

Leydig Cell Androgen Synthesis

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Introduction

Testicular Leydig cells are the primary source of androgens, which are indispensable for the development of the male reproductive system. Androgens synthesized by the interstitial Leydig cells include testosterone, 5 α -dihydrotestosterone, androstenedione, androstanediol, and androsterone (Payne and Hales, 2004; Teerds and Huhtaniemi, 2015). Among these, testosterone is the most prevalent male sex androgen. Testosterone induces masculinization before birth, drives the establishment of male secondary sexual characteristics after birth and supports spermatogenesis throughout life (Payne and Hardy, 2007).

There are two classes of enzymes involved in androgen biosynthesis: the cytochrome P450 heme-containing proteins and the hydroxysteroid dehydrogenases. These enzymes are located in mitochondria and smooth endoplasmic reticulum (SER), respectively, and they catalyze the conversion of cholesterol to androgens. Notably, the prevalent androgens produced by Leydig cells at each developmental stage are different due to the developmental, stage-specific expression of steroidogenic enzymes (Ye et al., 2017). Given that testosterone is considered to be the most important androgen for masculinization and spermatogenesis, we will mainly discuss testosterone biosynthesis in this article.

Source of Cholesterol

In Leydig cells, cholesterol is synthesized de novo from acetate. Besides de novo synthesis, plasma lipoproteins bring cholesterol to Leydig cells (Hu et al., 2010). The de novo pathway is preferably used in the rat and lipoproteins in humans (Payne and Hardy, 2007). Cholesterol can be stored in the form of cholesterol esters in lipid droplets or come from various membranes e.g. plasma membrane.

Acetate

Under normal physiological conditions, Leydig cells preferentially synthesize cholesterol de novo from acetate (Fig. 1). Among numerous enzymes required for the conversion of acetate to cholesterol, 3-hydroxy-3-methylglutaryl (HMG)-CoA-reductase (HMGCR) located in the SER is the rate-limiting enzyme that catalyzes the condensation of acetyl-CoA into mevalonate. De novo synthesis of cholesterol is also under the control of pituitary luteinizing hormone (LH). The newly formed cholesterol will be transferred from the endoplasmic reticulum to the plasma membrane. Excess cholesterol is converted to cholesterol esters by acetyl-CoA acetyltransferase (ACAT1) and packed into lipid droplets.

Plasma Lipoprotein and Intracellular Cholesterol Esters

Newly synthesized cholesterol esters are the source of cholesterol for androgen synthesis in response to trophic hormone stimulation. Depending upon conditions, however (e.g., LH-induced desensitization of the steroidogenic response), Leydig cells may utilize plasma lipoprotein as the source for cholesterol synthesis (Fig. 1). In this scenario, cholesterol may come either from plasma low-density lipoprotein (LDL) or high-density lipoprotein (HDL). Cholesterol esters released from LDL are delivered to the plasma membrane or to lipid droplets and mitochondria for androgen synthesis. Cholesterol esters from HDL are hydrolyzed and the released free cholesterol is used for androgen synthesis.

Plasma Membrane

In mammalian cells, free cholesterol is abundant in the plasma membrane (Maxfield and Wustner, 2002). The origin of this cholesterol could be from either de novo synthesis or lipoprotein import and processing. Hormone-treatment of Leydig cells induces rapid mobilization of free cholesterol from the inner leaflet of the plasma membrane to mitochondria for steroid synthesis (Freeman, 1989; Venugopal et al., 2016). This cholesterol constitutes the pool of free cholesterol used for steroidogenesis in response to LH stimulation until de-esterification of stored cholesterol provides large amounts to sustain steroidogenesis.

