STUDIES ON THE MECHANISMS OF MITOCHONDRIAL CHOLESTEROL TRANSPORT AND STEROIDOGENESIS

A Dissertation

Presented to the Faculty of the Graduate School

of Cornell University

In Partial Fulfillment of the Requirements for the Degree of

Doctor of Philosophy

By

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August 2019

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Cornell University 2019

Cholesterol is among one of the most decorated molecules in biology today, the study of which produced not only a number of Nobel Prizes but also key findings that influence our study of physiology and medicine today. It is the single starting substrate of the steroidogenic pathway, the synthesis of steroid hormones such as progesterone, testosterone, and corticosterone, which control a variety of essential physiological and metabolic functions including salt and water balance, spermatogenesis, follicular development and maintenance of pregnancy.

Steroidogenesis is a unique process in which all of the steroid hormones are derived from a single substrate, cholesterol, by the CYP11A1 enzyme located on the matrix side of the inner mitochondrial membrane. Steroidogenesis is rapidly triggered in response to induction by tropic hormones such as ACTH or LH binding to their respective G-protein coupled receptors on the plasma membrane, triggering a rise in intracellular cAMP. This process, however, requires that cholesterol be trafficked across the aqueous intermembrane space, a substantial obstacle for the steroidogenic cell due to cholesterol's extreme hydrophobicity.

My studies provide evidence that this intramitochondrial trafficking is facilitated by the steroidogenic acute regulatory protein (STAR) and not the translocator protein

(TSPO). My studies conclusively eliminate TSPO from our current models of the steroidogenic process and demonstrate that a TSPO ligand that has been shown to reduce progesterone synthesis is, in fact, an inhibitor of 3β-HSD, a downstream enzyme of progesterone synthesis. During my studies, I also generated STAR knockout MA-10 Leydig cell lines, the first *in vitro* model of STAR deletion in a steroidogenic cell line, providing evidence that induction of STAR expression is required for the steroidogenic process and that this role cannot be compensated for by TSPO.

My studies also demonstrate that extracellular cholesterol sources, such as serum lipoproteins, can contribute to the total pool of cholesterol substrate utilized by the cell to synthesize steroid hormones. I also characterize a group of hCG-responsive MA-10 cells, an improved model system for the ongoing study of steroidogenic pathways beyond the mitochondria. I also provide evidence that changes in ER cholesterol synthesis in MA-10 cells can influence expression of the STAR protein, pointing toward the possible involvement of the sterol regulatory element binding protein (SREBP) family of transcription factors.

These studies set the stage for the study of cholesterol trafficking pathways beyond the mitochondria and characterize models that can be used as a tool to study cholesterol sourcing and trafficking proteins that support steroidogenesis.

BIOGRAPHICAL SKETCH

Amy Hengyi Zhao was born in Beijing, China on May 10, 1992. At the age of three, she immigrated to the Edgewater, New Jersey in the United States. She attended elementary school in Edgewater until the third grade before moving to Fort Lee, New Jersey, where she completed her elementary, middle and high school years. Amy was an avid pianist starting at age eight and at age 17, completed all eight levels of the piano exams for the Associated Board of the Royal School of Music from the United Kingdom. She also joined the debate team, school choir, played clarinet in the school band, and, briefly, the viola. She also explored scientific research as part of a two-year course, presenting a short literature review on homologous DNA repair mechanisms.

Amy graduated high school in the top 10% of her class and was admitted to the Honors Program at Rutgers University. There, as a biology major, she gained experience in training guide dogs for the blind and decided to pursue a career in veterinary medicine. To pursue her dream, she transferred to the College of Agriculture and Life Sciences at Cornell University. In her junior year as an Animal Science major at Cornell, Amy joined the lab of Dr. Vimal Selvaraj and completed an undergraduate honors thesis focusing on the role of TSPO in heme synthesis. This work was published in The Journal of Biological Chemistry in 2016.

After completion of her B.S., Amy decided to stay in the Selvaraj lab to complete her Ph.D. in Animal Science, focusing on mitochondrial cholesterol transport and steroidogenesis. During her Ph. D., Amy also spent three years as a graduate teaching assistant for BIOG1445, an introductory level biology class. Amy is also the co-founder of Bailemos Latin Dance Club, a Cornell student club that provides instruction in salsa

dancing on campus and for the Ithaca community. Upon completion of her Ph. D., Amy will be relocating to Boston, Massachusetts to pursue a career in research and development of human therapeutics. She will also be joining Rumba y Timbal Dance Company as an instructor in salsa and bachata.

ACKNOWLEDGEMENTS

I have been at Cornell for eight years now and looking back, I am grateful for all of the opportunities and learning experiences I have encountered along the way. First, thank you to Dr. Vimal Selvaraj for all of his advice and understanding over the past eight years and giving me the opportunity to work with him on a variety of projects. I would also like to thank my committee members Dr. Susan Quirk, Dr. Mark Roberson, and Dr. Alex Travis for their support and guidance during my graduate career.

I also wanted to take this opportunity to thank several members of the Selvaraj Lab who have supported me during this adventure. Dr. Kanako Morohaku, for being the first person to teach me the basics of molecular biology and introducing me to the lab. To Lan Tu, Viju Vijayan Pillai, Kasey Schalich, Shailesh Gurung, and Dr. Prasanthi Koganti for their never-ending support and patience. I am inspired by all of you and look forward to meeting again in the future.

I would also like to express my deepest gratitude to my family, especially my mother, Xiaomei Liu, my father, Kefei Zhao, and my sister Annie Zhao for their support and all of the sacrifices they made to allow me to be where I am today. Lastly, I would like to thank Dr. Jose Rios for all of his love and support in this endeavor, and for always being there for me.

TABLE OF CONTENTS

LIST OF FIGURES

CHAPTER 1

INTRODUCTION AND REVIEW OF LITERATURE

Introduction

Cholesterol is a highly hydrophobic small molecule derived from the mevalonate pathway that has fascinated the scientific community since its initial isolation from gallstones in 1782. It is the precursor for a variety of essential metabolites including bile acids, steroid hormones and oxysterols, and plays a crucial role in the maintenance of cellular membrane fluidity. While cholesterol is essential to the survival of animal cells, its inability to dissolve in water also means that its erroneous accumulation results in a wide variety of severe pathologies, such as atherosclerosis and hypercholesterolemia.

Cholesterol plays crucial roles in several major physiological pathways, but, due to its chemistry, remains a challenge to study. Unlike proteins, cholesterol's small size and extreme hydrophobicity means that it is permanently incorporated into membranes or esterified in lipid droplets, making it difficult to visualize and track within the cell. In addition, the ability for cholesterol to quickly equilibrate within different cellular compartments has made the identification of the precise mechanisms used to regulate and traffic cholesterol within the cell extremely elusive.

Despite these challenges, some of the mechanisms that animal cells use to regulate cholesterol levels and traffic cholesterol within the cell have been identified. In steroidogenesis, cholesterol is trafficked to the mitochondrial matrix and cleaved by CYP11A1 to form pregnenolone, the first precursor of all steroid hormones. To distribute and retrieve cholesterol between the liver and rest of the body's tissues, cholesterol is esterified and packaged with circulating lipoproteins and can be imported by recipient cells using endocytic mechanisms. To regulate cellular cholesterol levels, the sterol regulatory binding proteins (SREBP) interact with the SREBP cleavage-activating protein

(SCAP) and, in low cholesterol conditions, are released from the endoplasmic reticulum to upregulate an entire cohort of cholesterol synthesis and acquisition genes.

This review looks back at the history of the cholesterol field and highlights the key findings and developments that led us to where we are today.

The History of Cholesterol

Cholesterol was first identified in 1782 by French chemist François Poulletier de la Salle, who isolated the crystallized substance from patient-derived gallstones. These findings were published and verified by Antoine Francois de Fourcroy, a French chemist, who later isolated the same substance from an aged, hardened human liver, describing it as “crystallizable adipocire”. In 1815, Michel Eugene Chevreul, another French chemist, published his findings establishing the difference between adipocire and “cholesterine”, the substance we now know as cholesterol[1]. These discoveries were quickly followed by many others as cholesterol was isolated from a wide variety of tissues including blood[2], liver cysts, and cerebral tumors[1] and by the 1860s, it was known that cholesterol was processed by the liver and discharged with the bile in the form of taurine or glycine-conjugated salts[3].

