

Sequential transformation of 4-androstenedione into dihydrotestosterone in prostate carcinoma (DU-145) cells indicates that 4-androstenedione and not testosterone is the substrate of 5 α -reductase

Melanie Samson, Fernand Labrie and Van Luu-The*

Oncology, Molecular Endocrinology and Genomics Research Center, Laval University and Centre hospitalier universitaire de Quebec, Quebec, Canada

Abstract

Background: Although it is well recognized that 5 α -reductases possess higher affinity for 4-androstenedione than testosterone, and the affinity of 4-androstenedione is higher for 5 α -reductases than 17 β -hydroxysteroid dehydrogenases, it is generally believed that dihydrotestosterone is necessarily produced by the transformation of testosterone into dihydrotestosterone, suggesting that the step catalyzed by 17 β -hydroxysteroid dehydrogenase precedes the step catalyzed by 5 α -reductase. This interpretation is in contradiction with the enzymatic kinetic law that suggests that the 5 α -reduction step that catalyzes the transformation of 4-dione into 5 α -androstane-3,17-dione precedes the 17keto-reduction step.

Materials and methods: To verify which of these two pathways is operative, we quantified mRNA expression levels of steroidogenic enzymes in prostate carcinoma DU-145 cells by real-time PCR and determined the metabolites produced after incubation with [¹⁴C]4-dione in the presence and absence of a 5 α -reductase inhibitor and analyzed the metabolites produced by thin layer chromatography and HPLC.

Results: Real-time PCR analysis strongly suggests that the new type 3 5 α -reductase is responsible for 5 α -reductase activity in DU-145 cells. Steroid profile analysis shows that in the absence of inhibitor 5 α -androstenedione is first produced, followed by the production of androsterone and dihydrotestosterone. The concentration of testosterone was not detectable. In the presence of Finasteride, an inhibitor of 5 α -reductase, there was no transformation of 4-androstenedione and also there was no production of testosterone. The present data clearly indicate that the biosynthesis of dihydrotestos-

terone in DU-145 cells does not require testosterone as intermediate, and the step catalyzed by 5 α -reductase precedes the step catalyzed by 17 β -hydroxysteroid dehydrogenase.

Keywords: 4-androstenedione; 5 α -androstenedione; 5 α -reductase; androgen biosynthetic pathway; DU-145; Finasteride.

Introduction

Androgens play a crucial role in the development, growth, and control of function of the prostate as well as the diseases associated with the prostatic gland, such as benign prostatic hyperplasia (BPH) and prostate cancer. It is well recognized that there are two androgens, namely testosterone (T) and dihydrotestosterone (DHT). T is produced in the testis by the conversion of 4-dione into T by the enzyme type 3 17 β -HSD (17 β -HSD3) (1) and promotes the formation of the internal male reproductive structures (epididymes, seminal vesicles, and vas deferens). A defect in the HSD17B3 gene causes disorders of human intersex called male pseudohermaphroditism. The production of DHT requires a 5 α -reduction step of 3-keto-4-enestroids catalyzed by the enzyme 5 α -reductase. A defect in 5 α -reductase also causes male pseudohermaphroditism (2) with ambiguous external genitalia. However, in contrast to HSD17B3 gene deficiency, the Wolffian structures are normally differentiated (3).

DHT is mainly produced in a long series of peripheral tissues, including the liver, skin, and prostate where 5 α -reductases are expressed (4–6). T and DHT act via the same androgen receptor (AR), because a defect in AR causes the X-linked androgen-insensitive syndrome (AIS) (3, 7), in which the development of both the internal and external male reproductive structures are altered.