Biosynthesis of Androgens From Cholesterol

Mobilization of Cellular Cholesterol

As noted above, cholesterol is released from the plasma membrane and then lipid droplets upon trophic stimulation by LH or human chorionic gonadotropin (hCG). LH/hCG binds to a G protein-coupled receptor that activates the adenylate cyclase leading

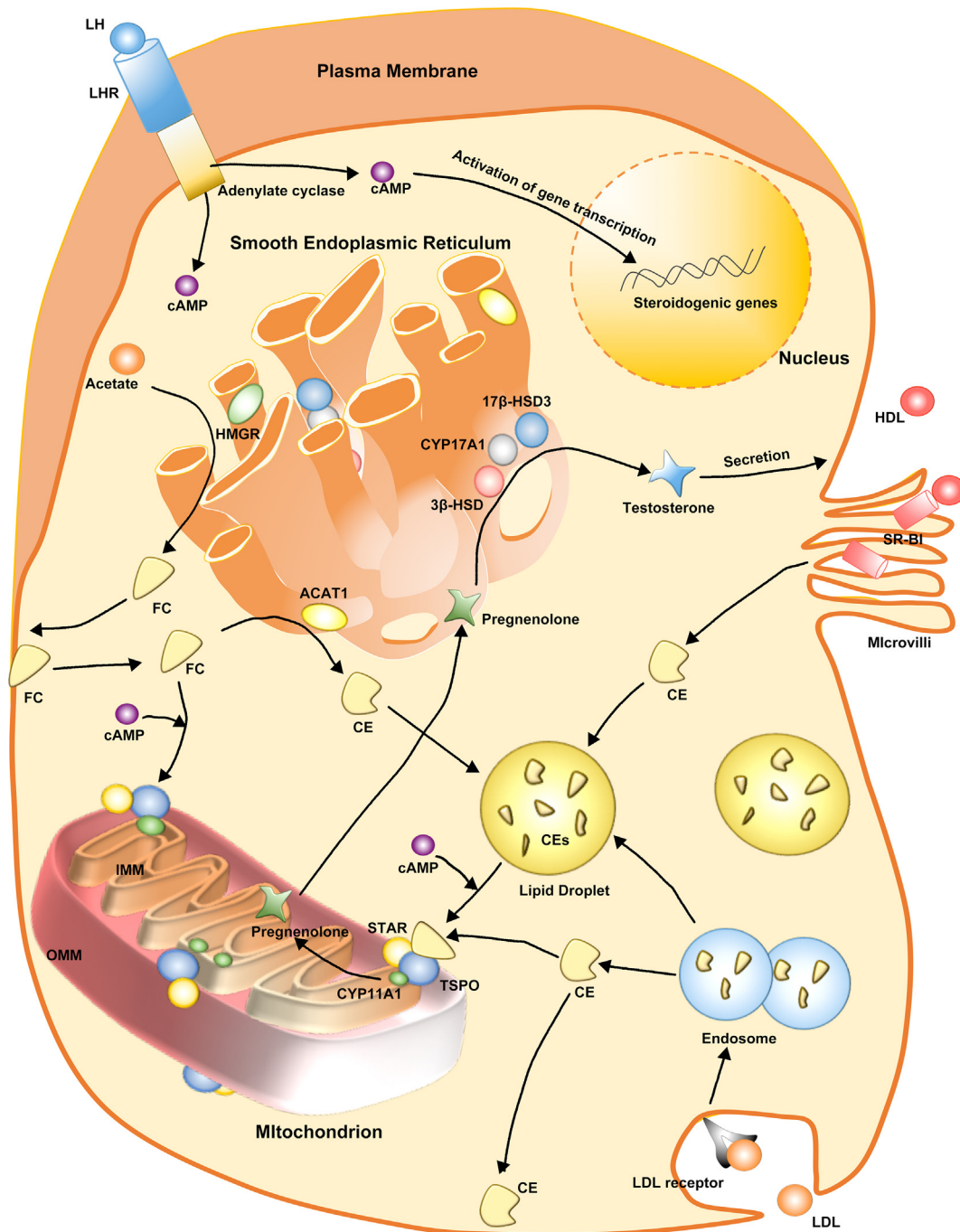


Fig. 1 Molecular and cellular events involved in testosterone biosynthesis in Leydig cells. In Leydig cells, androgens are derived from cholesterol. This figure depicts testosterone synthesis in Leydig cells. Cholesterol is either derived by de novo synthesis or from low-density lipoprotein (LDL) or high-density lipoprotein (HDL). Following this, cholesterol is delivered to mitochondria for the first step of testosterone biosynthesis. At the inner mitochondrial membrane (IMM), cholesterol is catalyzed to pregnenolone by CYP11A1. Pregnenolone is translocated from mitochondria to smooth endoplasmic reticulum, at which it is further processed to testosterone and other androgens. Then testosterone is secreted by Leydig cells. FC, free cholesterol; CE, cholesterol ester; SR-B1, scavenger receptor class B, type I.

