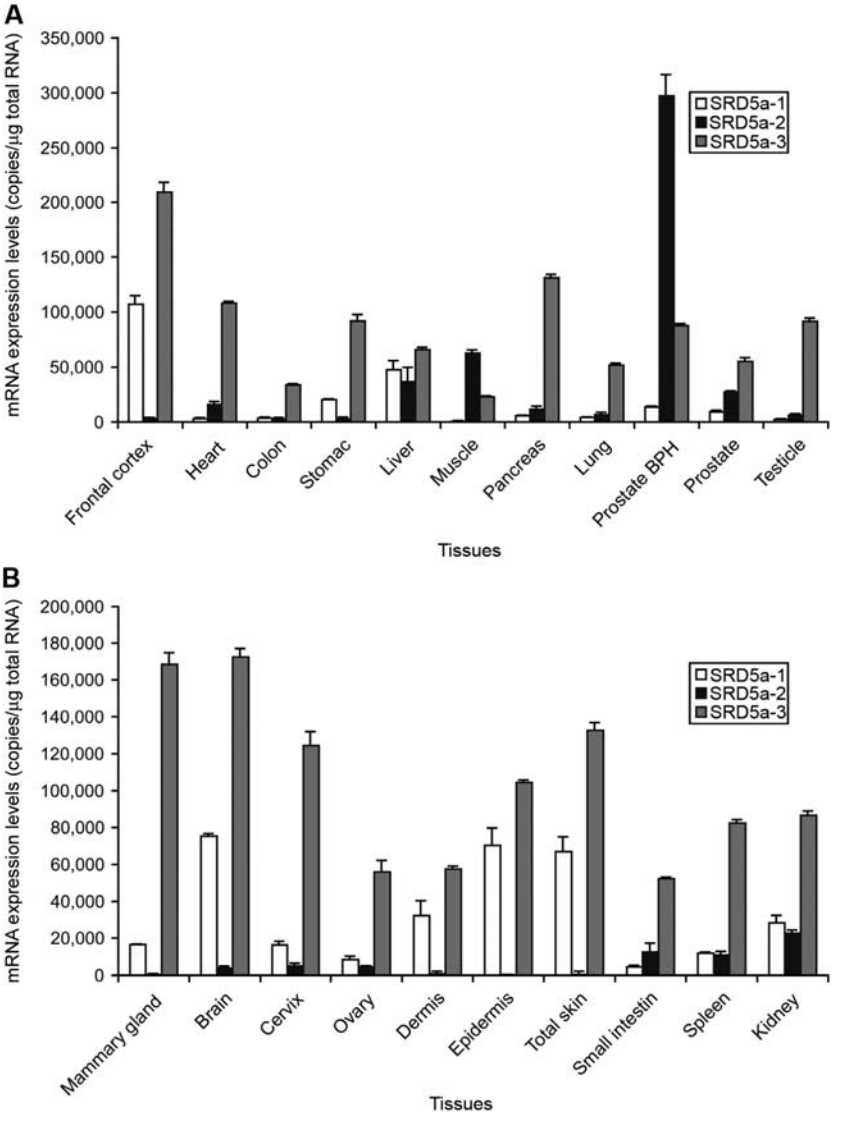

Figure 3 Comparison of mRNA expression levels of SRD5a-1, 2 and 3 in human tissues.
Commercially available total RNA of male (A) and female (B) tissues was used to quantify mRNA expression levels of SRD5a-1, 2 and 3 by real-time PCR as described in the Materials and methods section. Data are expressed as mean ± SEM of triplicate measurements.

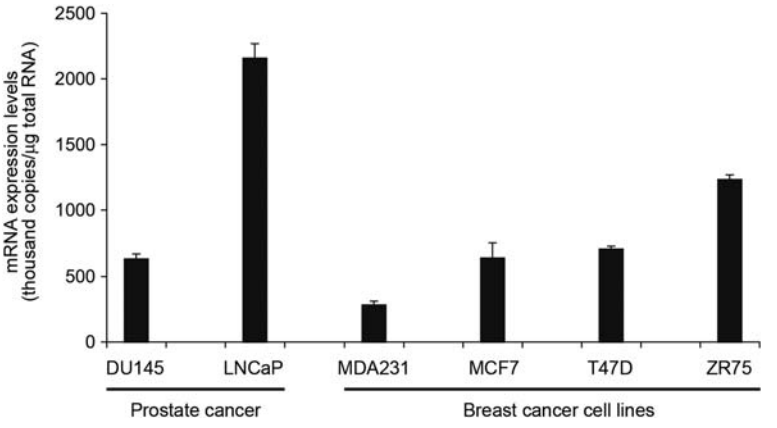

Figure 4 Comparison of mRNA expression levels of SRD5a-1, 2 and 3 in prostate and breast cancer cell lines.
Total RNA from prostate and breast cancer cell lines was extracted and used to quantify mRNA expression levels of SRD5a-1, 2 and 3 by real-time PCR method as described in the Materials and methods section. Data are expressed as mean ± SEM of triplicate measurements.