Despite extensive work on the subject, cholesterol synthesis was still a highly controversial topic. In the 1860s, work by Austin Flint in dogs had mistakenly concluded that the central nervous system was major site of cholesterol synthesis in the body[3] and up to the 1920s, whether cholesterol could be synthesized from non-sterol substances remained a mystery[4]. Studies using chickens and eggs by Mendel and Leavenworth in 1908 and Ellis and Gardner in 1909 concluded that there was no additional cholesterol synthesis as the chick developed[5,6] but this conclusion was soon overturned. By 1925,

several researchers, including Gardner, had published findings demonstrating that cholesterol output was significantly higher than cholesterol intake in human and animal subjects fed a low cholesterol diet[7–10], definitively demonstrating that cholesterol can, indeed, be synthesized *de novo*. By the 1930s, the search for the mechanisms responsible for the synthesis of cholesterol had begun.

At this point in time, scientists attempting to untangle the metabolic web of human and animal physiology were limited by the fact they could not distinguish between endogenous metabolites and the ones that they were introducing to the animal, making the task of elucidating the complex pathway of cholesterol synthesis impossible. It was not until the 1930s, with discovery of radioactive isotopes such as deuterium (for which Harold C. Urey was awarded the Nobel Prize in Chemistry in 1934) that Rudolf Schoenheimer, a German biochemist and pathologist, developed the revolutionary method of isotope tagging biomolecules, which finally enabled scientists to differentiate between endogenous metabolites and those they introduced into the body[11,12]. This method, employed by Bloch and Rittenberg after Schoenheimer's death in 1941, led them to the discovery that radiolabeled acetate became readily incorporated into both the side chain and the tetracyclic moiety of the cholesterol molecule[13,14]. Bloch then moved from Columbia University to University of Chicago, where he began to derive the sources of all of the carbons in the cholesterol molecule, work that would lead him and Feodor Lynen to the Nobel Prize in Physiology or Medicine in 1964.

Derivation of the Cholesterol Synthesis Pathway

Up until this point, the biosynthesis of cholesterol remained an enigma for chemists and biochemists due to its complex ring structure. Several key pieces of evidence

ultimately led to Bloch's breakthrough in this field. (1) Previous evidence from Heilbron, Kamm, and Owens had demonstrated that feeding squalene to animals would increase the cholesterol content of the tissues[15,16] along with (2) L. Ruzicka's hypothesis that terpenes and steroids were derived from a single origin[17] led to early speculations that perhaps cholesterol was synthesized by the folding of a "long-chain precursor", an idea that was ultimately proven to be true.

Bloch hypothesized that two-carbon acetate made up the majority, if not all of the 27 carbons present in the cholesterol molecule and, in 1950, published the findings in the Journal of Biological Chemistry with Henry N. Little[18]. This finding was further supported in 1951 when pyruvate-deficient red mold *Neurospora crasa* grown on radioactive acetate was shown to have no other major contributors to the total ergosterol pool[19]. How is acetate assembled into the hypothesized "long-chain precursor" then?

Fortunately, in 1949, Bonner and Arreguin had demonstrated that acetate was utilized for the synthesis of isoprene polymers in guayule plants and Bloch hypothesized that a similar mechanism was used to synthesize cholesterol[20]. Given the evidence from Heilbron, Kamm and Owens, it was likely that these isoprene units were assembled into squalene, the previously hypothesized "long-chain" precursor. The process of producing squalene from radiolabeled acetate proved difficult but was ultimately successful using liver tissue from white rats[21]. The completion of this work brought Bloch and the rest of the field to the task of dissecting the pathways that converted squalene to cholesterol. Accomplishing this task was a concerted effort by several research groups. Work by Cornforth, Hunter and Popjak[22] documented the precise position of each acetate-derived carbon in the cholesterol molecule, leading to the essential studies demonstrating that

squalene was converted to the intermediate lanosterol[23,24], a sterol that had previously been derived from wool fat, and that lanosterol was ultimately converted to cholesterol[25] by the mid 1950s. Over the next two decades, Bloch and other researchers began to derive all of the intermediate steps in both cholesterol and fatty acid synthesis pathways, and by the time he received his Nobel Prize in 1964, they had uncovered a large portion of the enzymes involved[26]. Included in this work is the discovery that cholesterol is among few cellular metabolites that requires O_2 for its synthesis[27].

Today, we group cholesterol synthesis into three phases. (1) The conversion of acetyl-coA units to an isopentenyl pyrophosphate, (2) the assembly of isopentenyl pyrophosphates to squalene, and (3) the conversion of squalene to cholesterol.

Identification of the Lipoproteins

Due to its near complete insolubility of cholesterol in water, specialized lipoprotein systems have evolved for their transport in circulation. The first concrete evidence of serum lipoproteins emerged in 1929 when Michel Macheboeuf, a French biochemist, fractionated horse serum using ammonium sulfate[28]. This particle, which we now identify as high-density lipoprotein (HDL) contained 59% protein and 41% lipid (18% cholesterol and 23% phospholipid). This particle was also capable of being resuspended in water, agreeing at the time with its role as a lipid transport particle[28]. In 1950, a second lipoprotein particle was identified, containing 23% protein, 8% free cholesterol and 39% cholesterol esters[29], roughly equal in profile to what is now defined as low density lipoprotein (LDL).

During this time, lipoproteins were classified into two classes--α- and β-lipoproteins. The introduction of the vacuum centrifuge by Ed Pickels and modified centrifugation

conditions allowed John Gofman and his student Frank Lindgren to separate multiple classes of lipoproteins based on their Svedberg flotation rates corresponding to very low-density lipoproteins (VLDLs), intermediate-density lipoproteins (IDLs), LDLs and HDLs[30,31]. Gofman and his team also demonstrated that increased cardiovascular disease risk correlated with increased levels of lipoproteins with Svedberg flotation rates from 0-20. They also demonstrated an increase in these lower density lipoproteins with increasing age, in males, and in patients with diabetes or myocardial infarctions. Using these new characterizations, an "atherogenic index" was used to predict the risk of developing cardiovascular disease[30]. Gofman continued to study the problem over the next several decades, completing a large-scale study evaluating 4914 men 40-59 years of age. He also came to the realization that these changes in lipoprotein profile were much more effective as a predictor of cardiovascular disease in younger individuals. These findings have played a pivotal role in our understanding of cardiovascular disease and, today, serum lipoprotein measurements continue to be used as an assessment of cardiovascular disease risk.

Over the next several decades, classification of the lipoproteins continued, differentiating the A and B proteins into several different classes based on their N-terminal residues[32,33], which were eventually redesignated as apoA-I and apoA-II. The discovery of a new lipoprotein, "C", by several groups later yielded identification of three novel apolipoproteins, apoC-I, apoC-II, and apoC-III[34,35]. Later, the Shore group and Utermann would isolate the three isoforms of apoE, apo-E2, -E3, and -E4[36–38].

Based on this new knowledge, Fredrickson, Levy, and Lees at the National Heart Institute in Bethesda, Maryland, systematically classified lipoprotein disorders into five

categories based on their lipoprotein profile[39–43]. Among the classified disorders, was familial hypercholesterolemia (FH). First identified by Carl Müller, FH was a hereditary condition of unknown cause that resulted in high plasma LDL at birth and increased incidence of heart attacks, beginning at 30 to 40 years of age[44,45]. It was the study of this disease model by Goldstein and Brown that drove cholesterol research forward once more, uncovering novel mechanisms of cholesterol trafficking and regulation.

Low Density Lipoprotein and the LDL Receptor

In 1972, Goldstein and Brown were presented with two patient siblings, ages 6 and 8, exhibiting the severe, homozygous form of FH. At the time, the consensus remained that all major cholesterol metabolism events occurred in the liver or intestine[46–48], which was nearly impossible to obtain from patients for experimentation. It was, however, postulated based on previous work that cultured fibroblasts may have the desired enzymatic pathways and feedback regulation machinery[49]. Cells cultured in medium supplemented with serum synthesized little cholesterol *de novo* but synthesis increased dramatically when serum lipoproteins were removed.