to cyclic adenosine 3',5'-monophosphate (cAMP) production. cAMP-dependent protein kinase then is activated to initiate a series of events that includes the mobilization of free cholesterol in the plasma membrane and phosphorylation of neutral cholesteryl ester hydrolase (nCEH), leading to the mobilization of cholesterol esters.

Translocation of Cholesterol in Mitochondria

Steroidogenesis begins with the transfer of free cholesterol from the plasma membrane and intracellular stores into mitochondria—the rate-limiting step in androgen biosynthesis. This initial rapid response to LH, and its second messenger cAMP, is mediated

by hormone-sensitive protein synthesis, protein–protein interactions, and organelle communications. Chronic stimulation by LH is required for optimal expression of steroidogenic enzymes leading to sustainable steroid formation.

The first catalytic reaction in androgen biosynthesis occurs in mitochondria. Before this reaction, cholesterol must be translocated from its various locations in the cell to the outer mitochondrial membrane (OMM). This may occur through either a vesicular or non-vesicular transport pathway (Fig. 1). An important mechanism of vesicular transport is the delivery of cholesterol through protein–protein interactions. There is evidence that there is increased association of mitochondria with the endoplasmic reticulum in Leydig cells in response to hormone treatment mediated via AAA+ ATPase domain 3 (ATAD3) protein (Issop et al., 2015). It is also possible that the soluble *N*-ethylmaleimide-sensitive factor attachment receptor (SNARE) complexes facilitate the delivery of cholesterol by promoting functional protein–protein interactions between lipid droplets and mitochondria (Lin et al., 2016). Indeed, there is strong interorganelle interactions that facilitate free cholesterol exchange in response to LH in Leydig cells (Issop et al., 2013). Cholesterol also can be delivered through a non-vesicular transport pathway, mediated by high-affinity cholesterol-binding proteins including steroidogenic acute regulatory protein (STAR). In particular, STARD4 has high affinity with free cholesterol and is involved in cholesterol transport (Mesmin et al., 2011).

Cholesterol targeting into mitochondria is mediated by the formation of a mitochondrial scaffold, the transduceosome, created by protein–protein interactions of cytosolic and OMM proteins (Rone et al., 2009). After cholesterol is translocated to the OMM, it will be delivered to the inner mitochondrial membrane (IMM), where it is converted to pregnenolone by CYP11A1 (Fig. 1). Cholesterol is imported and metabolized by the 800-kDa bioactive metabolon, composed of the OMM/IMM translocator protein (TSPO), voltage-dependent anion channel (VDAC), ATAD3, and CYP11A1 (Rone et al., 2012). The rapid induction of STAR formation by LH and its subsequent targeting and insertion into OMM leads to accelerated cholesterol delivery to CYP11A1 (Rone et al., 2012; Papadopoulos and Miller, 2012).

Biosynthesis of Testosterone From Cholesterol

The process of testosterone biosynthesis from cholesterol involves four enzymes: cytochrome P450 family 11 subfamily A1 (CYP11A1), 3 β -hydroxysteroid dehydrogenase/ $\Delta^5 \rightarrow \Delta^4$ isomerase (3 β -HSD), CYP17A1, and 17 β -HSD3 (Miller and Auchus, 2011; Payne and Hales, 2004). CYP11A1 is located in mitochondria, 3 β -HSD is present in mitochondria but predominantly found in SER, and CYP17A1 and 17 β -HSD3 are found only in SER (Figs. 1 and 2).

