REGULATION OF EXPRESSION OF 5 ALPHA-REDUCTASE TYPE 1
IN MOUSE LEYDIG CELLS

APPROVED BY SUPERVISORY COMMITTEE

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REGULATION OF EXPRESSION OF 5 ALPHA-REDUCTASE TYPE 1 IN MOUSE LEYDIG CELLS

by

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THESIS

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REGULATION OF EXPRESSION OF 5 ALPHA-REDUCTASE TYPE 1 IN MOUSE LEYDIG CELLS

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Testosterone is the principal androgen produced in the testis of most species. The enzyme 5α-reductase converts circulating testosterone to dihydrotestosterone in target tissues, a process that is necessary for development of the male phenotype in human beings. However, in some animals,

androstanediol, a 5α-reduced androgen, is the primary androgen produced by the testis during certain stages of development, indicating the presence of 5α-reductase in the testis itself. Studies in mice show that while both isozymes of 5α-reductase are absent in the fetal and adult testis, 5α-reductase type 1 is the only form present in the immature (prepubertal) testicular Leydig cells when androstanediol is their principal product. The high testosterone production by the fetal and adult mouse testis is driven by human chorionic gonadotropin and luteinizing hormone, respectively, which act through elevation of intracellular cyclic AMP. Consequently, we hypothesized that 5α-reductase type 1 is constitutively present in the mouse Leydig cell and repressed by cyclic AMP. We screened two mouse Leydig cell lines: MLTC-1 cells & MA-10 cells, for 5α-reductase type 1 activity. Both cell lines expressed abundant 5α-reductase type 1 activity under basal conditions and mmetabolized progesterone to allopregnanolone, but MA-10 cells yielded additional products. Treatment of MLTC-1 cells with forskolin or human chorionic gonadotropin dramatically decreased 5α-reductase type 1 activity in the cells after 1 hour. Future experiments will address the mechanism of this effect, including the changes in transcription, translation, and degradation.

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Abbreviations

4MA = 17β-N,N-diethylcarbamoyl-4-aza-5α-androstan-3-one

5αR1 = 5α-reductase type 1

5αR2 = 5α-reductase type 2

AC = adenylyl cyclase

Adiol = 5α-androstanediol

alloP = allopregnenolone

AMP = adenosine monophosphate

ATP = adenosine triphosphate

CAP = catabolite activator protein

CYP17 = cytochrome P450 CYP17

DHT = dihydrotestosterone

DHP = dihydroprogesterone

DMEM = Dulbecco's modified Eagle medium

DNA = deoxyribonucleic acid

dNTP = deoxynucleoside triphophate (e.g., dATP, dUTP, dGTP, dCTP)

EDTA = ethylenediaminetetraacetic acid

FSH = follicle stimulating hormone

hCG = human chorionic gonadotropin

HEPES = N-2-hydroxyethylpiperazine- N'-2-ethane sulfonic acid

HPLC = high performance liquid chromatography

HSD = hydroxysteroid dehydrogenase

ICER = inducible cyclic AMP early repressor

LH = luteinizing hormone

PBS = phosphate-buffered saline

PCR = polymerase chain reaction

PKA = cyclic-AMP-dependent protein kinase

Prog = progesterone

RNA = ribonucleic acid

SRD5αR2 = 5α-reductase type 2 deficiency

STAR = steroidogenic acute regulatory protein

T = testosterone

TLC = thin layer chromatography

Chapter 1: Background and Previous Studies

Introduction

The Jost model for sexual differentiation includes three distinct sequential stages during which differentiation occurs; these are the genetic, gonadal, and phenotypic stages [1,2]. At the genetic stage, chromosomal sex is established at fertilization, where XY indicates male while XX represents female. For the first two months of human gestation, the two sexes develop in an identical fashion. The gonadal stage is the period during which the indifferent gonads develop into either ovaries or testes. The phenotypic stage is where sexual phenotypes develop as a result of gonadal differentiation; the internal genital tract and the external genitalia develop into their characteristic male or female structures.

The anatomic events occurring in the embryo to define phenotypic sex are as follows [2,3]. The internal urogenital tract arise from two sets of ducts, the wolffian and mullerian ducts, present in the early embryos of both sexes. In females, the mullerian ducts give rise to the fallopian tubes, uterus, and upper vagina, and the wolffian ducts persist in vestigial form. In males, the wolffian ducts give rise to the epididymides, vas deferens, seminal vesicles, and ejaculatory ducts, and the mullerian ducts regress.

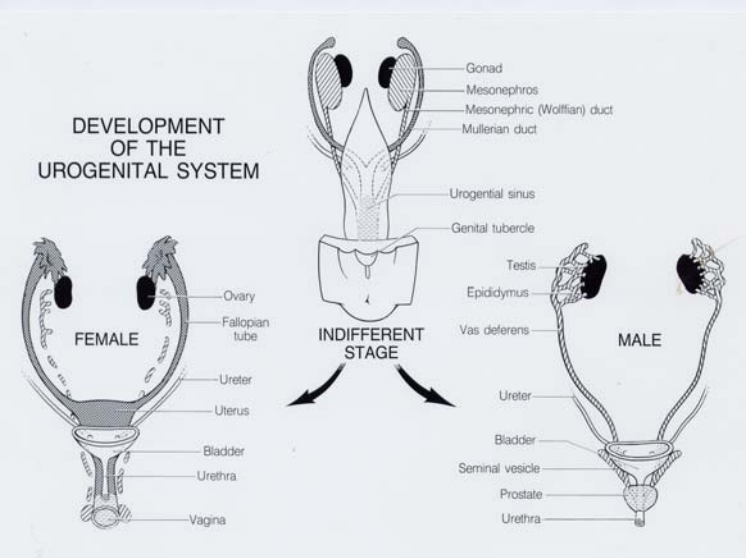


Figure 1: Development of the internal urogenital tract. Indifferent stage: bipotential gonads exist here. When no Y chromosome present, the bipotential gonads form the ovaries. In the presence of a Y chromosome, the bipotential gonads form the testis. (Figure courtesy of Dr. Jean Wilson.)

The external genitalia and urethra in both sexes develop from the common genital tubercle, the genital swellings, and the genital folds. In females, the genital tubercle becomes the clitoris, the genital swellings become the labia majora, and the genital folds become the labia minora. In males, the genital swellings fuse to become the scrotum, the genital folds elongate and fuse to form the shaft of the penis and the penile urethra which terminates in the glans penis formed from the genital tubercle, and the prostate forms in the walls of the urogenital sinus. Formation of the male phenotype is largely complete by week 12, except for testicular descent which takes place in the latter part of gestation.

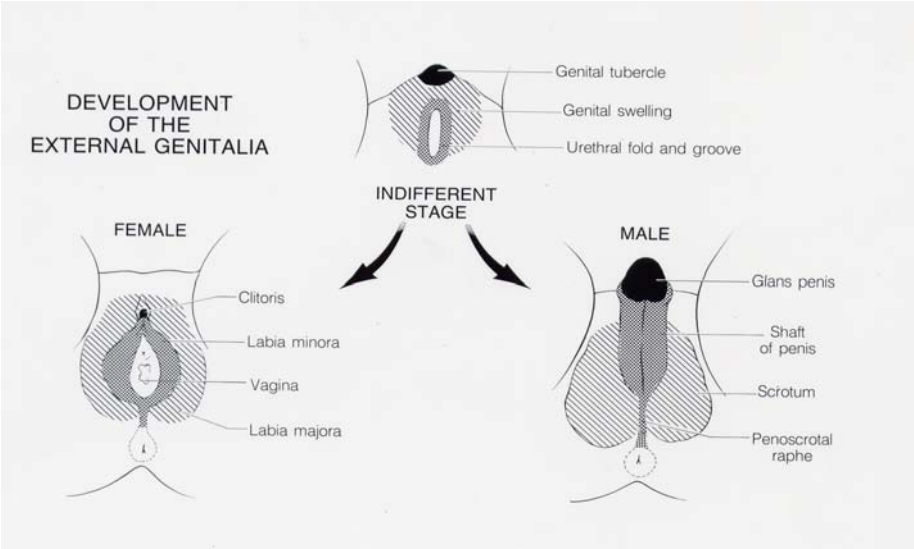


Figure 2: Development of the external genitalia. The constitutive pathway is female (meaning that even if gonadal differentiation does not occur, female external genitalia will form). Formation of male external genitalia primarily revolves around the genital tubercle forming the glans penis and the urethral groove closing up. (Figure courtesy of Dr. Jean Wilson.)

Looking specifically at male reproductive physiology, there are many hormones that control the formation of the male urogenital tract [3-5]. Antimullerian hormone is made by the testes and prevents development of the uterus. Testosterone (T) secreted by the testes induces development of the male ejaculatory system. 5α-dihydrotestosterone (DHT) in target tissues is required for the normal masculization of the external genitalia in utero.