The above-mentioned data strongly suggest that depending upon the steroid precursors and enzymes present in the cells or tissues, AR is modulated by T or DHT. This is, somehow, in contradiction with the general belief that DHT is globally produced by 5 α -reduction of T (6, 8). In addition, the higher affinity of T for AR ($K_m \sim 10^{-8}$, 10^{-9} M) than 5 α -reductases ($K_m \sim 10^{-6}$ M) strongly suggests that in tissues that express both AR and 5 α -reductases T will preferentially bind to AR. The reduction of T to DHT step does not seem substantiated by enzymatic kinetics. This is in agreement with the finding

*Corresponding author: Dr. Van Luu-The, Oncology, Molecular Endocrinology and Genomics Research Center (CREMOG), Laval University and Centre hospitalier universitaire de Quebec (CHUQ/CHUL), 2705 Laurier Boulevard, T3-52, Quebec, QC, G1V 4G2, Canada
Phone: +33-418-654-2296, Fax: +33-418-654-2761,
E-mail: Van.luu-the@crchul.ulaval.ca
Received May 28, 2009; accepted July 28, 2009;
previously published online September 17, 2009

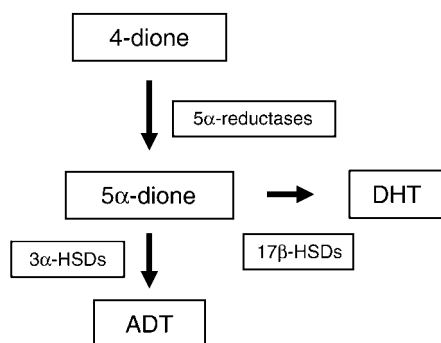


Figure 1 Schematic representation of the two last steps of 4-androstenedione (4-dione) metabolism. DHT, dihydrotestosterone; ADT, androsterone; HSD, hydroxysteroid dehydrogenase.

that 4-dione is a better substrate for 5 α -reductases than T (4, 9). Together, these data strongly suggest that DHT is produced by a pathway that does not require T biosynthesis, as suggested by Luu-The et al. (10).

According to this pathway (10), the 5 α -reductase activity step precedes the 17 β -HSD activity step (Figure 1). The profile of 5 α -reduced metabolites described in the literature (11) showing very high levels of 5 α -reduced steroids in the circulation, namely in the micromolar range, also supports the existence of a predominant pathway where the 5 α -reduction step precedes the 17keto-reduction step. Indeed, the concentration of 5 α -reduced steroids in men between 20 and 30 years (11) is highest for androsterone-sulfate (ADT-S) with \sim 1400 nM, followed by ADT-G (80 nM) > 5 α -androstane-3 β ,17 β -diol-G (3 β -diol) (\sim 47 nM) > 5 α -androstane-3 α ,17 β -diol-G (3 α -diol-G) > ADT (5 nM) > 3 β -diol (3 nM) > DHT (2.8 nM) > 3 α -diol (2.2 nM). The presence of large amounts of ADT derivatives (ADT-S and ADT-G) that contain a 17-keto group in the circulation compared with compounds having a 17 β -hydroxy group (DHT, 3 β -diol-G, 3 α -diol-G, 3 β -diol, and 3 α -diol) provides support for the pathway suggested by Luu-The et al. (10) in which ADT metabolites are produced from 5 α -dione obtained from 5 α -reduction of 4-dione.

It is worth noting that the amount of steroids measured in the blood circulation represent most probably the amount "remained after transformation" and not the amount "available for transformation". Thus, a steroid amount available for transformation is equal to the amount found in the blood plus the sum of all the metabolites produced downward in the pathway having this steroid as precursor. Controversy between the general belief and the exact steroidogenic pathway could be due to misinterpretation of this concept.

In the present report, to verify the importance of the pathway of DHT biosynthesis that does not require the T production step, we analyzed metabolites produced during incubation of [14 C]4-dione and a 5 α -reductase inhibitor and quantified the expression levels of steroidogenic enzymes present in prostatic carcinoma (DU-145) cells. Our data clearly indicate that DHT biosynthesis from 4-dione in DU-145 does not require T biosynthesis.

Materials and methods

Cell culture

The prostate carcinoma cells DU-145 obtained from ATCC (HTB-81) (Manassas, VA, USA) were maintained in MEM (Invitrogen Life Technologies, Inc.) supplemented with 10% (vol/vol) FBS (Wisent Inc., St-Bruno, QC, Canada) at 37°C under a 95% air/5% CO₂ humidified atmosphere.

5 α -reductase inhibitor

Finasteride (1 μ M), synthesized in our laboratory under the name EM-351, was used as 5 α -reductase inhibitor. It was added directly in cell culture media 10 min before addition of 4-dione.