Using this system, Goldstein and Brown began to uncover the components controlling cholesterol homeostasis in cultured skin fibroblasts, an effort that would eventually lead them to the Nobel Prize in Physiology or Medicine in 1985. Assays for HMG-coA reductase activity revealed that the enzyme was subject to negative feedback regulation and that cells cultured in serum-free conditions have 50-fold higher HMG-coA reductase activity which could be suppressed by reintroduction of serum lipoprotein[50,51]. In addition, this increase could be suppressed only LDL, not HDL[51,52]. In FH patient fibroblasts, however, cell cultured in serum-supplemented medium nevertheless retained

50- to 100- fold elevated HMG-coA reductase activity[50,52]. It was hypothesized that perhaps FH was caused by a gene in the HMG-coA reductase gene which prevented it from being downregulated. This was quickly disproved because when cholesterol was introduced directly into the cell FH fibroblasts using an alcohol-protein mixture, HMG-coA activity was reduced to normal levels[52].

This led to a second working hypothesis that perhaps FH cells cannot extract cholesterol from lipoproteins and led to the proposal that there was a receptor for LDL on the plasma membrane of cells[45]. The existence of the LDL receptor was confirmed by incubating normal and FH fibroblasts with ^{125}I-labeled LDL. It became clear that normal fibroblasts could bind LDL but FH cells could not, likely because they lacked the functioning receptor[53,54]. Further work identified several key characteristics of the LDL receptor. LDLR gathers at coated pits on the cells surface and is quickly internalized via clathrin-mediated endocytosis[55,56]. These endosomes are then exposed to the low pH and lipases of the lysosomes which separates the LDL from its receptor and breaks LDL down to its components and the receptor is recycled back to the plasma membrane[57–60].

Statins

The growing evidence linking elevated blood cholesterol to heart disease drove many scientists, both in academia and industry, to search for molecules that could inhibit cholesterol synthesis. Many molecules that resembled intermediates of the cholesterol synthesis pathway failed to be effective. Triparanol was briefly introduced in 1959 only to be later withdrawn due to side effects such as cataracts[61]. Nicotinic acid and cholestyramine, a resin that binds bile acids in the digestive tract, were both used in an

attempt to regulate plasma cholesterol in hypercholesteroemia patients but with only moderate success[62–64].

In 1973, Japanese biochemist Akira Endo at Sankyo Research Laboratories in Tokyo discovered a compound from a fungal extract, later named compactin, marking the first introduction of the statin, a now widely used treatment for high blood cholesterol. The compound was quickly put to the test and, as hypothesized by Goldstein and Brown, the compound was able to lower circulating LDL levels by preventing hepatocytes from synthesizing cholesterol *de novo* and therefore upregulating surface LDL receptors to increase LDL uptake, lowering plasma LDL levels[65].

Endo began his search in 1971 and screened over 3800 fungal strains before finally identifying a mold broth with strongly inhibited the conversion of HMG-coA to mevalonate. The ultimate identification of compactin (ML-236B) from a blue-green mold, *Penicillium citrinum* marked the beginning of a successful journey to one of the most widely used drugs today. Compactin's potent inhibitory activity on HMG-coA reductase is a result of its structural similarity to HMG-coA, competing and blocking the enzyme active site[66,67]. These findings ultimately led to the development of multiple statins by both Endo and Merck and produced lovastatin, which became the first commercially available statin and was approved by the FDA in 1987[68]. Since then, several other statins have been commercialized—simvastatin, pravastatin, fluvastatin, pitavastatin, rosuvastatin, and atorvastatin, the most widely used statin today[61].

The Sterol Regulatory Element Binding Proteins

The discovery of the statins and their efficacy confirmed the importance of LDL's feedback on cholesterol balance in the cell but the exact mechanism of control still

remained a mystery. After receiving their Nobel Prize, Goldstein and Brown continued their work in the cholesterol field, elucidating the key system that controls cholesterol homeostasis at the cellular level over the next two decades.

The group identified a 42-bp sequence of the LDL receptor promoter that could confer sterol-regulatory characteristics when inserted into a heterologous promoter[69]. This activity was narrowed down to a 10-bp sequence and named the sterol regulatory element (SRE)[70]. Purified from HeLa cells, a ~60kDa protein was found bound to this sequence and was given the name SRE binding protein (SREBP)[71]. In a successful attempt to clone the cDNA, it was revealed that SREBP was, in fact, not a 60kDa protein but a larger 125kDa protein[72]. Over the next several years, two more proteins were identified. SREBP-1c, another isoform of the SREBP1 gene which primarily acts on fatty acid synthesis, and SREBP-2, which preferentially acts on cholesterol-related pathways[72–75]. It was determined that the SREBPs were integral membrane proteins that resided in the endoplasmic reticulum and, in conditions of low cholesterol availability, the protein underwent two proteolytic cleavages[76], releasing the ~60kDa N-terminal portion from the endoplasmic reticulum to be translocated to the nucleus, controlling gene synthesis[77].

Despite this discovery, how sterols regulated this process still remained a mystery. Through the use of sterol-resistant CHO cells, the cDNA for SCAP, initially named SREBP cleavage-activating protein, was cloned[78]. The SCAP protein contains eight transmembrane helices and multiple copies of the WD40 sequence on its C terminus[79] which interacts with the C-terminal of SREBP proteins on the cytosolic face of endoplasmic reticulum[80]. Fusion of green fluorescent protein with the membrane portion

of SCAP also demonstrated how it regulates the cleavage of SREBP. In high-sterol conditions, SCAP and SREBP are localized to the ER but when sterols are depleted, SCAP and its bound SREBP is sorted into COPII coated vesicles and delivered to the Golgi, where the proteases that cleave the SREBP protein, S1P and S2P reside[77,81,82].

The last piece of the puzzle came when immunoprecipitation experiments revealed a new SCAP binding partner, Insig, a previously identified protein of unknown function named for its upregulation in response to insulin[83]. Insig-1 and Insig-2 retained the SREBP/SCAP complex in the endoplasmic reticulum when sterol levels were high by binding to SCAP in its low sterol-conformation[84–86]. These findings by the Goldstein and Brown groups identified and elucidated a key regulatory system of cholesterol homeostasis that controls nearly every single protein of the cholesterol synthesis pathway and several key players in cholesterol acquisition, an influential collection of findings that greatly informs our current knowledge of intracellular cholesterol balance.

Intracellular Cholesterol Transport Proteins

Cholesterol's complete insolubility in water poses not only a challenge to trafficking the substance in circulation, but also in the aqueous environment of the cytosol. Moving cholesterol between cellular compartments requires highly specific protein machinery, some of which have been identified and will be reviewed here.

Steroidogenic Acute Regulatory Protein (STAR): The mitochondrial steroidogenic acute regulatory protein consists of two portions, a 62-amino acid mitochondrial targeting signal at its N-terminus and a STAR-related lipid transfer, or START, domain that binds to cholesterol and facilitates its transfer to the CYP11A1 enzyme[87]. STAR is known to be crucial for steroidogenesis and disruption of STAR function results in almost complete

loss of steroid production in both mouse models[88–90] and human patients[91–93]. While the exact mechanism of action of STAR is unclear, several different mechanisms have been proposed which are discussed in more detail in the context of mitochondrial cholesterol transport below. STAR was the first protein identified containing this lipid-binding START domain and, since its discovery, several other proteins containing the same domain have been identified, 15 of which have been identified in humans so far[94]. START domains bind cholesterol in a 1:1 ratio[95].

STARD3/MLN64: STARD3/MLN64 consists of an N-terminal membrane tethering MENTAL domain and a C-terminal START domain and has been proposed to play a role in mitochondrial cholesterol transport in the human placenta, which does not express STAR, but only the START domain has measurable steroidogenic capabilities while expression of the full protein imparts no steroidogenic capabilities[96]. STARD3 deletion in mice also results in minimal changes to the steroidogenic pathway[97]. The MENTAL domain of STARD3 anchors it to the endosomal compartment, projecting the START domain into the cytoplasm[98]. More recent data have shown that STARD3 interacts with vesicle-associated protein (VAP) and mediates cholesterol transfer between endoplasmic reticulum and endosomal contact sites[99].