In normal androgen physiology, T from the testis is converted via 5α-reductase type 2 (5αR2) to form DHT in target tissues [4,6]. This conversion involves reduction at the 5 carbon position of the A ring of the steroid molecule. This reaction changes the configuration of DHT, allowing it to fit into the

androgen receptor in a manner slightly different than testosterone. Thus, DHT, the most potent androgen, is bound selectively to the androgen receptors in genital skin and fibroblasts, making its action necessary for the development of normal male genital anatomy in the fetus. Testosterone is involved in spermatogenesis, regulation of gonadotopin, and Wolffian stimulation. However, DHT is responsible for external virilization and sexual maturation at puberty [4,5].

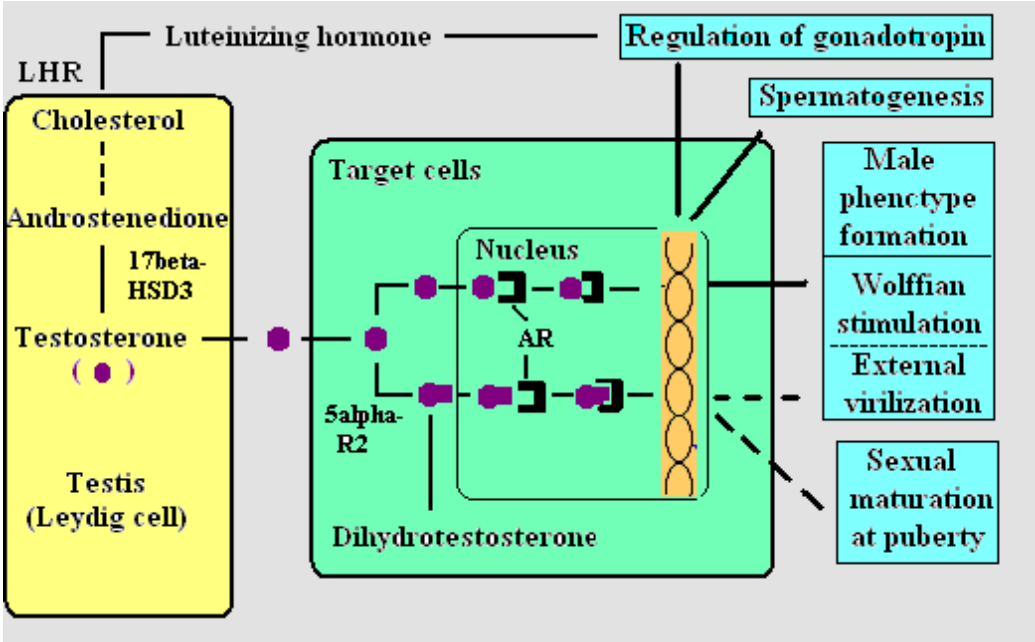


Figure 3: Normal androgen physiology shows that testosterone, secreted by the testis, binds to the androgen receptor in a target cell, either directly or after conversion to dihydrotestosterone. Dihydrotestosterone binds more tightly than testosterone, and the complex of DHT and the androgen receptor can bind more efficiently to chromatin. The major actions of androgens are mediated by testosterone or dihydrotestosterone. (Figure adapted from Griffin 1992.)

There are many causes of virilization defects in genetic males [5]. Impairment of testosterone synthesis can occur due to single gene defects (such as

those in 17βHSD, 3β-HSD2, and CYP17) or developmental defects in the testes . Defective 5α-reduction of testosterone is one cause of virilization defects [6-8]. Abnormalities of the androgen receptor can occur, causing complete and partial androgen insensitivity [9].

Looking specifically at defective 5α-reduction of testosterone, the condition 5α-reductase type 2 deficiency (SRD5α2) is an autosomal recessive sex-limited condition resulting in the inability to convert T to DHT [6-8]. Through observations of humans with SRD5α2, it is known that DHT is required for the male phenotype [4]. Because DHT is required for the normal masculization of the external genitalia in utero, genetic males with SRD5α2 are born with ambiguous genitalia (i.e., male pseudohermaphroditism) [10]. Described clinical abnormalities range from infertility with normal male genital anatomy to underdeveloped male genitalia with hypospadias to predominantly female external genitalia, most often with mild clitoromegaly [10,11]. The uterus and fallopian tubes are absent because of the normal secretion of antimullerian hormone. Testes are intact, as are Wolffian structures (the epididymis, vas deferens, seminal vesicles). Male internal ducts are present but terminate either in a blind pseudovaginal pouch or on the perineum. A hypoplastic prostate may be present, regardless of the degree of undervirilization of the external genitalia.

Previous Studies

The model proposed above, in which T is converted to DHT by 5αR2 in target tissues, predicts that testosterone, not dihydrotestosterone, is the principal circulating androgen during sexual differentiation [12]. These experiments are not feasible in human male fetuses; studies were instead done in an experimentally approachable marsupial model, the wallaby [13]. Extensive studies revealed that there were two pathways for steroid biosynthesis and that 5α-androstanediol (Adiol) was the major circulating 19-carbon steroid during sexual differentiation [13-15]. In the classical pathway testosterone is made from progesterone in the testis, and 5αR2 is located in target tissues. This pathway is used for some of the Adiol production from testicular testosterone. The back-door pathway also involves 5α-reductase, but, this 5α-reductase is present in the testis, although it was unclear which isozyme was present [16]. The back-door pathway for steroid biosynthesis is utilized the most in the wallaby.

Pathways for Steroid Biosynthesis in the Wallaby

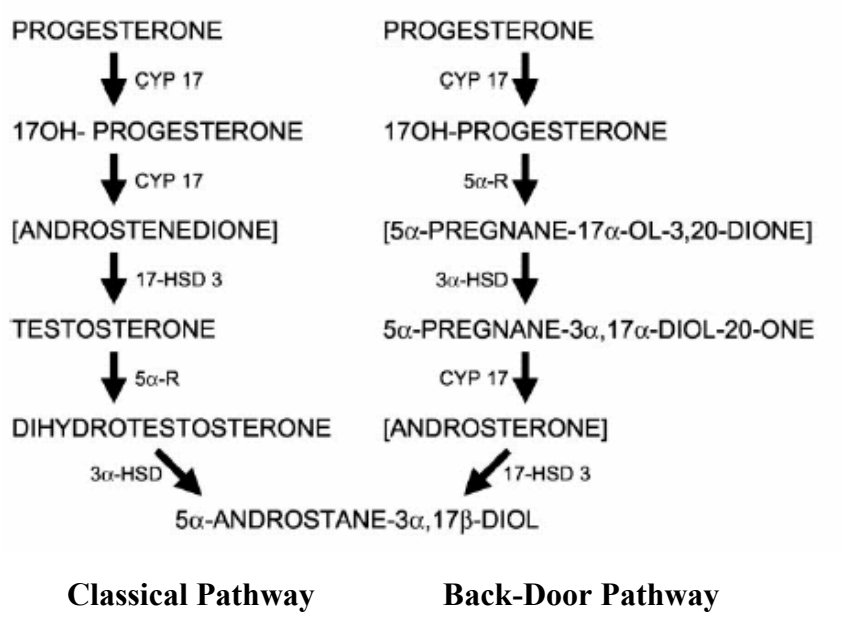


Figure 4: Classical and back-door pathways for steroid biosynthesis in the wallaby. Classical pathway: left, 5α-reductase is type 2 located in target tissues, & this is used for some of the androstandiol production. Back-door pathway: right, we don't know which isozyme of 5α-reductase this is but we know it is present in the testis, & this is the pathway utilized the most in the wallaby. (Figure courtesy of Dr. Richard Auchus.)

Two genes encoding for 5α-reductase have been identified in the mouse and human, each encoding a slightly different isoenzyme [17-20]. The two isoforms of 5α-reductase are very hydrophobic 30 kDa microsomal proteins that share 50% identity. The product of 5α-reductase type 1 (5αR1) is expressed only in nongenital skin and liver at low levels from the time the individual is aged 3 years to puberty, at which time enzyme expression is measurable in sebaceous glands and scalp [21]. It is not expressed well in the developing fetus, which

explains why a deficiency of 5αR2 is not compensated by 5αR1. Linkage analysis has demonstrated that 5αR1 is unrelated to the clinical syndrome of SRD5α2. The other isoenzyme, 5αR2, found on chromosome 2, correlates with clinical symptoms. It is expressed in high levels in the prostate and other androgen-sensitive issues [22]. 5αR2 is responsible for most of the hepatic 5α-reduction. Additionally, progesterone (prog) is a better substrate for both isoforms than testosterone; 5αR1 has better activity with prog and 17-hydroxyprogesterone than 5αR2 [18].