CYP11A1

CYP11A1, which is generically termed cytochrome P450 (P450_{scc}), is a 56-kDa enzyme located in the IMM (Fig. 1). It catalyzes the conversion of cholesterol to pregnenolone (Fig. 2). This conversion includes three sequential oxidation reactions: hydroxylation of cholesterol at C22, hydroxylation of 22(R)-hydroxycholesterol at C20, and cleavage of the C20–22 bond of 20(R), 22(R)-dihydroxycholesterol to yield pregnenolone and isocaproaldehyde. All three reactions are catalyzed in the same site of CYP11A1. The mitochondrial environment is required for the catalytic activity of CYP11A1 since CYP11A1 is lacking mitochondrial leader and when targeted to the ER is inactive. During the conversion, CYP11A1, a monooxygenase, catalyzes oxidation by utilizing electrons from reduced nicotinamide adenine dinucleotide phosphate (NAPDH). A mitochondrial electron transport system consisting of ferredoxin reductase (FdR) and ferredoxin (FdX) facilitates the electron transport between CYP11A1 and NAPDH.

CYP11A1 determines the biosynthetic capacity of Leydig cells. The amount of CYP11A protein is primarily decided by transcription of the *CYP11A* gene, which can be regulated in a hormonally responsive fashion. The transcription of the *CYP11A* gene is induced by both cAMP and cAMP-dependent protein kinase. Upon LH stimulation, the activation of cAMP is necessary for the expression of involved enzymes in androgen synthesis. Steroidogenic factor 1 (SF1) also is essential for androgen synthesis, involved in the regulation of the expression of CYP11A and other steroidogenic enzymes.

3 β -HSD

Pregnenolone diffuses passively from the mitochondria to SER for further processing. Although 3 β -HSD is found in mitochondria, it is abundant in SER (Fig. 1) in proximity to CYP17A1 and 17 β -HSD3 so that each intermediate of testosterone synthesis can move directly to the next enzyme. Depending upon the species, Leydig cells use either the Δ^4 or Δ^5 pathway to produce testosterone (Fig. 2). Rodent Leydig cells preferentially follow the Δ^4 pathway while human and rabbit Leydig cells follow the Δ^5 pathway. In the Δ^4 pathway, pregnenolone is converted to Δ^4 steroids by 3 β -HSD. 3 β -HSD is a 42-kDa enzyme that converts pregnenolone to progesterone (Fig. 2). Additionally, it can also convert 17 α -hydroxypregnenolone to 17 α -hydroxyprogesterone (17OHP), dehydroepiandrosterone (DHEA) to androstenedione, and androstenediol to testosterone. Two chemical transformations are catalyzed by 3 β -HSD to stimulate these conversions (Fig. 2). First, the 3 β -hydroxyl group of the substrate is oxidized to the ketone group, when NAD⁺ is converted to NADH. Next, NADH activates the second activity of 3 β -HSD, the $\Delta^5 \rightarrow \Delta^4$ isomerase activity, i.e. catalyzing the isomerization of the double bond from carbon 5 and 6 to carbon 4 and 5, to yield Δ^4 steroid.

CYP17A1

Human and rabbit Leydig cells synthesize testosterone via the Δ^4 pathway, in which the first reaction is catalyzed by CYP17A1. CYP17A1 is a 57-kDa enzyme that expresses both 17 α -hydroxylase and 17, 20-lyase activities. In the Δ^5 pathway, CYP17A1 converts pregnenolone to 17 α -hydroxypregnenolone (Fig. 2). Then it converts 17 α -hydroxypregnenolone to DHEA by its 17, 20-lyase activity (Fig. 2). The presence of cytochrome *b*₅ increases the rate of lyase reaction more than 10-fold. In addition to the two-step conversion