NPC1/NPC2: Niemann-Pick C1 and C2 are proteins located in lysosomes that facilitate cholesterol export from the lysosomal compartment[100]. In the lysosome, NPC2 binds cholesterol and transfers it to NPC1[101–106]. Given their localization, it is currently thought that NPC1 and NPC2 are responsible for cholesterol efflux from the lysosome after LDL is internalized[100]. While the exact mechanism of cholesterol efflux from the lysosomal compartment is unknown, it is known that both NPC1 and NPC2 are required for this

process. Inactivating mutations in either protein results in Niemann-Pick Type C disease, characterized by accumulation of cholesterol in lysosomes which is most apparent in the liver, spleen and brain and can result in death at an early age[107].

Aster-A, -B, -C: Aster proteins possess a single transmembrane helix that anchors them to the endoplasmic reticulum and recent data demonstrates that these proteins facilitate the internalization of HDL-derived cholesterol from the plasma membrane to the endoplasmic reticulum in adrenal cortical cells[108], a link in cholesterol trafficking mechanisms that had been missing up until this point.

High-Density Lipoproteins

Since the 1960s, when the link between circulating lipoproteins and cardiovascular health was first determined, it was known that higher levels of high density lipoprotein (HDL) was associated with lower rates of cardiovascular events[109]. This knowledge brought scientists around the world to study the physiological role of HDL particles. Like LDL, HDL is made up of both apolipoproteins and the lipids it carries. Unlike LDL, however, which generally deposits cholesterol in tissues, HDL plays a major role cholesterol efflux from tissues[110].

HDL particles are first formed when Apo A-I is synthesized by the liver or intestine and secreted from the cell into circulation where they acquire cholesterol from peripheral cells[111]. In order to maintain the ability to uptake cholesterol onto the particle, acquired cholesterol is esterified by circulating lecithin cholesterol acyl transferase (LCAT) and moved to the center of the particle. These more mature particles are spherical in shape, rather than discoid like the nascent particles[112].

ATP-binding cassette A1 (ABCA1) is currently the major protein documented responsible for loading cholesterol onto HDL particles. ABCA1 mediates the transfer of cholesterol across plasma membranes to the nascent HDL protein, a mechanism discovered from studying patients with Tangier disease, a condition characterized by Dr. Donald Fredrickson in 1956 resulting from the loss of ABCA1 function. Patients with Tangier disease present with low serum HDL and a resulting accumulation of cholesterol ester in tissues such as the tonsils, liver, spleen and intestine[113]. Although the importance of ABCA1 in this role is well-documented, the exact molecular mechanism by which ABCA1 performs this function still remains controversial[112].

Removal of lipids from HDL is primarily mediated by the scavenger receptor BI (SR-BI). SR-BI binds preferentially to mature, spherical HDL particles and selective uptake of cholesterol esters, free cholesterol and phospholipids[114]. The receptor is expressed in a variety of tissues[115] but is most highly expressed in major sites of cholesterol uptake, such as the liver and steroidogenic tissues[116,117]. Studies have defined that presence of Apo A-I protein is required for SR-BI mediated cholesterol ester uptake[118] but the exact mechanism by which this happens remains to be uncovered.

Intracellular Cholesterol Storage

Cholesterol is an essential component of cellular membranes, and thus indispensable for cellular function. Cholesterol inside the cells is distributed amongst a few different pools—unesterified plasma membrane cholesterol, unesterified cholesterol in intracellular membranes, and esterified cholesterol, usually stored in the form of lipid droplets. Estimates consistently show that most of the cell's cholesterol (64%-90% depending on the cell type and method of estimation) is contained in the plasma

membrane pool[119,120]. Plasma membrane cholesterol is moved continuously to the endoplasmic reticulum and equilibrates rapidly and consistently[121]. While this mechanism was previously unknown, recent identification of the Aster proteins (discussed above) has provided an explanation on how plasma membrane cholesterol reaches the ER compartment[108]. In steroidogenic cells, work by Dale Freeman demonstrated that this cholesterol is rapidly internalized upon induction of steroidogenesis and is used by the cell as steroidogenic substrate to synthesize progesterone[122]. Free cholesterol content of the inner cell organelle membranes is very low compared to the plasma membrane. The ER, despite being the site of cholesterol synthesis contains 0.5%-1% of the total cellular free cholesterol, surprising when taking into account its relatively large surface area[123].

In recent years, cholesterol ester storage in lipid droplets has become an area of interest, especially in the context of adipocytes and steroidogenic cells, where these organelles provide a major storage function and source of metabolic substrate[124]. Lipid droplets are formed as an extension of the ER from the accumulation of neutral lipids between the ER membrane leaflets, which then buds from the ER membrane to form a single membrane organelle[125]. Release of cholesterol esters from the lipid droplet requires the action of hormone-sensitive lipase (HSL), a neutral cholesterol ester hydrolase that has been shown to localize to the lipid droplet after phosphorylation by protein kinase A on multiple serine residues[126]. HSL activity is essential for cholesterol ester hydrolysis and in the context of adrenalcortical cells, HSL deletion results in a dramatic decrease in corticosterone production[127].

Steroidogenesis and Mitochondrial Cholesterol Transport

One of the most unique pathways in which cholesterol exits the cell is in the form of steroid hormones, synthesized mainly by the adrenal cortex, the Leydig cells of the testes, and the thecal cells of the ovarian follicle. First isolated in the 1920s and 1930s steroid hormones are an essential part of mammalian life and the Adolf Betenandt, Edward C. Kendall, and Tadeus Reichstein would go on to receive multiple Nobel Prizes for their work in the identification and isolation of steroid hormones[128–130]. Today, we know that all of the steroid hormones are derived from a single precursor, cholesterol, and that cholesterol is committed to the steroid hormone pathway when it is converted to pregnenolone on the matrix side of the inner mitochondrial membrane by the P450 side chain cleavage enzyme (CYP11A1)[131]. The product, pregnenolone, then diffuses out of the mitochondria and is converted to the other steroid hormones by the steroidogenic enzymes in other compartments of the cell[132].

The main challenge faced by the cell is the trafficking of cholesterol from its source of acquisition across the cytosol and the aqueous intermembrane space to the CYP11A1 enzyme. Over the past several decades, work in the steroidogenesis field has focused largely on mitochondrial cholesterol transport. Essentially, how does cholesterol cross the aqueous intermembrane space? This is thought that the delivery of cholesterol to the inner mitochondrial membrane (IMM) remains the rate-limiting step of the entire process[133,134]. Experimental evidence has demonstrated that steroidogenic cells can be acutely stimulated by tropic hormones such as ACTH or LH[135] and that the synthesis of these hormones requires the synthesis of new proteins[136–139]. Based on this knowledge, many candidate proteins were proposed to be the key regulator of mitochondrial cholesterol transport, including sterol carrier protein 2 (SCP2), the steroidogenesis

activator polypeptide (SAP), the translocator protein (TSPO) and the steroidogenic acute regulatory protein (STAR). SCAP2 and SAP were soon after ruled out from consideration for this role. SCP2 knockout mice were not defective in steroidogenesis[140] and SAP was later as part of the larger protein glucose-regulated protein 78 (GRP78), a key protein involved in the unfolded protein response of the endoplasmic reticulum[141,142]. TSPO and STAR remained candidates of interest and research on these two targets intensified.