Mice were also used as an experimental model in order to determine which isozyme of 5α-reductase is present in the testis and back-door pathway [23]. Mice were used for two reasons: previous literature indicates that Adiol is produced in the immature mouse testis, therefore some form of 5α-reductase is present. The other reason mice were studied was due to the ability to make knockouts specific to the two 5α-reductase isozymes. Studies were performed to look at progesterone metabolism in mouse testes at different stages: fetal, neonatal (also known as prepubertal or immature), and adult; these studies looked at 5α-reduced versus non-5α-reduced metabolites. These studies found that in the fetal and adult stages, no 5α-reduced metabolites were produced in the testis; however, during the immature stage (occurring in day 24 to 26 mice), 5α-reduced metabolites were present, and specifically, Adiol is the predominant androgen in

the testis during this stage. Also, using 5α-reductase knockout mice, these studies determined which isozyme was present in the immature stage where the 5α-reduced metabolites were present, therefore determining which isozyme of 5α-reductase was involved in the back-door pathway for steroid biosynthesis.

In order to determine which 5α-reductase isozyme is present in the immature testis, minces from testis of day 24 or 25 immature mice were incubated with tritium-labeled progesterone [23]. The pattern of metabolism was similar in wild type and 5αR2 deficient mice in that the radioactivity was recovered in areas corresponding to T, DHT, and Adiol. Adiol was the principal androgen recovered testes from both animals. In contrast, no 5α-reduced metabolites were recovered after incubation of testes deficient in 5αR1, and the only 19-carbon steroids formed were androstenedione and testosterone. It was concluded therefore that 5αR1 appears to be the only 5α-reductase isoenzyme expressed in the mouse testis at this stage of development.

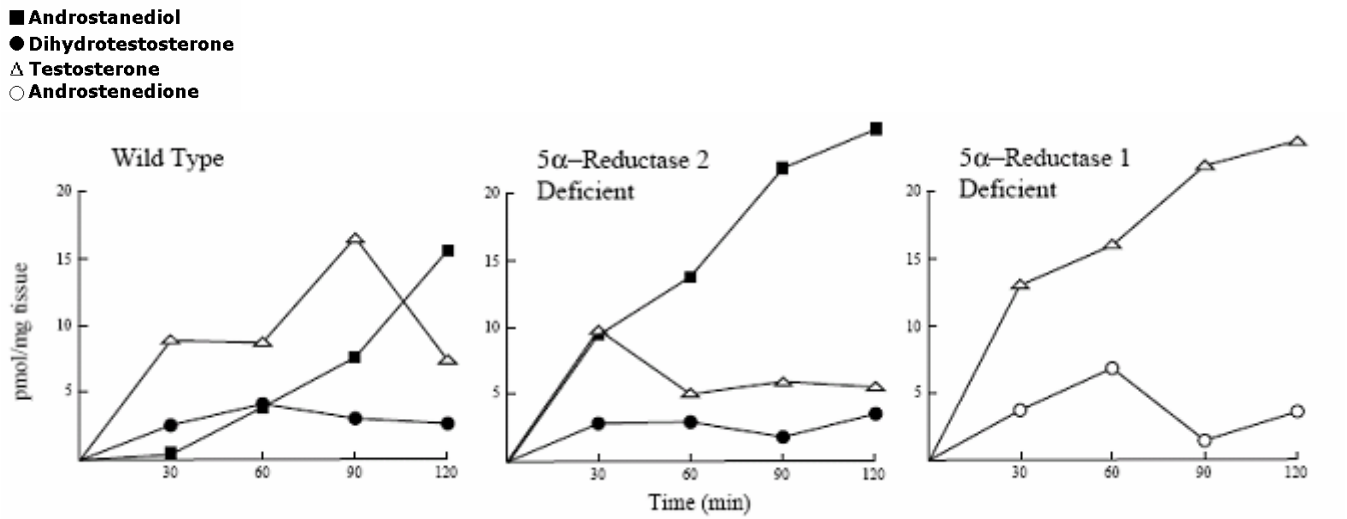


Figure 5: Conversion of tritium-labeled progesterone to androgens by testes from immature wild type and 5α-reductase deficient animals. [Testes minces from day 25 or 26 mice were added to tubes containing 50 μl DMEM, 1% fetal calf serum, and 2 μM [^{3}H]-labeled prog. The tubes were then gassed with 95% oxygen-5% carbon dioxide, capped, incubated with shaking at 37^{o}C for the times indicated, and analyzed by HPLC.] (Figure courtesy of Dr. Richard Auchus.)

To determine the cellular localization of 5αR1 in the immature mouse testis, testes from 24 day old wild type and 5αR1 deficient mice were subjected to in situ mRNA hybridization using radiolabelled sense and antisense strand probes [23]. 5αR1 mRNA was detected with the antisense probe in wild type testes in Leydig cells in the interstitium between the seminiferous tubules, and this labeling was absent in a testis from a day 24 mouse deficient in 5αR1. [The specificity of this expression is supported by the absence of specific hybridization signal with a 5αR1 mRNA sense probe in the wild type day 24 mouse testis.]

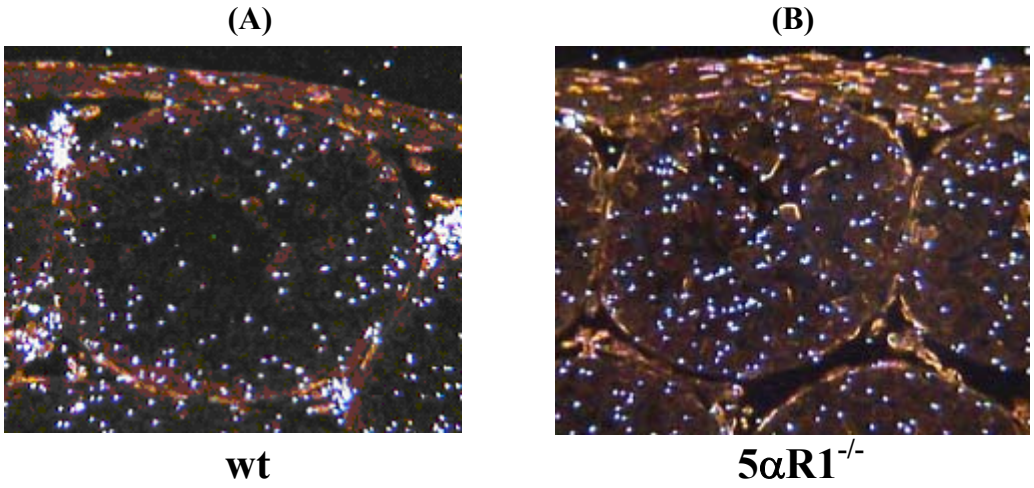


Figure 6: Hybridization of 5αR1 antisense- and sense-strand [^{35}S]-RNA probes to 24 day old immature mouse testes. A) wild-type 24 day mouse testis probed with antisense-strand probe to 5αR1-> shows presence of 5αR1 mRNA-mRNA is localized to the interstitium between the seminiferous tubules- these are where Leydig cells are found. B) 5αR1 deficient 24 day mouse testis probed with antisense-strand probe for 5αR1 shows no 5α-reductase present in Leydig cells. (Figure courtesy of Dr. Richard Auchus.)

The hybridization studies proved that 5αR1 is present in the Leydig cell, and that 5αR1 is indeed the isoform that is present. In summary, studies in day 24-26 immature male mouse testis show similar patterns of metabolism of progesterone to 5α-reduced androgens in wild type and 5αR2-deficient mice. Studies also determined that 5αR1 is the only isoenzyme expressed in day 24-26 mouse testes. In mice, the two pathways for steroid biosynthesis consist of the classical pathway, where the 5αR2 isozyme is present and is located in target tissues, and the back-door pathway where the 5αR1 isozyme is present and is

located in the testis. The classical pathway is utilized in fetal and adult mice while the back-door pathway is utilized in the immature mouse.