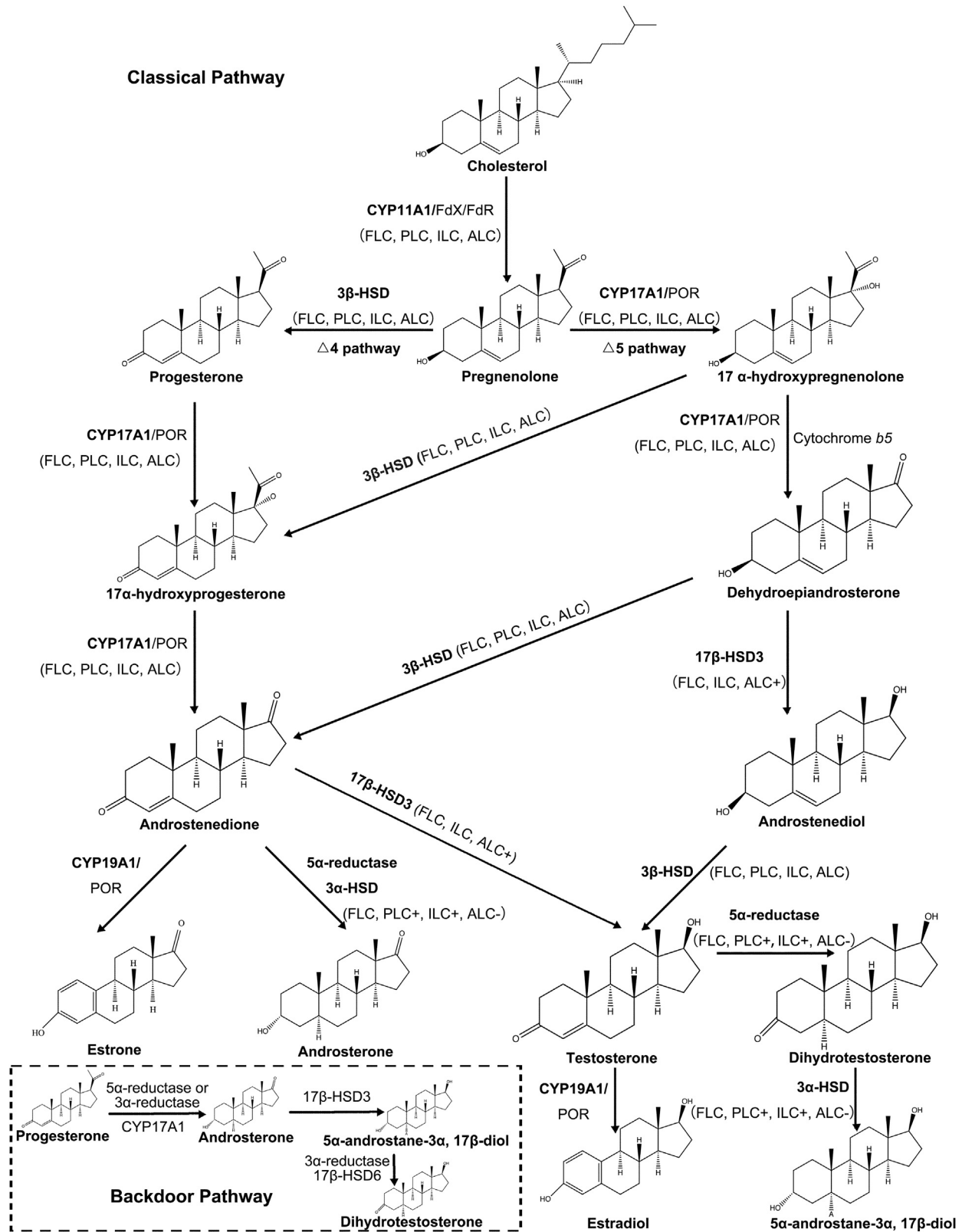


Fig. 2 Androgen biosynthetic and metabolic pathways in Leydig cells. (Top) In adult Leydig cells (ALCs), androgens are synthesized via the classical pathway. The major steps involved in the classical pathway include conversion of cholesterol to pregnenolone and synthesis of testosterone from pregnenolone by following two species-dependent pathways (Δ^4 and Δ^5). (Bottom left) Testosterone is further metabolized to dihydrotestosterone (DHT), 5α -androstane- 3α , 17β -diol and estradiol. In fetal Leydig cells (FLCs), using the backdoor pathway. The critical enzyme employed by the backdoor pathway is 3α -reductase, which converts 5α -androstane- 3α , 17β -diol to DHT. All the enzymes, co-enzymes, electron transport systems and direction of reactions are shown. Androgens are emphasized by larger structural images. The developmental stages during which key enzymes are expressed are included in parentheses. “+” and “-” represent significant and negligible expression levels of enzymes, respectively.