TSPO, previously known as the peripheral benzodiazepine receptor (PBR), is an 18kDa protein located on the outer mitochondrial membrane and expressed in a various tissues but highest in those that produced steroids[143,144]. TSPO knockout mice were believed to be embryonically lethal[145] and multiple studies linked the use of synthetic TSPO ligands to steroid synthesis in multiple cell types[146,147]. In addition, evidence was presented that TSPO knockdown reduced steroid synthesis in R2C rat Leydig cells[148]. In fact, literature spanning nearly thirty years has suggested that TSPO is the key mitochondrial cholesterol transporter[149–152]. Since 2014, however, the introduction of several key studies removed TSPO from consideration as a key player in steroidogenesis. First, deletion of the *Tspo* gene had no effect on steroid hormone synthesis *in vivo*[153]. In addition, knockdown of TSPO in steroidogenic cell lines had no effects on steroidogenesis and TSPO-binding ligands such as PK11195 continue to stimulate steroidogenesis despite lack of TSPO expression[154], suggesting an alternative explanation for their action. These findings have since then been replicated by several other groups who have independently-generated TSPO knockout mice[155]. In addition to PK11195, several other TSPO ligands have been synthesized, including 19-atriol (3,17,19-androsten-5-triol)[156], that have been shown to have effects on steroid production and be of potential therapeutic

interest[157]. TSPO plays no role in steroidogenesis. Its so-called ligands, albeit non-specific, however, are of potential therapeutic value and continue to be studied.

The last protein, the steroidogenic acute regulatory protein (STAR), first identified the 1980s by Orme-Johnson[158–160], was isolated/sequenced by Stocco[31] from the mitochondria of MA-10 mouse Leydig cells after stimulation to induce steroidogenesis[161]. STAR was rapidly synthesized as a novel protein in response to induction with tropic hormones such as ACTH, LH or second messenger Bt_2cAMP. In addition, the synthesis of these proteins could be prevented by cycloheximide treatment, consistent with previous work that induction of steroidogenesis could be prevented by interrupting protein synthesis[136,137,139,161]. Initially, several different hypotheses were proposed for STAR's mechanism of action. First, it was hypothesized that STAR created contact sites between the outer and inner mitochondrial membranes, allowing the transfer of cholesterol between the two[162]. Another proposed that STAR acted as a shuttle to transport cholesterol between the membranes[95]. More recent data suggests, however, that STAR was capable of performing its function even when limited to the outside of the mitochondria. When STAR was fused to TOM20, an outer mitochondrial membrane protein, steroids were produced at a maximal level in COS1 cells expressing steroidogenic enzymes[87]. Currently, STAR remains the most likely candidate as the mitochondrial cholesterol transporter responsible for delivering cholesterol to the CYP11A1 enzyme for steroidogenesis.

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CHAPTER 2

19-ATRIOL (3,17,19-ANDROSTEN-5-TRIOL) INHIBITION OF STEROIDOGENESIS IS MEDIATED BY ACTION ON 3B-HSD AND CYP11A1, NOT TSPO

Abstract

Recent reports that refute the role for the translocator protein (TSPO) in mitochondrial cholesterol import and steroidogenesis have prompted reevaluation of TSPO-associated pharmacology. Previously, it was reported that 3,17,19-androsten-5-triol (19-Atriol) could bind to TSPO and inhibit steroidogenesis in MA-10 Leydig cells (1). Using CRISPR/Cas9-mediated *Tspo* gene deleted MA-10 Leydig cells, we reexamined whether TSPO was indeed the target mechanism for 19-Atriol effect on steroidogenesis. Our results confirmed that 19-Atriol suppressed progesterone production as previously reported; however, this effect was independent of TSPO. By testing the associated enzymatic steps, 19-Atriol was observed to be converted to 19-hydroxytestosterone by 3β-hydroxysteroid dehydrogenase (3β-HSD) and therefore competitively inhibit the conversion of pregnenolone to progesterone. In addition, 19-atriol prevents the conversion of cholesterol to pregnenolone by CYP11A1. Based on these data, we conclude that 19-Atriol is a substrate of 3β-HSD and an inhibitor of CYP11A1 and does not influence mitochondrial cholesterol import. These findings rectify a core error in describing the mechanism of 19-Atriol action and highlight the need for rigorous target validation in TSPO pharmacology.

Introduction

Discovery of the peripheral benzodiazepine receptor (PBR, now called the translocator protein/TSPO) in the late 1970s was by distinct specificity of certain pharmacological agents in that they did not bind to the central benzodiazepine receptor (gamma amino butyric acid receptor A/$GABA_A$) (2,3). TSPO localization to the outer mitochondrial membrane (4) and the ability of some TSPO-binding drugs to induce of transient low levels of steroidogenesis (5-7) led to its association with the mitochondrial cholesterol import function, a rate limiting step in steroid hormone biosynthesis (8). This functional supposition together with substantial pharmacological interest on TSPO as a diagnostic/therapeutic target for neuroinflammation and neuropsychiatric disorders have fueled plentiful publications focusing on beneficial outcomes rather than mechanism in both model systems and humans (9-13). Nevertheless, recent emergence of *Tspo*-gene deleted models, both *in vivo* and *in vitro* have contradicted its postulated steroidogenic function (14-17), and have raised numerous questions (18-20).

Expression of TSPO is not restricted to steroidogenic tissues (21), and high expression is observed in lipid enriched cells (22). Emerging evidence suggests TSPO deficiency has an effect on lipid metabolism that appears conserved across kingdoms (22,23). As precise pathways/mechanisms are yet to be uncovered, delineating pharmacological effects of drugs that bind TSPO has confounded interpretations. The prototypical TSPO binding drug PK11195 [N-butan-2-yl-1-(2-chlorophenyl)N-methylisoquinoline-3-carboxamide], considered as an agonist at the foundation of its link to steroidogenesis (5-7), has shown contradicting responses in other studies that directly examined steroid production (24-26). Recently, using TSPO-deleted MA-10 (MA-

10:*Tspo*$^{\Delta/\Delta}$) cells, we demonstrated that the PK11195 effect on Leydig cell steroidogenesis is an effect independent of TSPO (16). As complete loss of PK11195-binding has been observed in TSPO-deficient tissues (17), potential for TSPO-independent effects has been associated with its insertion into the membrane bilayer that alters biophysical properties (27).

As discovery of a TSPO antagonist, it was reported that 3,17,19-androsten-5-triol (19-Atriol) could bind to TSPO and inhibit steroidogenesis in MA-10 Leydig cells (1). Specifically, 19-Atriol was identified as capable of binding to the cholesterol recognition amino acid consensus (CRAC) motif, suggesting that it may compete for the same site as cholesterol on TSPO (1). The inhibitory activity was further refined by examining structural changes through steroid hydroxylations at C19, C11, C7 and C4 (28). Effect of 19-Atriol *in vivo* has also been demonstrated to inhibit Leydig cell testosterone production in rats (29).

Given recent evidence that TSPO is not involved in mitochondrial cholesterol import function essential for steroidogenesis, we decided to reexamine the effect of 19-Atriol in MA-10:*Tspo*$^{\Delta/\Delta}$ cells. Our results demonstrate that 19-Atriol action to suppress progesterone synthesis in MA-10 Leydig cells as previously indicated (1), is independent of TSPO. Additionally, we uncover that 19-Atriol is either an inhibitor or an alternate substrate of 3β-hydroxysteroid dehydrogenase (3β-HSD).

Materials and Methods

Cell culture

MA-10 Leydig cells (30) were cultured in DMEM and 10% fetal bovine serum and 1% penicillin-streptomycin and 1% non-essential amino acids as previously described (21).

Clones of TSPO-deleted MA-10 (MA-10:*Tspo*$^{\Delta/\Delta}$) cells were previously generated using clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9-mediated gene targeting and validated (16).

Treatments and hormone assays

For all experiments, MA-10 cells were plated at 5 x 10^4 cells per well in a 0.1% gelatin-coated 96-well plate and allowed to attach overnight. Steroidogenesis was induced in serum free DMEM for 3 hours for experiments using 0.5mM Bt_2cAMP (Sigma) or 20μM 22(R)-hydroxycholesterol (Sigma). For experiments intended to bypass the first enzymatic step, pregnenolone (Sigma) was added at different concentrations (1, 5 and 50 μM) and incubated for 3 hours. Vehicle (DMSO, 0.1% v/v final) or 19-Atriol (10 μM) was incorporated into the above experimental conditions to test the specific effects. Cell culture supernatant was collected for quantification of progesterone by radioimmunoassay as previously described (16). Progesterone values were normalized to total protein content in each well. Experiments were repeated as four independent trials.