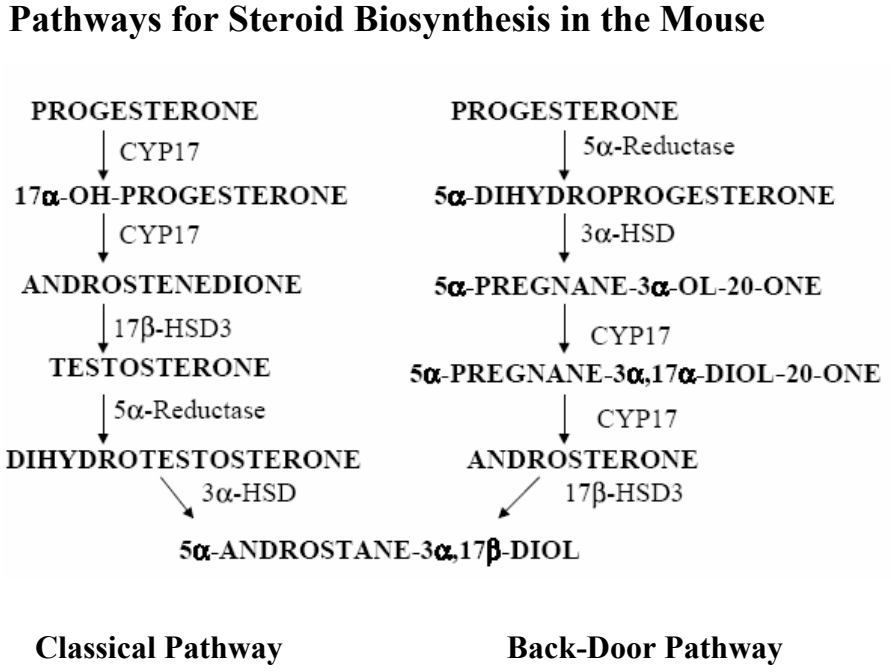


Figure 7: Classical and back-door pathways for steroid biosynthesis in the mouse. Classical pathway: left, 5α-reductase is type 2 located in target tissues, & this is what is utilized in fetal & adult mice. Back-door pathway: right, 5α-reductase is type 1 located in testis, & this is utilized in immature mouse. (Figure courtesy of Dr. Richard Auchus.)

Chapter 2: Regulation of 5α-reductase type 1 expression

Introduction

Based on the previous studies, we asked the question: "What regulates the expression of 5αR1 in the testis?" It is known in mice that 5αR1 is expressed only in the immature animal; this suggests negative regulation by luteinizing hormone (LH) via cyclic AMP (cAMP). In the fetal and adult stages of mice, high levels of testosterone production occur; this is driven by human chorionic gonadotropin (hCG) and LH, which upregulate the machinery/enzymes necessary to make testosterone and act through the elevation of intracellular cyclic AMP [23]. Since 5αR1 is only expressed in the immature animal when neither hCG or LH is present, this is indicative of an inverse relationship between testosterone biosynthesis and 5αR1 activity. Therefore, the working hypothesis for my studies was that 5αR1 is constitutively expressed in the mouse testis, and that it is repressed by cyclic AMP.

Many water soluble hormones do not cross the cell membrane, but instead cause effects within the cell via a second messenger. There are several known second messenger systems, of which the most ubiquitous is adenylyl cyclase/cAMP/cAMP-dependent protein kinase. Cyclic AMP is a small cyclized monophosphate that is produced from ATP by the enzyme adenylyl cyclase [24]. For a second messenger to work effectively, it must have the following

characteristics: (1) Amplification- when a few hormone molecules deliver the signal to a cell, the cell needs to respond with a signal that is strong enough to change its physiology without repeated high levels of stimulation, (2) Control- eventually a response must be shut down, and there are instances when it is necessary to modulate the response- so control is needed, and (3) Specificity- when a hormone is released, target cells should respond in a specific way; not all cells will respond to this hormone and the hormone may not have the same effect in all of the cells that do respond to it. All of these criteria are met in the cyclic AMP system.

Cyclic AMP is a key and widespread mediator in the conversion of extracellular signals into intracellular events, and many of the components of its signaling pathway have been well characterized. The cAMP concentration in a cell is a function of the ratio of its rate of synthesis from ATP by adenylyl cyclase and its rate of breakdown to AMP by a specific phosphodiesterase. Adenylyl cyclase (AC), a transmembrane protein, is stimulated by the binding of certain hormones to their cell-surface receptors, to catalyze the synthesis of cAMP inside the cell. A classic example used to explain the actions of cyclic AMP is when glycogen acts on receptors in the liver, cyclic AMP is produced. The second messenger then goes on to activate a cascade of enzymes that allows the mobilization of glucose from glycogen. Many different hormones working in many different cells utilize cyclic AMP. For example: adipose tissue, epinephrine

increases triglyceride hydrolysis; cardiac muscle, epinephrine increases contraction rate; kidney, vasopressin increases reabsorption of water; and bone cells, parathyroid hormone, stimulates reabsorption of calcium from bone.

Although cyclic AMP can directly activate certain types of channels in the plasma membrane of some highly specialized cells, in most animal cells it exerts its effects mainly by activating cyclic-AMP-dependent protein kinase (PKA). In response to the binding of cAMP to regulatory subunits of PKA, the catalytic subunits are released and catalyze phosphorylation of target proteins [24]. Cyclic AMP is required for the activity of PKA; this enzyme catalyzes transfer of the terminal phosphate group from ATP to specific serines or threonines of selected cellular target proteins, including phosphorylase kinase and glycogen synthase, thereby regulating their activity. PKA is found in all animal cells and is thought to account for the effects of cyclic AMP in most of these cells. The intracellular concentration of cAMP therefore determines the fraction of PKA in its active form and thus the rate at which it phosphorylates its substrates. The substrates for PKA differ in different cell types, which explains why the effects of cyclic AMP vary so markedly depending on the cell type. The targets of PKA include enzymes involved in glycogen metabolism. Thus, when epinephrine binds to the β-adrenergic receptor of a muscle cell, for example, the sequential activation of a G-protein, AC, and PKA leads to the activation of glycogen phosphorylase,

thereby making glucose-6-phosphate available for glycolysis in a “fight-or-flight” response.

It is classically believed that cAMP acts through activation of the PKA, but there are some reports demonstrating that cAMP also induces some PKA-independent effects. Some authors speculate that AC, responsible for the production of cAMP, is arranged in such a way that cAMP is “channeled” from the AC to PKA by a molecular mechanism. Conversely, in a few cell types, some effects of cAMP have been described to be independent of PKA.

Gene regulatory proteins rescue poorly functioning promoters by binding to a nearby site on the DNA and dramatically increase the probability that RNA polymerase will initiate transcription. Because the active, DNA-binding form of such a protein turns genes on, this mode of gene regulation is called positive control, and the gene regulatory proteins that function in this manner are known transcriptional activators or gene activator proteins. A transcriptional activator can operate as part of a simple on-off genetic switch in combination with a transcriptional repressor. The bacterial activator protein CAP (catabolite activator protein), for example, activates genes that enable *E. coli* to use alternative carbon sources when glucose, its preferred carbon source, is not available [25]. Falling levels of glucose induce an increase in cyclic AMP, which binds to the CAP protein, enabling it to bind to its specific DNA sequence near target promoters and thereby turn on the appropriate genes. In this way the expression of a target

gene is switched on or off, depending on whether cyclic AMP levels in the cell are high or low, respectively.

Cyclic AMP can upregulate expression of various genes; several positive acting cAMP response elements have been described. The expression of steroidogenic acute regulatory protein (StAR) is regulated by cAMP-dependent signaling in steroidogenic cells [26,27]. hCG has been used to study the regulation of StAR protein expression in mouse Leydig cells. Ovarian and testicular steroid hormone production is under the control of the gonadotropins, luteinizing hormone and follicle stimulating hormone, or LH and FSH, respectively [28]. Testosterone production by Leydig cells is under the control of LH. LH is secreted by the anterior pituitary and travels via the blood stream to the testes where it binds to receptors on the surface of the Leydig cells and stimulates testosterone production. LH binds to receptors on the outside surface of the cell and activates an intracellular second messenger system inside the cell to provoke cellular responses. LH actions in Leydig cells are signaled via the production of cAMP, which activates the enzyme protein kinase A, and protein kinase A activation promotes testosterone production. Cyclic AMP therefore stimulates gene expression by activating PKA, which in turn phosphorylates members of the CREB/ATF family of transcription factors, thereby increasing their transactivation potential [29]. Studies have shown that LH, hCG, and analogs of cAMP (dibutyryl cAMP, 8-bromo-cAMP), coordinately increase StAR

protein and StAR mRNA levels in a time frame that is consistent with increased steroid production in mouse Leydig cells [26,27].