Assay of enzymatic activity

Enzymatic activity was determined using intact cells in culture as previously described (12). Briefly, 0.1 μ M [14 C]-labeled androstenedione (specific activity 56 mCi/mmol) (American Radiolabeled Chemicals Inc., St Louis, MO, USA) was added to 6-well culture plates containing 2 mL culture medium per well. After 24 h incubation the medium was removed, steroids were extracted twice with 1 mL ethyl-ether and metabolites were analyzed by thin layer chromatography (TLC). Organic phases were pooled and evaporated to dryness. Steroids were solubilized in 100 μ L methylene chloride and separated on Silica gel 60 TLC plates (Merck, Darmstadt, Germany) using the chloroform/ethyl acetate (4:1) solvent system. Substrates and metabolites were identified by comparison with reference steroids and quantified by a PhosphorImager Storm 860 system (Molecular Dynamics, Inc., Sunnyvale, CA, USA). In our system, DHEA, 4-dione, T, E1, and estradiol (E2) are very well separated, whereas DHT and ADT are poorly resolved. They were analyzed further by high performance liquid chromatography (HPLC) as previously described (13). Briefly, using intact cells in culture, 0.2 μ M [14 C]-labeled 4-dione (American Radiolabeled Chemicals Inc.) was added to 6-well culture plates containing 2 mL culture medium per well. After 24 h incubation, metabolites were extracted and analyzed by HPLC/MS and radiolabeled HPLC profiles were recorded by the online liquid scintillation counter (Flo One Radiomatic 500TR Series; Canberra Packard). Steroids were identified using commercial standard.

RNA quantification by real-time PCR

Total RNA was extracted from 1×10^6 cells using an RNeasy mini kit (Qiagen, Mississauga, Canada) according to the manufacturer's protocol. Total RNA (5 μ g) was converted to cDNA by incubation at 42°C for 2 h with 200 U SuperScript II reverse transcriptase (Invitrogen Life Technologies, Inc.), using oligo-d(T)24 as primer in a reaction buffer containing 50 mM Tris-HCl pH 8.3, 75 mM KCl, 3 mM MgCl₂, 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 quantitative real-time PCR method on a LightCycler Realtime 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 (14). In brief, 30 ng 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 as follows: denaturation at 94°C for 15 s, annealing at 55°C for 10 s, and elon-

gation at 72°C for 35 s. Data were normalized using mRNA expression levels of a housekeeping gene, namely ATP5o (subunit O of ATPase) as internal standard. mRNA expression levels are expressed as numbers of copies/ μg total RNA using a standard curve of Cp versus 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

mRNA expression levels of steroidogenic enzymes in DU-145 cells

To determine the enzymes possibly involved in the metabolism of 4-dione and biosynthesis of DHT in DU-145 cells, we quantified mRNA expression levels of steroidogenic enzymes in these cells using real-time PCR. As shown in Figure 2, P450 dependent enzymes, such as P450scc, P450 C17, and P450 aromatase, as well as 3 β -HSDs are absent, whereas types 1 and 3 5 α -reductases and types 5, 7, and 10 17 β -HSDs are present.

Metabolism of [^{14}C]4-dione in DU-145 cells

DU-145 cells were incubated with 0.2 μM [^{14}C]4-dione and the radiolabeled steroids were analyzed at 12 and 24 h incubation using TLC and HPLC. Use of TLC permits quantification using a PhosphoImager system, whereas use of HPLC presents better separation and identification of steroids. As illustrated in Figure 3, at 12 h incubation, 4-dione

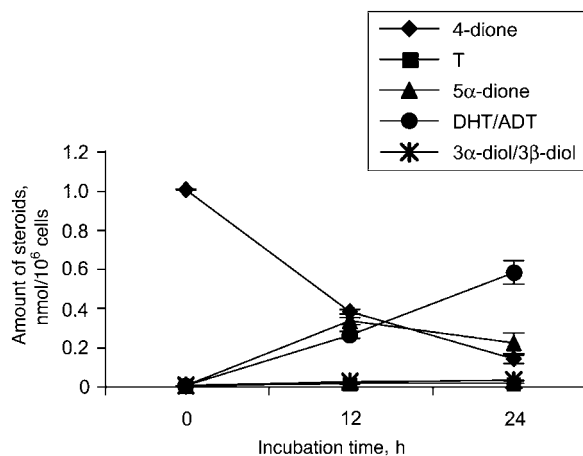


Figure 3 Quantification of [^{14}C]metabolites of 4-dione in DU-145 cells in culture.