Pregnenolone assays

Cell culture supernatants were incubated with 0.1U cholesterol oxidase from Streptomyces sp. (Sigma) in phosphate-buffered saline for 6 hours at 37C at 300rpm on an orbital shaker. Duplicate samples were incubated with phosphate buffered saline for progesterone measurement.

Immunoblots

Cells were collected in SDS buffer containing protease inhibitors (Sigma). Proteins were separated by SDS-PAGE, transferred to a PVDF membrane and immunoblotted using a

rabbit monoclonal primary antibody against TSPO (Abcam) and an affinity purified rabbit polyclonal antibody against STAR (31). Membranes were also blotted using a monoclonal mouse antibody for β-Actin (LI-COR) as a loading control. Detection of TSPO and STAR was using a (Poly-HRP) secondary antibody and IRDye800 (LiCor) labeled secondary antibodies for Actin using quantitative imager (C600, Azure Biosystems).

Structural modeling and ligand docking

Structural information for CYP11A1 was obtained through Protein Data Bank (3N9Y). 3β-HSD structure was modeled based on homology calculated using Phyre2 with progesterone 5β-reductase (32). Ligands cholesterol, pregnenolone, 19-atriol, and 19-hydroxytestosterone were docked using AutoDock Vina (http://vina.scripps.edu) (33) and interacting amino acids were modeled and binding affinities were calculated using LigPlot (https://www.ebi.ac.uk/thornton-srv/software/LIGPLOT/).

Results

19-atriol inhibition of progesterone synthesis is TSPO-independent

We examined the ability for 19-atriol to reduce progesterone synthesis in MA-10 cells expressing (MA-10:*Tspo*$^{+/+}$) or in two subclones lacking (MA-10:*Tspo*$^{\Delta/\Delta}$) the TSPO protein. Addition of 19- atriol with 0.5mM Bt_2cAMP (Figure 2.1A) or 20μM 22(R)-hydroxycholesterol (Figure 2.1B) resulted in a dose-dependent decrease in progesterone synthesis in both MA-10:*Tspo*$^{+/+}$ and MA-10:*Tspo*$^{\Delta/\Delta}$ cells.

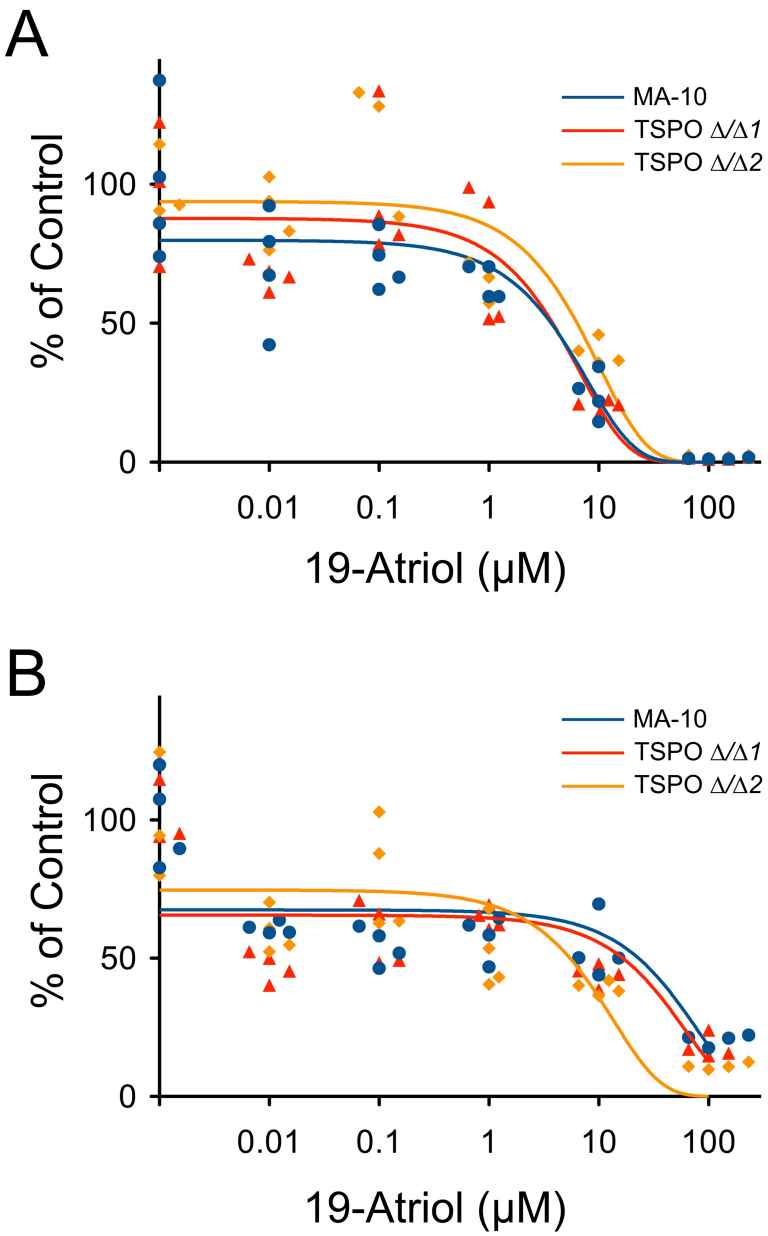

Figure 2.1 Inhibition of progesterone production by 19-atriol is unrelated to TSPO. (A) Progesterone (P4) production by MA-10 Leydig cells stimulated with Bt_2cAMP was decreased by 19-Atriol in a dose-dependent manner irrespective of TSPO presence or absence. The dose-response inhibitory activity was similar between MA-10:*Tspo*$^{+/+}$ and MA-10:*Tspo*$^{\Delta/\Delta}$ cells. **(B)** P4 production in response to treatment with 22(R)-hydroxycholesterol (20 μM) was also inhibited in a dose-dependent manner by 19-Atriol in both MA-10:*Tspo*$^{+/+}$ and MA-10:*Tspo*$^{\Delta/\Delta}$ clones.

19-atriol inhibits the conversion of pregnenolone to progesterone by 3β-HSD

Next, we examined whether 19-atriol inhibition of progesterone synthesis was due to competition for the 3β-HSD enzyme. MA-10:*Tspo*$^{+/+}$ and MA-10:*Tspo*$^{\Delta/\Delta}$ cells treated with 1μg/mL pregnenolone are able to convert the substrate to progesterone without the need for additional stimulation due to the consistent presence of the 3β-HSD enzyme. 19-atriol treatment significantly reduced progesterone synthesis compared to controls treated only with pregnenolone. Trilostane, a 3β-HSD inhibitor, caused an almost complete inhibition of progesterone synthesis in response to pregnenolone treatment. 19-atriol's ability to reduce the conversion of pregnenolone to progesterone demonstrates that it is an inhibitor of 3β-HSD and is effectives regardless of TSPO expression (Figure 2.2A). Treatment of MA-10:*Tspo*$^{+/+}$ and MA-10:*Tspo*$^{\Delta/\Delta}$ cells with increasing doses of pregnenolone in the presence of 10μM 19-atriol resulted in increasing levels of progesterone synthesis (Figure 2.2B).

19-atriol is converted to 19-hydroxytestosterone by 3β-HSD

19-atriol's structure along with its ability to competitively inhibit 3β-HSD make it a likely candidate as a substrate for 3β-HSD. Conversion of the 3β-hydroxyl group to a carbonyl group would yield 19-hydroxytestosterone. Treatment of MA-10 cells with 19-atriol in the absence of stimulation resulted in an accumulation of 19-hydroxytestosterone (Figure 2.3A). 19-hydroxytestosterone treatment of MA-10 cells in the presence of 1ug/mL pregnenolone resulted in modest reduction in progesterone synthesis by 3β-HSD (Figure 2.3B).