The negative regulation of gene transcription by cAMP is far less well documented than positive regulation. According to the most studied examples, inhibition of transcription by cAMP can be achieved through various mechanisms, all of which ultimately result in the alteration of the binding properties of transcription factors to promoter elements. In the gene for L-type pyruvate kinase, the L4 element binds major late transcription factor; it is the glucose/insulin response element and is required for inhibition by cAMP. Inhibition by cAMP also requires the contiguous L3 element, an element that binds hepatic nuclear factor 4. Cyclic AMP also inhibits transcription of the genes for IL-2 and IL-2R in EL4 cells; the inhibition requires an AP1 site [30] this case, cAMP increases the binding of Jun/Fos heterodimers to the AP1 site and alters the composition of Jun proteins that participate in the AP1 complex. A third example of inhibition of transcription by cAMP involves the hepatic gene for fatty acid synthase; insulin-induced transcription of this gene is inhibited by cAMP. The cis-acting element required for the inhibitory effect is an inverted CAAT box. The proteins that bind to this element have not been identified. As demonstrated for other genes, including that encoding α1-AR, the inhibitory effect of cAMP on transcription can be achieved through increased binding to CRE of repressors of the CRE modulator protein family, such as CREM α and β

(CRE modulator) or ICER (inducible cAMP early repressor) [31]. Another example of negative regulation of gene expression by cAMP is malic enzyme: glucagon, acting via cAMP, inhibits transcription of the malic enzyme gene in chick embryo hepatocytes [32]. The chicken malic enzyme gene is expressed primarily in hepatocytes and is subject to regulation by various hormones and nutritional states. It has been shown that increased intracellular levels of cAMP markedly decrease transcription of the gene for malic enzyme in chicken embryo hepatocytes in culture via the classical PKA signaling pathway. In the nucleus, the phosphorylation states of transcription factors and related proteins appear to modulate function and expression of cAMP-responsive genes directly.

In order to determine if cyclic AMP suppresses 5αR1 activity and mRNA, metabolism studies were conducted using forskolin and hCG. Forskolin acts primarily by directly activating the enzyme adenylyl cyclase which then results in increased cyclic AMP in cells. Hormone and neurotransmitters also activate AC through the action of GTP-binding proteins, primarily $G_{\alpha s}$ subunits. Thus, forskolin can increase cyclic AMP without the assistance of hormones or neurotransmitters in preparations of membranes, cells, or tissues. The ability of forskolin to interact with AC has been exploited to develop affinity reagents for labeling or purifying the enzyme, sometimes termed C, the catalyst that generates cyclic AMP.

hCG is an agonist of the LH receptor, which controls testosterone production in Leydig cells [24,27]. Endocrine regulation of Leydig cell functions occurs predominantly through action of LH/hCG, interacting with its cognate receptor and coupling to the adenylyl cyclase signal transduction system. Adding hCG indirectly increases intracellular levels of cyclic AMP.

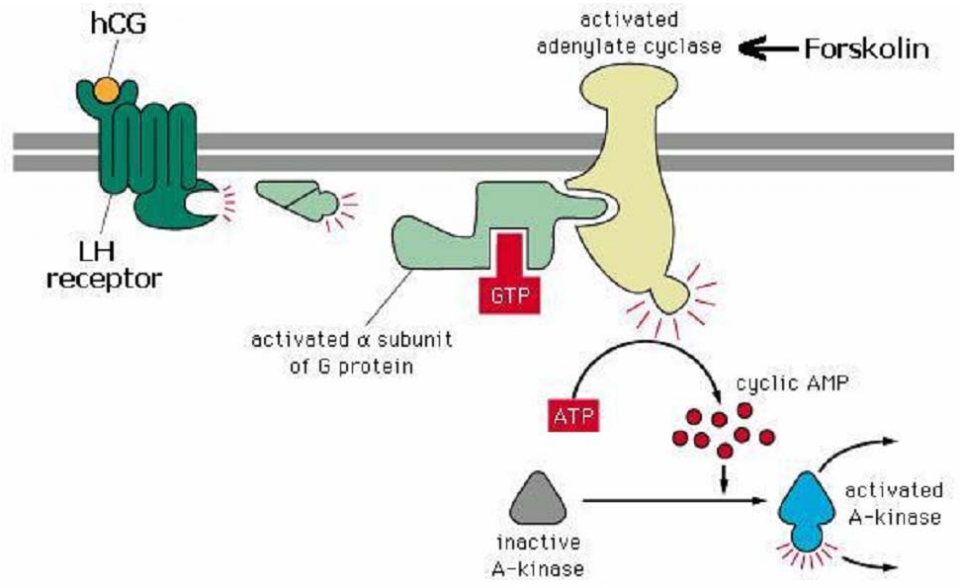


Figure 8: Forskolin & hCG effects on cyclic AMP. Forskolin directly activates adenylyl cyclase, raising intracellular levels of cyclic AMP. Alternatively, hCG indirectly increasing cyclic AMP by binding the LH receptor, which is coupled to the adenylyl cyclase signal transduction system.

Metabolic studies conducted were looking specifically at the conversion of progesterone to dihydroprogesterone (DHP) and then to allopregnenolone alloP).

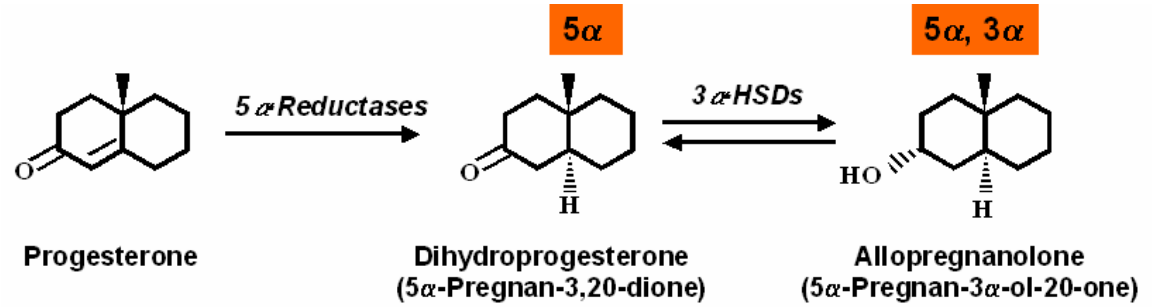


Figure 9: Metabolic conversion being studied in cell assays: progesterone is 5α-reduced to dihydroprogesterone, which is then further reduced via 3α-hydroxysteroid dehydrogenases to allopregnanolone. (Figure courtesy of Dr. Richard Auchus.)

Two mouse Leydig cell lines were screened: MLTC-1 cells and MA-10 cells, for 5α-reductase activity. Both cell lines expressed abundant 5α-reductase activity under basal conditions and metabolized prog to alloP, but MA-10 cells yielded additional products due to induction of CYP17 (cytochrome P450 CYP17) activity. Timecourses were then conducted in MLTC-1 cells in order to look at metabolism under various conditions: incubation with forksolin, hCG, and 4MA (17β-N,N-diethylcarbamoyl-4-aza-5α-androstan-3-one, a known inhibitor of 5α-reductase).

Methods

MLTC-1 timecourses: Mouse Leydig tumor cells were the primary cell line used in metabolic studies. Cells were thawed and maintained until confluent in complete media (DMEM, 10% bovine serum, and 1% penicillin/streptomycin). Flasks were then split into 12 well plates (1 ml/well) and wells were treated with a) 5 μl ethanol- control, b) 2 μl 10 mM forskolin, c) 5 μl hCG (1000u/ml), or d) 5 μl 1 mM 4MA for 1, 2, 4 and 24 hours. After each of these pre-incubation times, cells were assayed with 100 μl of master mix (300,000 cpm [^{3}H]-prog, 2 μl cold 0.01 mM prog, and 100 μl complete media) per well, and 500 μl aliquots were sampled at 20 and 40 minutes after addition of steroid. Each 500 μl aliquot was then extracted via addition of 1 ml of ethyl acetate:isooctane (1:1), and the organic phase was then dried down in a nitrogen evaporator. After drying, 35 μl of methylene chloride was added to each tube, which was then vortexed, and then spotted to plastic labeled TLC plate with a 4 μl microcapillary tube. Plates were run in a TLC chamber with 3 parts chloroform and 1 part ethyl acetate until complete. Plates were then dried and exposed to a phosphoimager screen. After retrieving results, plates were then sprayed with scintillation fluorescent spray and exposed to film, to allow for identification of steroids, which were cut and quantitated by scintillation counting.

MA-10 timecourses: MA-10 cells were also used in metabolic studies. Assays were conducted in a similar manner as those in MLTC-1 cells (see above protocol). MA-10 cells differed in two ways: cells were grown in a specific growth medium (Waymouth MB752/1 with 20mM HEPES, 15% horse serum, and 50 μg/ml gentamicin, pH 7.7), and flasks/plates used to store the cells were first coated with gelatin (0.5 g DIFCO bactogelatin was added to 500 ml Ca^{+2}/Mg^{+2}-free PBS and autoclaved, gelatin solution was added to flasks/plates and incubated at room temperature for 45 minutes and then aspirated off prior to addition of cells).