Curves showing the metabolic profile of 0.2 μM [^{14}C]4-dione incubated for 12 and 24 h with DU-145 cells cultured in 12-well plates. Experimental procedures are as described in the Materials and methods section.

concentration decreased with a concomitant increase in 5 α -dione, and to a lesser extent ADT and DHT (these two compounds are not distinguishable in our TLC system) levels increased. Analysis at 24 h incubation indicates that 5 α -dione decreases with a concomitant increase in DHT and ADT. HPLC analysis confirms formation of both DHT and ADT with a formation of a higher amount of ADT with no detectable quantity of T (Figure 4). These data strongly suggest that ADT and DHT are produced from 5 α -dione.

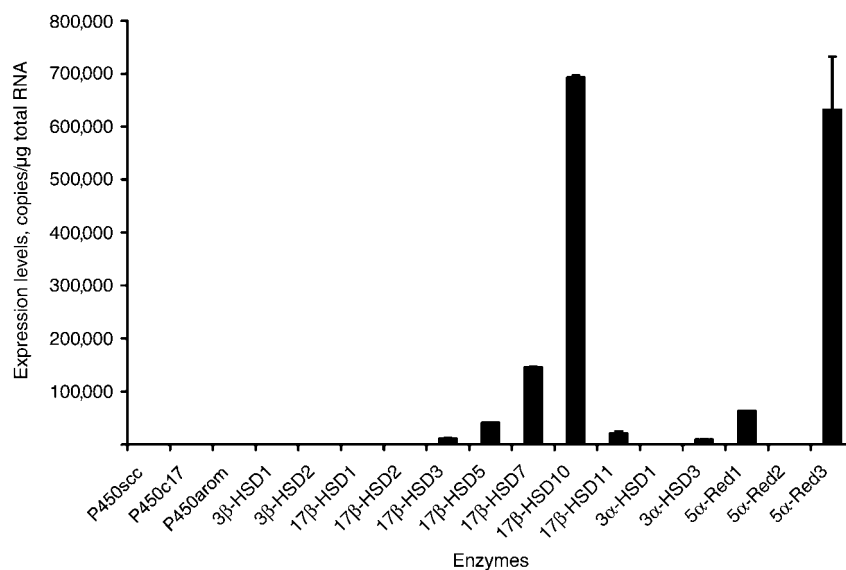


Figure 2 mRNA expression levels of steroidogenic enzymes in DU-145 cells.

Graph showing mRNA expression levels of steroidogenic enzymes in DU-145 cells quantified by a real-time PCR method described in the Materials and methods section. a and b following the numbers 3, 5, and 17 represent Greek symbols α and β , respectively. Data are expressed as means \pm SD of triplicate measurements.

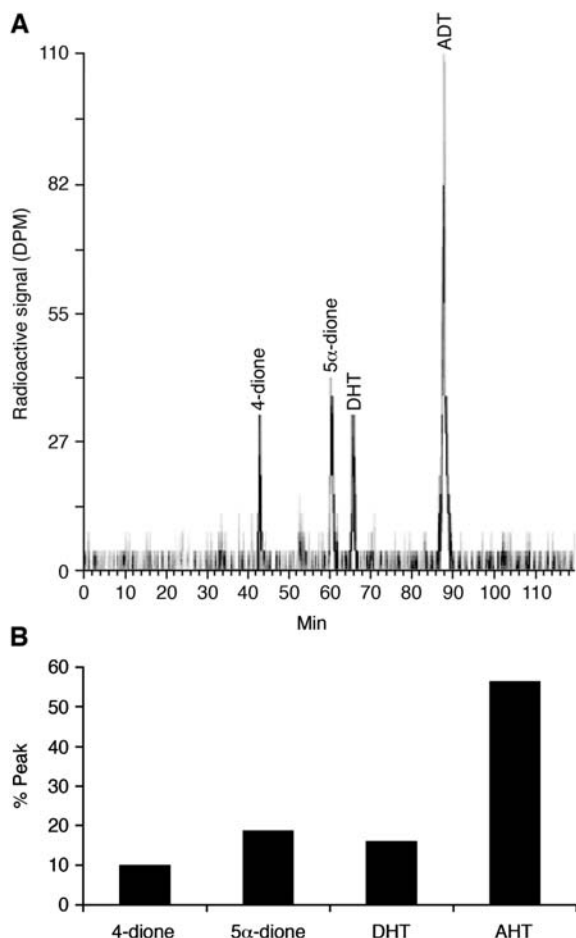


Figure 4 Identification of [^{14}C]metabolites of 4-dione in DU-145 cells in culture by high performance liquid chromatography (HPLC).