of pregnenolone to DHEA, CYP17A1 can also catalyze the transformation of progesterone to 17OHP. However, under normal conditions, 17OHP is not a critical precursor for androgen synthesis; the conversion of 17OHP to androstenedione catalyzed by CYP17A1 is 50-fold less efficient than the conversion of 17 α -hydroxypregnenolone to DHEA. The reaction catalyzed by CYP17A1 also requires the microsomal electron transport system termed flavoprotein P450 oxidoreductase (POR). POR is a single membrane-bound flavoprotein that receives two electrons from NADPH and delivers them at once to CYP17A1. In human cells, the interaction between POR and CYP17A1 is facilitated by cytochrome *b*₅ and the serine phosphorylation of CYP17A1. As the sole electron transfer system serving for all microsomal P450s, POR also delivers electrons to CYP19A1 for metabolism of androgens.

17 β -HSD3

17 β -HSD3 is a 34.5-kDa enzyme specifically expressed in the testis. It can catalyze DHEA transformation to androstenediol, and androstenedione to testosterone (Fig. 2). Androstenediol derived from DHEA may contribute to testosterone biosynthesis in Leydig cells.

Other Androgens and Estrogens in Leydig Cells

As noted above, in addition to testosterone, Leydig cells synthesize androgens that are intermediate products of testosterone biosynthesis, including DHEA, androstenediol and androstenedione (Fig. 2). Leydig cells also can metabolize androgens into dihydrotestosterone (DHT) and estrogens (Fig. 2).

DHT

DHT is a potent androgen for male sexual differentiation. It is derived from testosterone by a 30-kDa microsomal enzyme, 5 α -reductase. In the fetus, DHT and testosterone induce genital tubercle transformation into male external genitalia. In humans, two isoforms of 5 α -reductase have been identified. Type 1 5 α -reductase potentially contributes to pubertal virilization, as proven by the consequences of classic 5 α -reductase deficiency. Type 2 5 α -reductase, the more prevalent isoform in Leydig cells, is responsible for directing male sexual differentiation. Beyond converting testosterone to DHT, 5 α -reductase, especially the type I isoform, can also metabolize progesterone, 17OHP and related C₂₁ steroids. Moreover, type I isoform expressed in immature mouse Leydig cells participates in androstenediol synthesis via two distinct pathways (Fig. 2).

Estradiol and estrone

In addition to androgens, Leydig cells can also produce estrogens. 17 β -Estradiol derived from testosterone, is the main estrogen formed in Leydig cells. A microsomal enzyme, CYP19A1 (58-kDa), generically termed P450arom, catalyzes the aromatization of testosterone to estradiol. CYP19A1 aromatase also catalyzes the conversion of androstenedione to estrone (Fig. 2). In the male, androgen-derived estrogens contribute to numerous physiological processes, including skeletal maturation.

Developmental-Specific Expression of Enzymes

There are two distinct populations of Leydig cells identified in different stages of male sexual development. Fetal Leydig cells (FLCs) appear at the fetal stage, and a second population, the adult Leydig cells (ALCs), develop from stem cells after birth. The differentiation of ALCs can be divided into four stages: stem Leydig cells (SLCs), progenitor Leydig cells (PLCs), immature Leydig cells (ILCs), and mature adult Leydig cells (mature ALCs). The androgens formed at the various stages of Leydig cell development vary due to the differential expression of steroidogenic enzymes (Fig. 2) (Kotula-Balak et al., 2012; Ye et al., 2017).