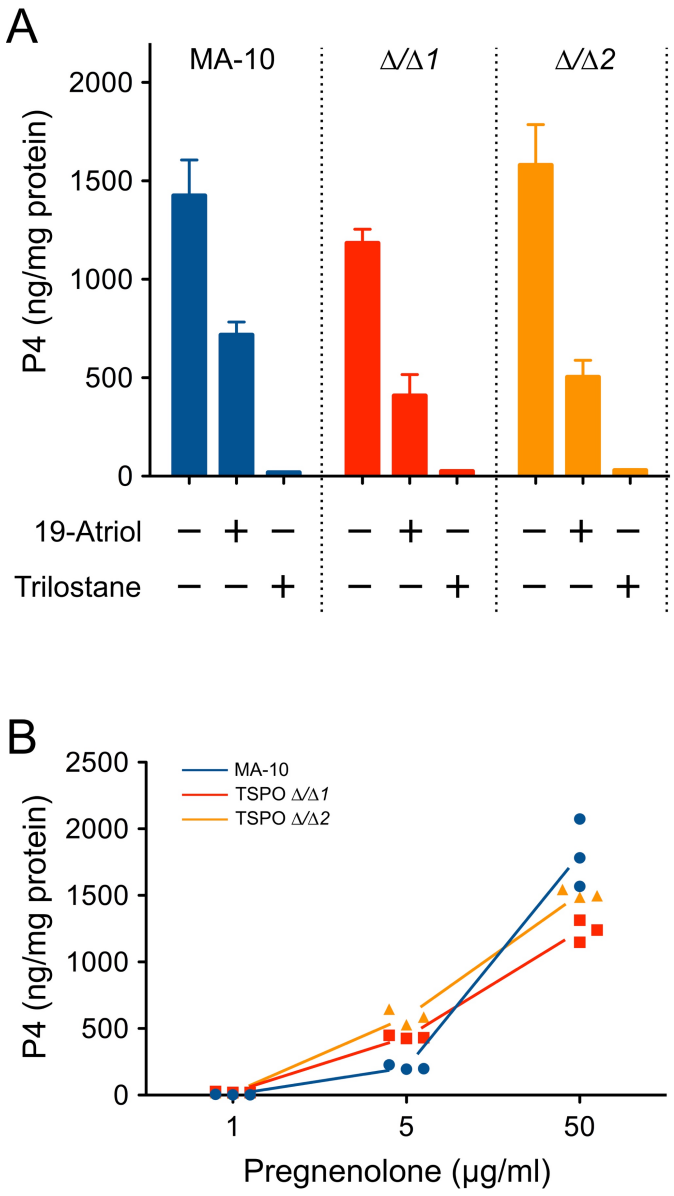

Figure 2.2 Conversion of pregnenolone to progesterone by 3β-HSD is inhibited by 19-atriol. (A) MA-10:*Tspo*$^{+/+}$ and MA-10:*Tspo*$^{\Delta/\Delta}$ cells treated with 1μg/mL pregnenolone can synthesize progesterone. Addition of 10μM 19-atriol decreases progesterone production in pregnenolone-treated MA-10:*Tspo*$^{+/+}$ and MA-10:*Tspo*$^{\Delta/\Delta}$ cells. The extent of inhibition observed was lower than that observed using trilostane (20 μM), a 3β-HSD inhibitor. **(B)** Addition of increasing concentrations of pregnenolone (1, 5, 50 μM) with 10μM 19-atriol could counter the inhibitory effect of 19-Atriol on P4 production.

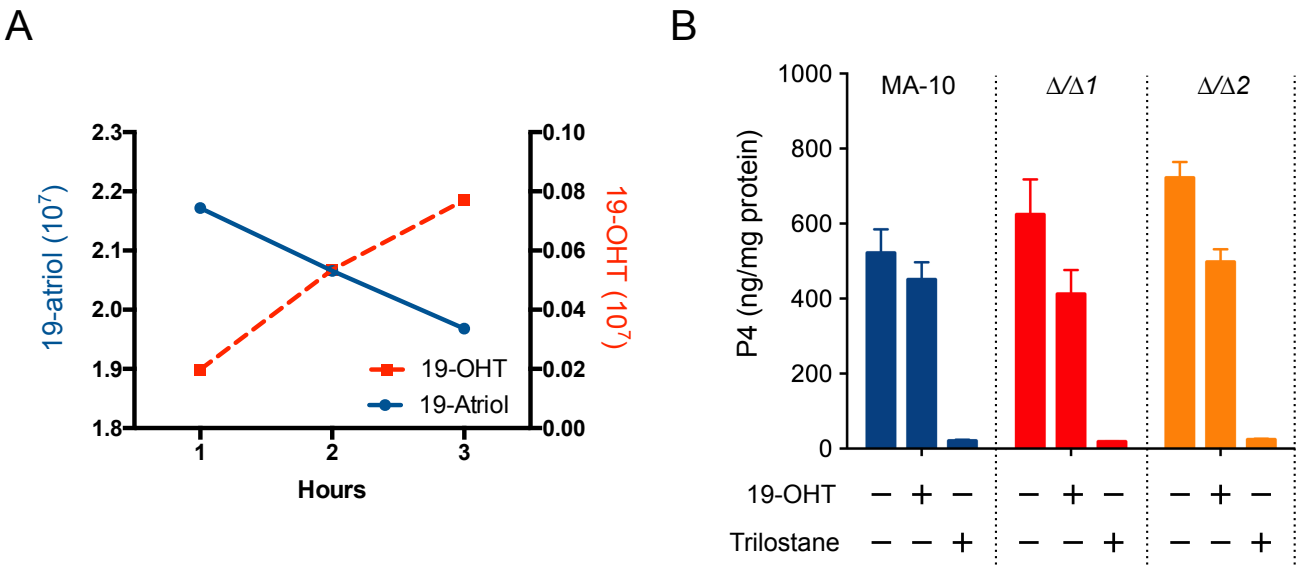

Figure 2.3 19-atriol is catalyzed to 19-hydroxytestosterone by 3β-HSD.
(A) MA-10:*Tspo*$^{+/+}$ cells treated with 100μM 19-atriol convert 19-atriol to 19-hydroxytestosterone. **(B)** Addition of 19-hydroxytestosterone (10μM) to pregnenolone-treated MA-10:*Tspo*$^{+/+}$ slightly reduced progesterone synthesis. This reduction was significantly weaker than that induced by trilostane.

19-atriol and 19-hydroxytestosterone docks 3β-HSD active site

19-atriol and 19-hydroxytestosterone are predicted to bind the active site of 3β-HSD in a similar orientation as pregnenolone (Figure 2.4A). ΔG of binding for pregnenolone, 19-atriol, and 19-hydroxytestosterone are 52.4 kcal/mol, 52.6 kcal/mol, and 54.6 kcal/mol, respectively. All three ligands bind to the 3β-HSD active site with similar interactions with Ala184, Arg 186, and Arg194 (Figure 2.4B).

19-atriol inhibits pregnenolone synthesis by CYP11A1

Pregnenolone was below our levels of detection in Bt_2cAMP-treated cells, likely because nearly all is converted in progesterone by 3β-HSD.