(A) HPLC/MS metabolic profile of 0.2 μM [^{14}C]4-dione incubated for 24 h with DU-145 cells cultured in 12-well plates. (B) Graphs showing principal metabolites obtained in (A). Data are expressed as means \pm SEM of triplicate measurements. Experimental procedures are as described in the Materials and methods section.

Metabolism of [^{14}C]4-dione in the presence of Finasteride, a 5 α -reductase inhibitor

As illustrated in Figure 5, in the presence of the 5 α -reductase inhibitor Finasteride, the production of 5 α -dione is blocked after 12 and 24 h incubations with a minimal production of T at 24 h. These data further confirm that, in DU-145 cells, the enzyme 5 α -reductase is involved in the transformation of 4-dione into 5 α -dione.

Discussion

The present data show that in the prostatic carcinoma cells DU-145, the pathway of the transformation of 4-dione first involves the enzyme 5 α -reductases which transform 4-dione into 5 α -dione, whereas subsequently, 5 α -dione is transformed into DHT and ADT by 17 β -HSD and 3 α -HSD,

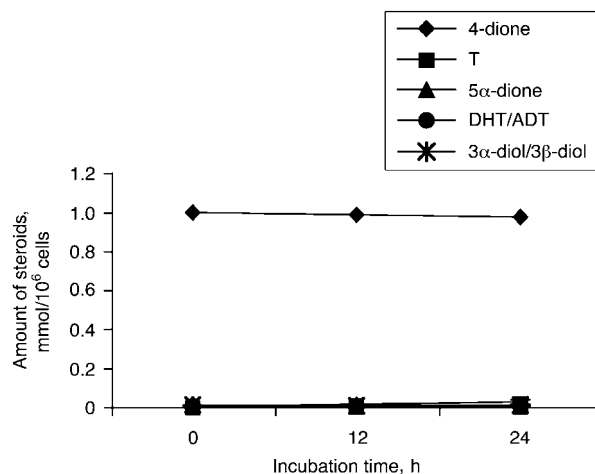


Figure 5 Effect of 5 α -reductase inhibitor on 4-androstenedione metabolism.

Experimental procedures are as described in Figure 2, except that 1 μM of the 5 α -reductase inhibitor Finasteride was added at the same time as the substrate. Data are expressed as means \pm SD of triplicate measurements.

respectively (Figure 1). This pathway provides particularly strong support for previous observations showing that 4-dione is a better substrate for 5 α -reductases than T (4, 9), and 5 α -reductases have higher affinity for the substrates ($K_m < 1 \mu\text{M}$) (4, 6) than 17 β -HSDs ($K_m > 1 \mu\text{M}$) (1, 12, 15).

These observations, however, are in contradiction with the pathway usually described in most publications which indicate that DHT is essentially produced from 5 α -reduction of T. Our data are confirmed further by experiments using transformed sebocytes, namely SZ-95, cell line (Samson et al. unpublished data), and by incubation in the presence of Finasteride, a 5 α -reductase inhibitor. In fact, after 24 h incubation, there is a very low decrease of 5 α -dione and a minimum increase of T formation. The low level of T formation is in agreement with the low efficiency of the 17 β -HSD enzymes compared with 5 α -reductases that possess much higher efficacy for the transformation of substrates (6).