FLCs express CYP11A1, 3 β -HSD, CYP17A1, 5 α -reductase, and 3 α -HSD. These enzymes continually increase during the fetal stage. The expression of 17 β -HSD3 is controversial and likely species-specific. In the mouse no 17 β -HSD3 has been detected in FLC; the enzyme is found in the tubular cells of the testis, indicating a possible cooperation between different testicular cells to produce androgens. However, the enzyme is present in rat FLC. At fetal day 15.5, the testis begins to produce testosterone. Its production gradually increases. DHT and androstenedione also are synthesized by FLCs. DHT is essential for the development of the external male genitalia. In FLCs, DHT is derived from progesterone by following the “backdoor” pathway (Fig. 2). Enzymes catalyzing this metabolic pathway include 5 α -reductase, 3 α -reductase, 17 β -HSD3 and 17 β -HSD6. The key enzyme of the “backdoor pathway,” 3 α -reductase, converts 5 α -androstane-3 α , 17 β -diol to DHT.

After the first postnatal week, testosterone declines due to reduced numbers of FLCs and their reduced steroidogenic activities. The level of testosterone is elevated again when ALCs start developing. In rats, ALC differentiation begins around postnatal day 14 and finishes around postnatal day 60. SLCs express no steroidogenic enzymes whereas PLCs express CYP11A1, 3 β -HSD, and CYP17A1 and therefore synthesize androstenedione. PLCs also express androgen-metabolizing enzymes, including 5 α -reductase and 3 α -HSD. These enzymes can metabolize androstenedione into androsterone, which is the main product of PLCs. When PLCs further differentiate into ILCs, CYP11A1, 3 β -HSD, and CYP17A1 are concomitantly increased. The appearance of 17 β -HSD3 enables ILCs to synthesize testosterone. However, due to the continuous presence of 5 α -reductase and 3 α -HSD, testosterone will be metabolized, leading to a high production of 5 α -androstane-3 α , 17 β -diol in ILCs. When ALCs become mature, testosterone production rises significantly as a consequence of increased numbers of LH receptors and thus elevated responsiveness to LH. At the same time, decline of 5 α -reductase activity also contributes to the accumulation of testosterone in mature ALCs. The

large quantity of testosterone secreted by mature ALCs is required for the initiation and the maintenance of spermatogenesis as well as the establishment of secondary sex characteristics in puberty and through adulthood.

Enzymes of Androgen Biosynthesis in Pathological Conditions

Every step of testosterone biosynthesis and the conversion of testosterone to DHT can be disturbed, leading to changes in androgen production and androgen balance. Disruption of androgen synthesis results in disorders of male sexual development (Miller, 2005; Miller and Auchus, 2011).

For example, STAR (STARD1) is a key protein regulating the transport of cholesterol into mitochondria. Mutations of the *STARD1* gene result in a severe genetic disorder of steroidogenesis called lipoid congenital adrenal hyperplasia (CAH). The loss of STAR in CAH leads to over 80% decline of steroids. The affected 46, XY fetuses CAH cannot undergo normal virilization and thus are born with female external genitalia. In patients with a less severe form of lipoid CAH, patients have 20%–25% of normal STAR activity. They have normal external male genitalia but mild hypergonadotropic hypogonadism. The patients with a *CYP11A1* mutation cannot be clinically and hormonally distinguished from those with lipoid CAH.

Mutations of 3 β -HSD1 have not been reported; such mutations would prohibit placental progesterone biosynthesis, leading to a spontaneous first-trimester abortion. The mutations of the *HSD3B2* gene cause 3 β -HSD2 deficiency and consequent decrease of testosterone production. The severely mutated form of 3 β -HSD2 results in lipoid CAH. In patients with this mutation, the level of Δ^5 steroids increases significantly while that of Δ^4 steroids increases slightly, leading to a high ratio of Δ^5 to Δ^4 steroids. Patients with the 17 β -HSD3 deficiency or 5 α -reductase 2 deficiency share the same clinical characteristics as those deficient for 3 β -HSD2; i.e. undermasculinization with hypoplastic-to-normal male internal genitalia, female external genitalia, and loss of the prostate. Additionally, 5 α -reductase 2 deficiencies may be associated with the male pattern baldness. Complete CYP17A1 deficiency includes the absence of both 17 α -hydroxylase and 17,20-lyase activities that inhibit the production of gonadal sex steroids. As a consequence, the affected 46, XY males present with the absence or abnormalities of male external genitalia. Selective deficiency of 17 α -hydroxylase and 17,20-lyase activities has also been identified.