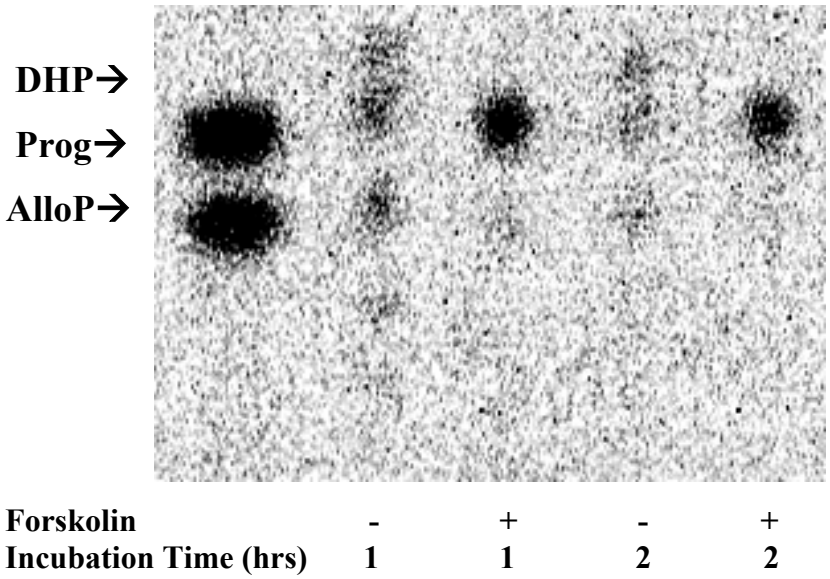
Real-Time PCR: Each of the MLTC-1 timecourses was done in triplicate with ethanol, forskolin, hCG, or 4MA. One set of wells was used for enzyme activity assays. One set of cells was harvested after various pre-incubation times for RNA analysis. Cells were harvested by aspirating off media, and incubating for 5 minutes with 400 μl 10 mM EDTA. After incubation, EDTA solution containing cells were centrifuged at 13,200 rpm for 1 minute, and supernatant aspirated off. Pellets were stored at –70^{o}C. RNA was harvested from cells using RNA STAT-60 Total RNA Isolation Reagent and protocol. DNA-free protocol was used then used to remove genomic DNA from total RNA in aqueous solution. Isolated RNA concentrations were then determined spectrometrically, and 2 μg total RNA was used to make cDNA using the TaqMan cDNA Synthesis Kit (Applied Biosystems). Twenty ng of cDNA was used to evaluate 5αR1 mRNA

expression by real-time PCR using the SYBR green detection system. Sample variation was accounted for by normalization to the housekeeping gene cyclophilin.

Results & Discussion

MLTC-1 control vs. forskolin timecourse

A)



B)

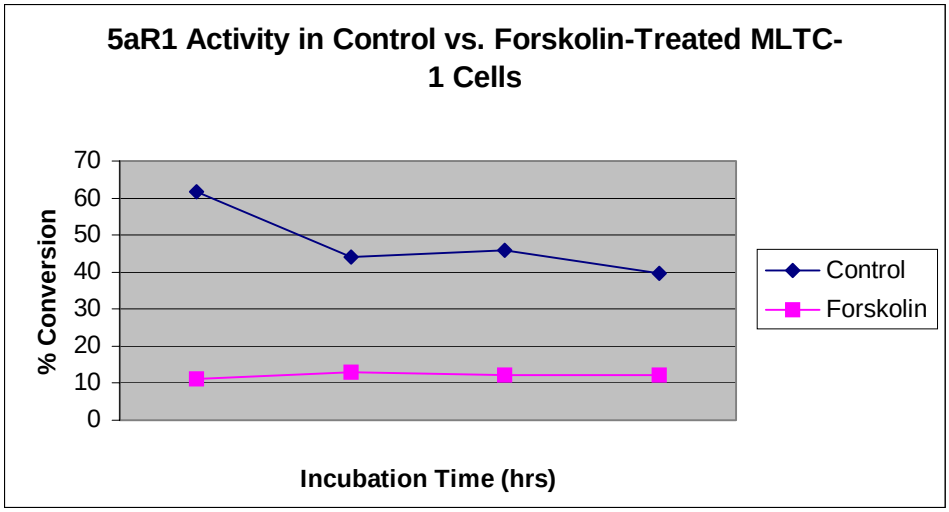
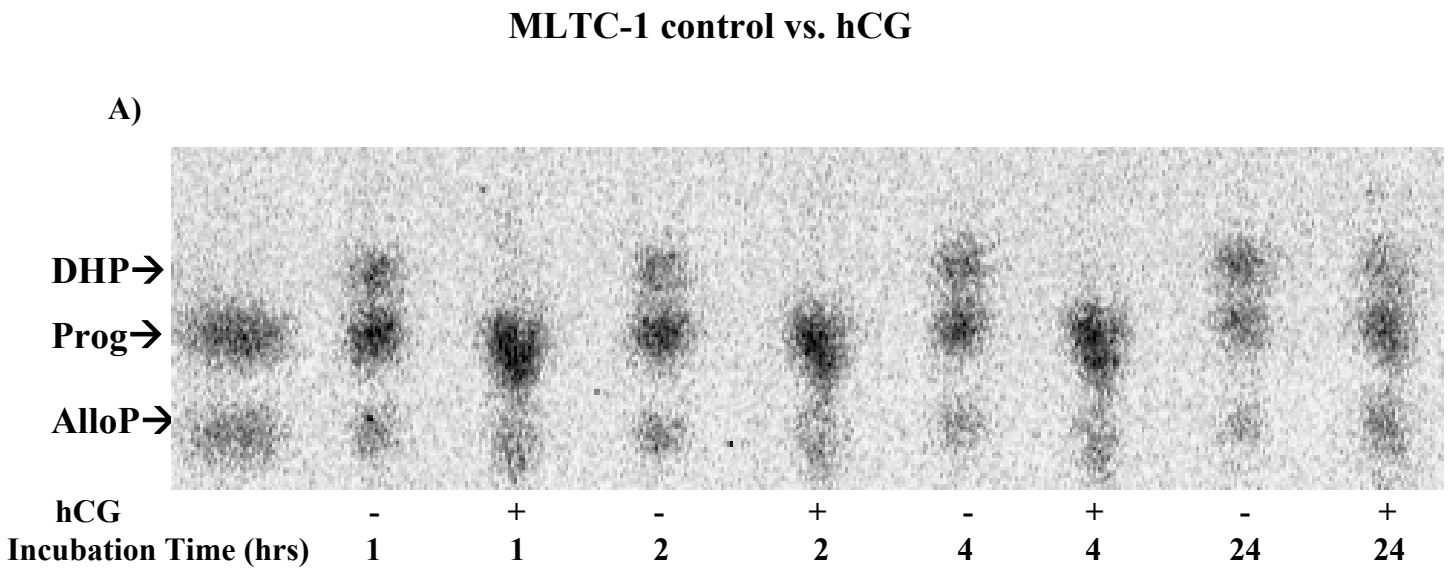


Figure 10: Timecourse results of MLTC-1 cells with and without forskolin treatment. A) TLC plate of control versus forskolin-treated cells incubated at periods of 1 or 2 hours. treatment of forskolin for 1 or 2 hours. After 1 hour pre-incubation with forskolin, 5α-reduction of prog decreases. B) % conversion of prog to its metabolites DHP and alloP is shown- 5α-reduction of prog is reduced up to 24 hours in cells treated with forskolin.

After only a 1 hour incubation with forskolin, the amount of 5α-reduction of progesterone that occurs is significantly reduced. The percent conversion of prog to 5α-reduced metabolites DHP and alloP ranges from approximately 60 to almost 100 percent in the control cells. However, after incubation with forskolin for only 1 hour, the conversion rate decreases to only about 10%. Therefore, within only 1 hour, forskolin dramatically decreases 5αR1 activity, and forskolin suppression of 5αR1 activity lasts 24 hours.