mRNA quantification of steroidogenic enzymes (Figure 2) using improved second derivative and the double correction real-time PCR method (16) indicates that 5 α -reductase type 3, a newly identified 5 α -reductase (17), which is expressed at 10-fold higher than type 1 5 α -reductase, is most probably the enzyme that catalyzes the conversion of 4-dione into 5 α -dione. The type 2 5 α -reductase, by contrast, is not significantly expressed. To estimate the number of copies/cell, it is worth noting that a single liver cell contains approximately 50 pg total RNA (18); thus, an expression level of 20,000 copies/ μg total RNA could correspond to approximately 1 copy/cell in a homogeneous liver cell population. Expression levels lower than 20,000 copies/ μg total RNA could indicate a non-significant expression level, a higher level of total RNA in a single cell or heterogeneous cell types in a tissue. These data also suggest that type 5 17 β -HSD (12) is probably the enzyme catalyzing the conversion of 5 α -

dione into DHT. Because the expression levels of types 1 and 3 3α -HSD are very low, the conversion of 5α -dione into ADT is most probably catalyzed by an unidentified 3α -HSD. The high level of ADT could also be due to oxidative 17β -HSD activity exerted by 17β -HSD10 and 11, the enzymes that facilitate NAD⁺ co-factor (19, 20) to transform 5α -androstane- $3\alpha,17\beta$ -diol into ADT.

For many decades, it has generally been believed that sex steroid hormones are produced exclusively from the gonads (21). However, recent data obtained from studies in aromatase knockout mice (22–24) and in men with aromatase deficiency (25–27) 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 circulation.

Similarly, studies in patients having a defect in androgen biosynthesis have identified that T and DHT are two androgens with different functions. T measured in the blood is produced mainly in the testis and acts directly to promote the formation of the internal male reproductive structures (epididymes, seminal vesicles, and vas deferens). A defect in 17β -HSD3 causes a disorder of human intersex termed male pseudohermaphroditism (1). The typical features of 17β -HSD deficiency show a 46,XY individual having ambiguous female external genitalia and marked virilization at puberty (21). At surgery, testes and epididymis are found in the inguinal canals, whereas lower Wolffian duct structures are male in character including seminal vesicles and ejaculatory ducts.

By contrast, a defect in 5α -reductase type 2 (SRD5a2) also causes male pseudohermaphroditism (2) with ambiguous external genitalia. However, as mentioned above, in contrast with type 3 17β -HSD deficiency, the Wolffian structures are normally differentiated (3). DHT and 5α -reductases thus play an important role in local androgen biosynthesis. The importance of local biosynthesis of sex steroids is in agreement with the intracrinology concept suggested by Labrie et al. (28–30).

Some discrepancy exists concerning identification of the substrate precursor for local biosynthesis of sex steroids. The general belief based on traditional literature suggests that the substrate of 5α -reductase and aromatase is T, although there is increasing evidence that suggests that the substrate of 5α -reductase and aromatase is 4-dione (10), especially in the human which produces high amounts of steroid adrenal precursors in the circulation (28, 31). The discrepancy is somewhat difficult to overcome because the mouse and the rat do not express 17α -hydroxylase/ 17β - 20β -lyase (CYP17) in the adrenals (32, 33), thus removing high levels of adrenal precursor in the circulation as found in the human (10, 34, 35). However, as mentioned above, the discrepancy is most probably due to the misinterpretation of the concentration of steroid measured in the blood circulation. The understanding is completely different if we consider that this concentration is the amount to be transformed or the concentration remaining after transformation. Traditional literature generally consid-

ered that the blood concentration of steroid is the concentration available to be used by the cells or tissues. However, it is more logical to consider that blood concentration of steroid is the amount remaining after transformation, and the amount available to be transformed is the sum of the blood concentration plus the concentrations of all metabolites found downward in the pathway. In this regard, T is almost not transformed. It reflects the physiological action of androgenic activity distributed through the endocrine system under the control of the hypothalamo-gonadal axis. One viewpoint is that blood concentration of 4-dione represents only a part of the available amounts. Indeed, it has been transformed into many 5α -reduced metabolites, including DHT. The androgenic activity DHT is thus controlled by the presence of enzymes involved in its biosynthesis and degradation, such as 5α -reductases, 17β -HSDs, 3α -HSDs, UDP-glucuronosyltransferases and sulfotransferases, and the concentration of precursor in the tissue.