Differences of Androgen Biosynthesis in Human and Rodent Leydig Cells

To date, most knowledge of androgen biosynthesis in Leydig cells has come from studies of rodent models, including steps of androgen synthesis and enzymes involved in this process. In contrast, we understand much less about androgen biosynthesis in humans in part because of the difficulty in obtaining samples. It remains unclear whether the factors identified in rodent Leydig cells play the same roles in human Leydig cells, and/or whether there are novel pathways in human Leydig cells that are involved in producing androgens. However, without doubt, differences of androgen biosynthesis between human and rodent Leydig cells do exist (Teerds and Huhtaniemi, 2015).

In contrast to rodents, three distinct populations of human Leydig cells, FLCs, neonatal Leydig cells (NLCs), and ALCs have been identified during male sexual development. NLCs appear between birth and the 6 months thereafter, concomitant with a unique peak of testosterone production in the human. This testosterone surge is thought to contribute to imprinting of many cell types of androgen-dependent organs. In rodents, no NLCs have been reported. There also are species-dependent differences in both the cholesterol source and the enzyme preference. Typically, Leydig cells utilize de novo synthesized cholesterol for androgen biosynthesis. However, in cases in which rodents use LDL or HDL as the cholesterol source for androgen biosynthesis, the Leydig cells heavily rely on the SR-BI/selective pathway. In contrast, human Leydig cells utilize the LDL/LDL-receptor endocytic pathway. Moreover, CYP17A1 in rodent Leydig cells acts on 17 α -hydroxyprogesterone (Δ^4), whereas in human Leydig cells it acts on 17 α -hydroxypregnenolone (Δ^5).

Summary

Testosterone biosynthesis is essential for the development of internal/external male genitalia, the establishment of secondary male characteristics, and spermatogenesis. Leydig cells are the primary source of testosterone in the testis. In addition to testosterone, Leydig cells produce other steroids that are essential for male development, including DHT and estradiol.

In Leydig cells, testosterone and other androgens are synthesized from cholesterol derived from different sources. The translocation of cholesterol from OMM to IMM is the hormone-sensitive and rate-limiting step in androgen biosynthesis. Four steroidogenic enzymes catalyze the biosynthesis of testosterone: CYP11A1, 3 β -HSD, CYP17A1, and 17 β -HSD3. After synthesis, testosterone can be further metabolized into DHT, estradiol, androsterone, and 5 α -androstenediol by three enzymes, 5 α -reductase, CYP19A1, and 3 α -HSD. Functionally different from ALCs, FLCs synthesize more DHT to support male sexual development in fetal life.

There are still many unknowns about Leydig cell androgen biosynthesis, particularly so in the human. For example, the origin of human Leydig cells, the identification of individual SLCs, and the cell fate of NLCs all remain to be clarified. Moreover, it is well known that the substrate specificity of cytochrome P450s is limited, and this suggests the presence of alternative unexplored yet substrates for androgen formation (Lieberman, 2008).

Investigating Leydig cell androgen biosynthesis is important in order to understand the underlying mechanisms of male sexual development and the origins of associated developmental defects. Moreover, reduced androgen formation is associated with aging.

Indeed, 20%–50% of men over age 60 are reported to have serum testosterone levels significantly below those of young men (Midzak et al., 2009). Whether in aging or young men, reduced serum testosterone is associated with a number of metabolic and quality-of-life changes, including infertility, erectile dysfunction, obesity, osteoporosis and cardiovascular disease. Greater understanding of the molecular, cellular and biochemical mechanisms of androgen formation could lead to the design of methods to induce and control androgen formation.

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