Discussion

Recently, a third type of 5α-reductase (SRD5a-3) was described (10). The enzyme efficiently catalyzes the transformation of T into DHT and was reported to be highly expressed in the pancreas, refractory prostate cancer, prostate cancer cell lines (10), as well as normal prostate samples (Figure 3A). Genomic sequence analysis (Genbank accession numbers: NM_024596 and NT_022853) indicates that the human SRD5a-3 possesses the same genomic structure as SRD5a-1 with genes having five exons separated by four introns with essentially identical intron/exon splicing positions. The results strongly suggest that these three genes derive from primordial gene duplication. However, 5α-Red-3 contains 48 additional amino acids in the NH2-terminal sequence which are most probably part of a secretory signal.

It is worth noting that most of the mutated amino acids in the 5α-Red-2 gene that reduce 5α-reductase activity in pseudohermaphrodite patients are conserved in the 5α-Red-3 sequence (Figure 1), thus suggesting that 5α-reductase activity is conserved in the SRD5a-3 enzyme. In addition, although altered SRD5a-1 and 2 genes in the mouse do not cause lethality, SRD5a-3 knockout mice, obtained by Lexicon Genetics, gene trap703 (MGI:1930252) produces post-

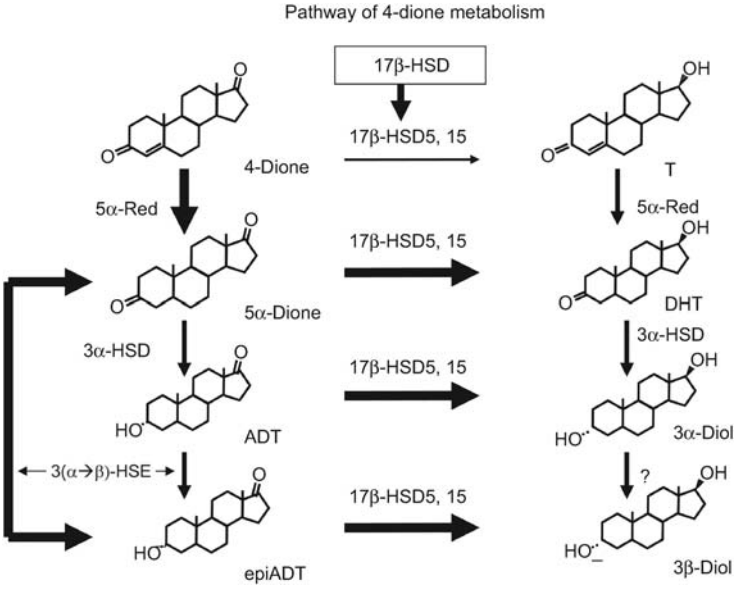

Figure 5 Proposed pathway of 4-androstenedione metabolism that does not require T as intermediate.
Only forward reactions are described in the present figure for clarity purpose. Thick arrows indicate preferred reactions. It is noteworthy that conventional 3β-HSD types 1 and 2 that catalyze the transformation of DHEA to 4-dione possess oxidative 3β-HSD activity. In cell culture, they catalyze the transformation of 3β-diol to DHT but not the reduction of T to 3β-diol in intact cells in culture (23). 3(α → β)-HSE is able to catalyze the transformation of ADT to epiADT as well as 5α-dione to epiADT (24). The 3β-HSD reductase activity of 3(α → β)-HSE that catalyzes the transformation of 5α-dione to epiADT is specific for 5α-dione. The activity of this enzyme for compounds having 17β-hydroxy group such as DHT and 3α-diol is negligible (V. Luu-The, unpublished data).

natal lethal death in mice. These data could suggest that SRD5a-3 possesses additional activity not involved in steroidogenesis but exists in ortholog enzymes in invertebrates, such as yeast and *Caenorhabditis elegans*, and is encoded by an unidentified common ancestor gene.

It is noteworthy that androgen and estrogen receptors and their ligands only appeared immediately before or during the precambrian period with vertebrates (17–19). Accordingly, enzymes expressed in invertebrates and able to transform androgens and estrogens are not necessarily involved in sex steroid biosynthesis. Similar situations have been observed for types 3 and 12 17β-HSD that catalyze the transformation of 4-dione to T and E_1 to E_2, respectively. These duplicated genes share 45% amino acid identity and the same genomic structure (11). In addition, whereas mutated type 3 17β-HSD genes cause pseudohermaphroditism but no lethality, altered type 12 17β-HSD gene in knockout mice causes embryonic lethality. The lethality is most probably caused by alteration of 3-ketoacyl-CoA reductase activity involved in long chain fatty acid elongation. This activity is encoded by ortholog genes (YBR159w and LET-767) found in the yeast *Saccharomyces cerevisiae* (20) and *C. elegans* (21), respectively. It is noteworthy that, although sex steroids are not functional in *C. elegans*, LET-767 catalyzes efficiently the transformation of 4-dione into T and E_1 into E_2 (22), two reactions catalyzed by types 3 and 12 17β-HSD, respectively.

The higher expression levels of SRD5a-3 than SRD5a-1 and SRD5a-2 in most of the peripheral tissues (Figure 3) and prostate and breast cancer cell lines (Figure 4) analyzed suggest that SRD5a-3 plays an important role in these tissues and cell lines. In addition, the higher affinity of the enzyme for 4-dione than T could also suggest that this enzyme is involved in the DHT biosynthetic pathway that does not require T as intermediate (Figure 5), as previously described (2, 3).

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