Knowledge of the exact biosynthetic pathway of DHT in peripheral tissue is important for a more specific design of inhibitor of androgen biosynthesis for the treatment of androgen-sensitive diseases, such as prostate cancer, BPH, alopecia, and acne. Data obtained in the present study in the DU-145 prostate cancer cell line strongly support the pathway of DHT biosynthesis that does not require T as intermediate.

Acknowledgements

The authors would like to thank Nathalie Paquet, Lucille Lacoste, and Patrick Belanger for helpful technical assistance. This work was supported by the Canadian Institute of Health Research (CIHR) strategic training program (grant number STP-53894 to F.L. and V.L.T.) and by Endorecherche Inc.

References

- Geissler WM, Davis DL, Wu L, Bradshaw KD, Patel S, Mendonca BB, Elliston KO, Wilson JD, Russell DW, Andersson S. Male pseudohermaphroditism caused by mutations of testicular 17β hydroxysteroid dehydrogenase 3. *Nat Genet* 1994;7:34–9.
- Andersson S, Berman DM, Jenkins EP, Russell DW. Deletion of steroid 5 alpha-reductase 2 gene in male pseudohermaphroditism. *Nature* 1991;354:159–61.
- Peterson RE, Imperato-McGinley J, Gautier T, Sturla E. Male pseudohermaphroditism due to steroid 5-alpha-reductase deficiency. *Am J Med* 1977;62:170–91.
- Andersson S, Russell DW. Structural and biochemical properties of cloned and expressed human and rat steroid 5 alpha-reductases. *Proc Natl Acad Sci USA* 1990;87:3640–4.
- Luu-The V, Sugimoto Y, Puy L, Labrie Y, Lopez Solache I, Singh M, Labrie F. Characterization, expression, and immunohistochemical localization of 5 alpha-reductase in human skin. *J Invest Dermatol* 1994;102:221–6.
- Russell DW, Wilson JD. Steroid 5 alpha-reductase: two genes/two enzymes. *Annu Rev Biochem* 1994;63:25–61.