B)

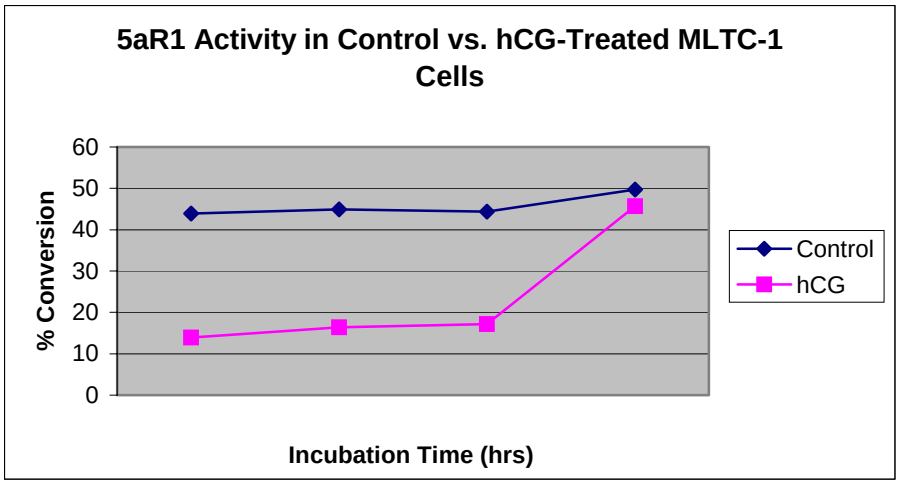


Figure 11: Timecourse results of MLTC-1 cells with and without hCG treatment. A) TLC plate of control versus hCG-treated cells incubated at periods of 1 or 2 hours. treatment of forskolin for 1 or 2 hours. After 1 hour pre-incubation with hCG, 5α-reduction of prog decreases. B) % conversion of prog to its metabolites DHP and alloP is shown- 5α-reduction of prog increases back to levels similar to that of control cells in cells treated for 24 hours with hCG.

After only a 1 hour incubation with hCG, the amount of 5α-reduction of prog that occurs is also significantly reduced. However, after 24 hours, the hCG-treated cells exhibit activity similar to that of the control cells. The percent conversion of prog to 5α-reduced metabolites DHP and alloP after pre-incubation with hCG for one hour was only 10-20%. However, again after 24 hours, the conversion rate increases back up to nearly 50%. The reappearance of 5αR1 activity in the timecourses may be due to the LH receptor being downregulated, therefore hCG is not able to exert its effects in increasing cyclic AMP concentration, so 5αR1 is no longer repressed. To summarize, within 1 hour, hCG dramatically decreases 5αR1 activity. After 24 hours however, the 5αR1 activity returns. These results help support the overall hypothesis that 5αR1 is constitutively expressed but that during fetal and adult stages it is repressed due to increased LH activity attributing to the large amounts of testosterone produced during those stages.

MLTC-1 control versus 4MA

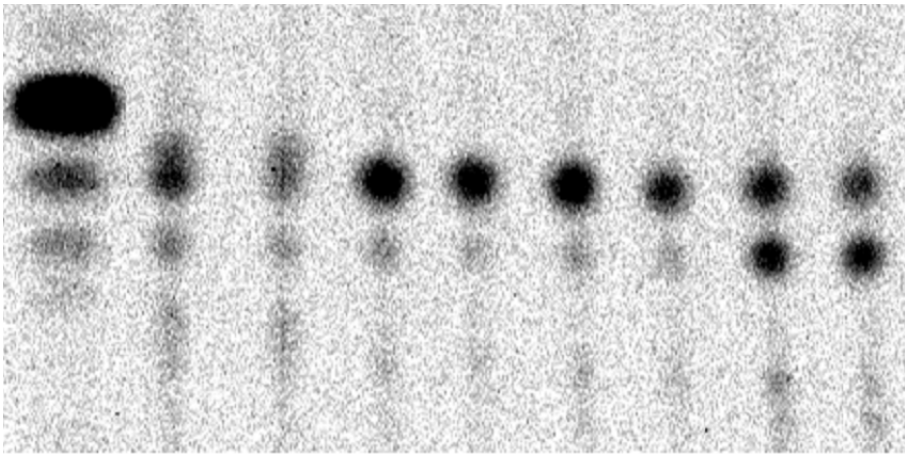


Figure 12: Timecourse results of MLTC-1 cells with and without treatment with forskolin, hCG, or 4MA. In the forskolin & hCG-treated cells, the amount of prog metabolites DHP & alloP are reduced, while in the 4MA-treated cells, the amount of alloP present seems to have increased to more than even that of the control cells.

Cells treated with 4MA, a known 5α-reductase inhibitor, gave an unexpected result. In forskolin and hCG-treated cells, the amount of prog converted to DHP and alloP decreased, while in the 4MA-treated cells, the amount of conversion to alloP appeared to increase to an amount even more so than in the control cells. Since this was an unusual result, HPLC analysis of the extracts from all of the assays was done in order to verify if the product was indeed alloP being produced. Though there are metabolites with mobilities that correspond to both DHP and alloP on the TLC assays, HPLC analysis showed that the metabolites produced are not the same. Therefore, more experiments were then done in order to determine whether these compounds were 5α-reduced.

The previous experimental timecourses in MLTC-1 cells treated with ethanol (control), forskolin, hCG, or 4MA were repeated but instead of assaying with [^{3}H]- prog, they were assayed directly with [^{14}C]-DHP, which is already 5α-reduced, to determine which progesterone metabolites are also 5α-reduced. After HPLC analysis of cells treated with forskolin, hCG, or 4MA, and assayed with prog or DHP, it was determined that there is one 5α-reduced metabolite decreased by forskolin and hCG, and blocked by 4MA.

Prog → A + B	**DHP → B**
Prog + F/hCG → Ø	**DHP + F/hCG → Ø**
Prog + 4MA → A↑	**DHP + 4MA → B**

Figure 13: Schematic representing metabolites detected upon HPLC analysis. Progesterone assays on control cells produce two metabolites are produced: A & B. Prog assays done with forskolin or hCG-treated cells block both of these metabolites. Prog assays done on 4MA-treated cells produce A. Dihydroprogesterone assays on control cells produce metabolite B. DHP assays done with forskolin or hCG-treated cells block both of these metabolites. DHP assays done on 4MA-treated cells produce B. Therefore, B is a 5α-reduced metabolite being produced.Therefore the MLTC-1 experiments conducted show that this 5α-reduced metabolite is suppressed in the presence of forskolin and hCG, thereby supporting the hypothesis that 5αR1 activity is repressed by cyclic AMP.

The next major question is where this cyclic AMP repression of 5αR1 activity is regulated: at the site of transcription, translation, or both. Immunoblots were performed to assess protein abundance, but the results were not conclusive due to poor signal from the only available 5αR1 antibody. In order to look at

transcription, real-time PCR was done in order to quantitate the amount of mRNA produced. Real-time PCR is the most sensitive technique for mRNA detection and quantitation currently available and can be used to quantify mRNA levels from much smaller samples; this technique is sensitive enough to enable quantitation of RNA from a single cell. From all of the previous MLTC-1 timecourses done in triplicate, one set of wells for each pre-incubation treatment of ethanol (control), forskolin, hCG, and 4MA was harvested, and RNA was isolated from the cells, from which cDNAs were made, and real-time PCR performed.

	Cyclophilin	5αR1			
	Ave Ct	Ave Ct	dCt	ddCt	2^ddCt
C1	22.50	21.29	-1.21	0.00	1.00
C24	22.23	22.12	-0.11	1.10	0.47
F1	23.40	22.51	-0.89	0.32	0.80
F24	23.63	25.26	1.63	2.84	0.14
H1	23.13	23.09	-0.05	1.16	0.45
H24	22.87	22.93	0.06	1.27	0.41
4MA1	22.96	23.05	0.08	1.29	0.41
4MA24	22.93	23.16	0.22	1.43	0.37

Expression relative to time-matched controls

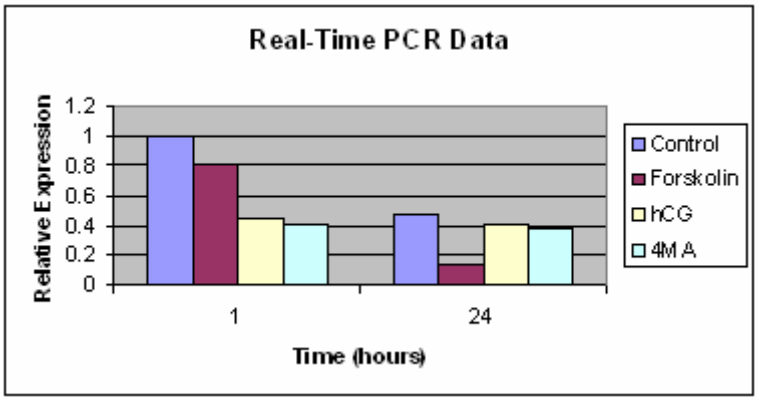


Figure 14: Real-time PCR data showing expression of 5αR1 (normalized to cyclophilin) in control cells and cells treated with forskolin, hCG, and 4MA for 1 or 24 hours. Cyclophilin: Ave Ct reflects the number of cycles it takes to get to half max (the more cycles = less RNA present). 5αR1: Ave Ct is the number of cycles it takes for 5αR1 to be expressed, dCt= 5αR1 Ave Ct normalized to their respective cyclophilin Ave Ct, ddCt = dCt number normalized to 5αR1 dCt.

The relative expressions shown in the last column confirm that 5αR1 mRNA is reduced by ~1 log unit by forskolin or hCG. This result indicates that at least part of the action of forskolin and hCG is to reduce 5αR1 mRNA transcription.

Future Directions

The first experiments necessary to continue this project are to return to the MA-10 cell line and repeat the previous timecourse experiments but at shorter time intervals. MA-10 cells will be studied because literature shows that MA-10 cells definitely produce allopregnenolone, therefore product identification will be straightforward. Shorter time courses will be used because the increase in CYP17

activity that interfered with the our previous metabolism studies in these cells takes several hours, so short time courses should provide cleaner results.

The next experiments to do would allow us to determine if regulation occurs at the site of translation. Western blot analysis would allow for quantitation of the amount of protein present. Since only one relatively weak antibody exists for mouse 5αR1, a positive control must be used, from protein obtained directly from term mouse cervix (a tissue with abundant 5αR1 expression) or by transfecting HEK293 cells with a 5αR1 expression plasmid. Alternatively, pulse-chase experiments where [^{35}S]-methionine is used to label proteins then chase with methionine and immunoprecipitate proteins could be performed; though 5αR1 antibody may be too weak to give a clean Western blot, it may work sufficiently for immunoprecipitation.

Finally, the regulatory elements controlling the 5αR1 gene must be characterized. To begin, the promoter region can be cloned upstream of a luciferase cassette for reporter gene assays. These assays permit direct and facile analysis of how hormones and second messengers affect 5αR1 expression. Variable regions of the promoter can be deleted or added to identify elements within the promoter that regulate gene expression.

Bibliography

1. M. B. Renfree, J. D. Wilson, and G. Shaw (2002). The hormonal control of sexual development. Novartis Found Symp 244: 152-6, 203-6, 253-7.

2. J. D. Wilson (1989). Sexual differentiation of the gonads and of the reproductive tract. Biol Neonate 55: 322-30.

3. J. D. Wilson, G. Shaw, M. L. Leihy, and M. B. Renfree (2002). The marsupial model for male phenotypic development. Trends Endo Metab 13: 78-83.

4. J. D. Wilson (1996). Role of dihydrotestosterone in androgen action. Prostate Suppl 6: 88-92.

5. J. D. Wilson (1979). Sex hormones and sexual behavior. N Engl J Med 300: 1269-70.

6. J. D. Wilson (2001). The role of 5alpha-reduction in steroid hormone physiology. Reprod Fertil Dev 13: 673-8.

7. J. D. Wilson, J. E. Griffin, and D. W. Russell (1993). Steroid 5α-Reductase 2 Deficiency. Endocrine Reviews 14: 577-593.

8. A. E. Thigpen, D. L. Davis, A. Milatovich, B. B. Mendonca, J. Imperato-McGinley, J. E. Griffin, U. Francke, J. D. Wilson, and D. W. Russell (1992). Molecular genetics of steroid 5α-reductase 2 deficiency. J Clin Invest 90: 799-809.

9. J. E. Griffin (1992). Androgen resistance- the clinical and molecular spectrum. N Engl J Med 326: 611-8.

10. B. B. Mendonca, M. Inacio, E. M. Costa, J. J. Arnhold, F. A. Silva, W. Nicolau, W. Bloise, D. W. Russel, and J. D. Wilson (1996). Male pseudohermaphroditism due to steroid 5alpha-reductase 2 deficiency. Diagnosis, psychological evaluation, and management. Medicine (Baltimore) 75: 64-76.

11. R. I. Silver and D. W. Russell (1999). 5α-reductase type 2 mutations are present in some boys with isolated hypospadias. Journal of Urology 162: 1142-1145.

12. J. D. Wilson, M. W. Leihy, G. Shaw, and M. B. Renfree (2002). Androgen physiology: unsolved problems at the millennium. Mol Cell Endocrinol 198: 1-5.

13. J. D. Wilson, R. J. Auchus, M. W. Leihy, O. L. Guryev, R. W. Estabrook, S. M. Osborn, G. Shaw, and M. B. Renfree (2003). 5alpha-androstane-3alpha,17beta-diol is formed in tammar wallaby pouch young testes by a pathway involving 5alpha-pregnane-3alpha,17alpha-diol-20-one as a key intermediate. Endocrin 144: 575-80.

14. J. D. Wilson, M. W. Leihy, G. Shaw, and M. B. Renfree (2003). Unsolved problems in male physiology: studies in a marsupial. Mol Cell Endocrinol 211: 33-6.

15. R. J. Auchus (2004). The backdoor pathway to dihydrotestosterone. Trends Endo Metab 15: 432-438.

16. M. W. Leihy, G. Shaw, J. D. Wilson, and M. B. Renfree (2004). Penile development is initiated in the tammar wallaby pouch young during the period when 5alpha-androstane-3alpha,17beta-diol is secreted by the testes. Endocrinology 145: 3346-52.

17. E. P. Jenkins, S. Andersson, J. Imperato-McGinley, J. D. Wilson, and D. W. Russell (1992). Genetic and pharmacological evidence for more than one human steroid 5α-reductase. J Clin Invest 89: 293-300.

18. M. S. Mahendroo and D. W. Russell (1999). Male and female isoenzymes of steroid 5α-reductase. Reviews of Reproduction 4: 179-183.

19. S. Andersson, R. W. Bishop, and D. W. Russell (1989). Expression cloning and regulation of steroid 5α-reductase, an enzyme essential for male sexual differentiation. J Biol Chem 264: 16249-16255.

20. S. Andersson and D. W. Russell (1990). Structural and biochemical properties of cloned and expressed human and rat steroid 5α-reductases. Proc Natl Acad Sci 87: 3640-3644.

21. S. Issa, D. Schnabel, M. Feix, L. Wolf, H.-E. Schaefer, D. W. Russell, and H.-U. Schweikert (2002). Human osteoblast-like cells express predominantly steroid 5α-reductase type I. Journal Clinical Endocrinol Metabol 87: 5401-5407.

22. W. D. Steers (2001). 5α-Reductase activity in the prostate. Urology 58: 17-24.

23. M. Mahendroo, J. D. Wilson, J. A. Richardson, and R. J. Auchus (2004). Steroid 5α-reductase 1 promotes 5α-androstane-3α,17β-diol synthesis in immature mouse testes by two pathways. Mol Cell Endocrinol 222: 113-120.

24. G. M. Fimia and P. Sassone-Corsi (2001). Cyclic AMP signaling. J Cell Sci 114: 1971-2.

25. C. L. Lawson, D. Swigon, K. S. Murakami, S. A. Darst, H. M. Berman, and R. H. Ebright (2004). Catabolic activator protein: DNA binding and transcription activation.
Curr Opin Struct Biol 14: 10-20.

26. P. R. Manna, I. T. Hihtaniemi, X.-J. Wang, D. W. Eubank, and D. M. Stocco (2002). Mechanisms of epidermal growth factor signaling: regulation of steroid biosynthesis and the steroidogenic acute regulatory protein in mouse Leydig tumor cells. Biol Reprod 67: 1393-1404.
27. P. R. Manna, I. P. Huhtaniemi, and D. M. Stocco (2004). Detection of hCG responsive expression of the steroidogenic acute regulatory protein in mouse Leydig cells. Biol Proced Online 6: 83-93.

28. A. Dierich, M. R. Sairam, L. Monaco, G. M. Fimia, A. Gansmuller, M. LeMeur, and P. Sassone-Corsi (1998). Proc Natl Acad Sci 95: 13612-7.

29. D. De Cesare, G. M. Fimia, and P. Sassone-Corsi (1999). Signaling routes to CREM and CREB: plasticity in transcriptional activation. Trends Biochem Sci 24: 281-5.

30. W. A. Wuyts, B. M. Vanaudenaerde, L. J. Dupont, M. G. Demedts, and G. M. Verleden (2003). Modulation by cAMP of IL-1βinduced eotaxin and MCP-1 expression and release in human airway smooth muscle cells. Eur Respir J 22: 220-226.

31. V. Morales, I. Gonzalez-Robayna, I. Hernandez, J. Quintana, P. Santana, C. M. Ruiz de Galarreta, and L. F. Fanjul (2003). The inducible isoform of CREM (inducible cAMP early repressor, ICER) is a repressor of CYP19 rat ovarian promoter. J Endocrinol 179: 417-425.

32. C. Mounier, W. Chen, S. A. Klautky, and A. G. Goodridge (1997). Cyclic AMP-mediated inhibition of transcription of the malic enzyme gene in chick embryo hepatocytes in culture: characterization of a cis-acting element far upstream of the promoter. J Biol Chem 272: 23606-23615.

Vitae

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