7. Xu W, Robert C, Thornton PS, Spinner NB. Complete androgen insensitivity syndrome due to X chromosome inversion: a clinical report. *Am J Med Genet A* 2003;120:434–6.
8. Wilson JD. Dihydrotestosterone formation in cultured human fibroblasts. Comparison of cells from normal subjects and patients with familial incomplete male pseudohermaphroditism, type 2. *J Biol Chem* 1975;250:3498–504.
9. Sugimoto Y, Lopez-Solache I, Labrie F, Luu-The V. Cations inhibit specifically type I 5 alpha-reductase found in human skin. *J Invest Dermatol* 1995;104:775–8.
10. Luu-The V, Belanger A, Labrie F. Androgen biosynthetic pathways in the human prostate. *Best Pract Res Clin Endocrinol Metab* 2008;22:207–21.
11. Labrie F, Belanger A, Cusan L, Gomez JL, Candas B. Marked decline in serum concentrations of adrenal C19 sex steroid precursors and conjugated androgen metabolites during aging. *J Clin Endocrinol Metab* 1997;82:2396–402.
12. Dufort I, Rheault P, Huang X-F, Soucy P, Luu-The V. Characteristics of a highly labile human type 5 17beta-hydroxysteroid dehydrogenase. *Endocrinology* 1999;140:568–74.
13. Dufort I, Soucy P, Lacoste L, Luu-The V. Comparative biosynthetic pathway of androstenol and androgens. *J Steroid Biochem Mol Biol* 2001;77:223–7.
14. Samson M, Labrie F, Luu-The V. Inhibition of human-type 1 3beta-hydroxysteroid dehydrogenase/Delta(5)-Delta(4)-isomerase expression using siRNA. *J Steroid Biochem Mol Biol* 2005;94:253–7.
15. Lin HK, Jez JM, Schlegel BP, Peehl DM, Pachter JA, Penning TM. Expression and characterization of recombinant type 2 3 alpha-hydroxysteroid dehydrogenase (HSD) from human prostate: demonstration of bifunctional 3 alpha/17 beta-HSD activity and cellular distribution. *Mol Endocrinol* 1997;11:1971–84.
16. Luu-The V, Paquet N, Calvo E, Cumps J. Improve real-time RT-PCR method for high throughput measurements using second derivative calculation and double correction. *BioTechniques* 2005;32:287–93.
17. Uemura M, Tamura K, Chung S, Honma S, Okuyama A, Nakamura Y, Nakagawa H. Novel 5 alpha-steroid reductase (SRD5A3, type-3) is overexpressed in hormone-refractory prostate cancer. *Cancer Sci* 2008;99:81–6.
18. Raymond K. Light Cycler technique manual. Paper presented at the Light Cycler Presentation, Quebec, Canada, 2002.
19. Li KX, Smith RE, Krozowski ZS. Cloning and expression of a novel tissue specific 17beta-hydroxysteroid dehydrogenase. *Endocr Res* 1998;24:663–7.
20. He XY, Merz G, Mehta P, Schulz H, Yang SY. Human brain short chain L-3-hydroxyacyl coenzyme A dehydrogenase is a single-domain multifunctional enzyme. Characterization of a novel 17beta-hydroxysteroid dehydrogenase. *J Biol Chem* 1999;274:15014–9.
21. Wilson JD. Sexual differentiation. *Annu Rev Physiol* 1978;40:279–306.
22. Fisher CR, Graves KH, Parlow AF, Simpson ER. Characterization of mice deficient in aromatase (ArKO) because of targeted disruption of the *cyp19* gene. *Proc Natl Acad Sci USA* 1998;95:6965–70.
23. Miyaura C, Toda K, Inada M, Ohshiba T, Matsumoto C, Okada T, Ito M, Shizuta Y, Ito A. Sex- and age-related response to aromatase deficiency in bone. *Biochem Biophys Res Commun* 2001;280:1062–8.
24. Oz OK, Zerwekh JE, Fisher C, Graves K, Nanu L, Millsaps R, Simpson ER. Bone has a sexually dimorphic response to aromatase deficiency. *J Bone Miner Res* 2000;15:507–14.
25. Morishima A, Grumbach MM, Simpson ER, Fisher C, Qin K. Aromatase deficiency in male and female siblings caused by a novel mutation and the physiological role of estrogens. *J Clin Endocrinol Metab* 1995;80:3689–98.
26. Bilezikian JP, Morishima A, Bell J, Grumbach MM. Increased bone mass as a result of estrogen therapy in a man with aromatase deficiency. *N Engl J Med* 1998;339:599–603.
27. Carani C, Qin K, Simoni M, Faustini-Fustini M, Serpente S, Boyd J, Korach KS, Simpson ER. Effect of testosterone and estradiol in a man with aromatase deficiency. *N Engl J Med* 1997;337:91–5.
28. Labrie F. Intracrinology. *Mol Cell Endocrinol* 1991;78:C113–8.
29. Labrie F, Luu-The V, Labrie C, Pelletier G, El-Alfy M. Intracrinology and the skin. *Horm Res* 2000;54:218–29.
30. Labrie F, Luu-The V, Labrie C, Simard J. DHEA and its transformation into androgens and estrogens in peripheral target tissues: intracrinology. *Front Neuroendocrinol* 2001;22:185–212.
31. Labrie F, Luu-The V, Labrie C, Belanger A, Simard J, Lin SX, Pelletier G. Endocrine and intracrine sources of androgens in women: inhibition of breast cancer and other roles of androgens and their precursor dehydroepiandrosterone. *Endocr Rev* 2003;24:152–82.
32. Brock BJ, Waterman MR. Biochemical differences between rat and human cytochrome P450c17 support the different steroidogenic needs of these two species. *Biochemistry* 1999;38:1598–606.
33. Luu-The V, Pelletier G, Labrie F. Quantitative appreciation of steroidogenic gene expression in mouse tissues: new roles for type 2 5alpha-reductase, 20alpha-hydroxysteroid dehydrogenase and estrogen sulfotransferase. *J Steroid Biochem Mol Biol* 2005;93:269–76.
34. Belanger B, Belanger A, Labrie F, Dupont A, Cusan L, Monfette G. Comparison of residual C-19 steroids in plasma and prostatic tissue of human, rat and guinea pig after castration: unique importance of extratesticular androgens in men. *J Steroid Biochem* 1989;32:695–8.
35. Labrie F, Dupont A, Belanger A. Complete androgen blockade for the treatment of prostate cancer. *Important Adv Oncol* 1985;193–217.