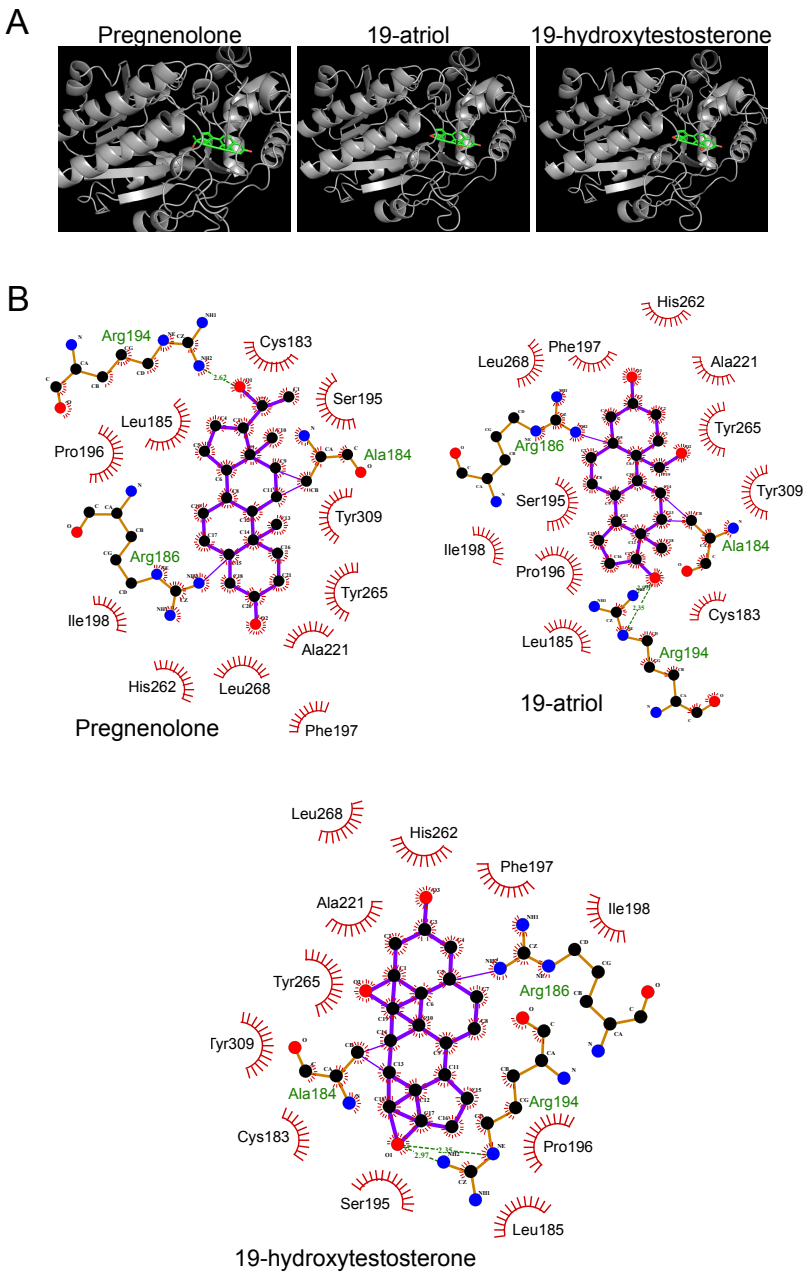

Figure 2.4 Modelling of 19-atriol and 19-hydroxytestosterone binding to 3β-HSD. **(A)** 19-atriol, 19-hydroxytestosterone predicted to bind the active site of 3β-HSD in highly similar orientations to pregnenolone. **(B)** Ligplot predictions for pregnenolone, 19-atriol, 19-hydroxytestoterone showing interacting amino acid residues.

Pregnenolone secretion in 19-atriol-treated samples revealed significantly decreased pregnenolone levels compared to trilostane-treated samples, suggesting a lack of pregnenolone synthesis by CYP11A1 despite Bt_2cAMP stimulation (Figure 2.5A). 19-atriol treatment also inhibits synthesis of pregnenolone in 22(R)-hydroxycholesterol-treated samples (Figure 2.5B). Analysis of cell culture supernatants confirms that both 19-atriol and 19-hydroxytestosterone inhibit pregnenolone formation in Bt_2cAMP-stimulated MA-10:*Tspo*$^{+/+}$ cells (Figure 2.5C).

Expression of STAR, CYP11A1 unchanged by 19-atriol treatment

STAR protein expression is unchanged by 19-atriol treatment (1-10μM) in both MA-10:*Tspo*$^{+/+}$ and MA-10:*Tspo*$^{\Delta/\Delta}$ cells. In addition, CYP11A1 protein expression is also unchanged by 19-atriol treatment, suggesting inhibition of CYP11A1 activity is not due to changes in enzyme abundance (Figure 2.5D).

19-atriol and 19-hydroxytestosterone favorably bind CYP11A1 active site

Structural modeling demonstrates 19-atriol and 19-hydroxytestosterone binding in the CYP11A1 active site in similar orientations as cholesterol. Cholesterol binding to the CYP11A1 active site orients its side chain closest to the heme group. Both 19-atriol and 19-hydroxytestosterone are predicted to bind in the identical active site, in a highly similar orientation, with the hydroxyl group present on carbon-19 oriented towards the interior of the protein, closest to the heme group (Figure 2.6A). ΔG of binding for cholesterol, 19-atriol, and 19-hydroxytestosterone is -12.4 kcal/mol, -10.2 kcal/mol, and -9.4 kcal/mol,

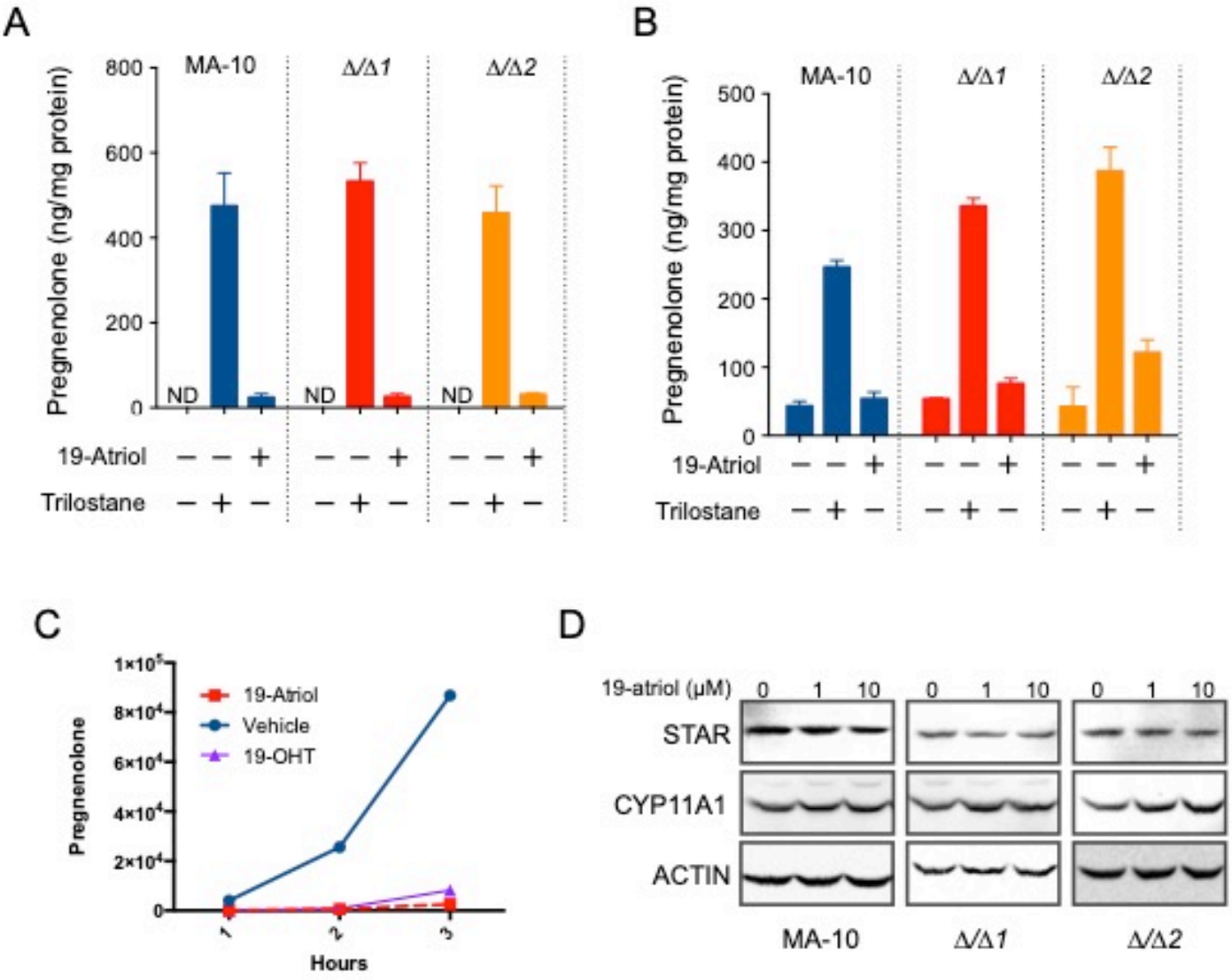

Figure 2.5 19-atriol treatment inhibits pregnenolone production by CYP11A1. **(A)** MA-10:*Tspo*$^{+/+}$ and MA-10:*Tspo*$^{\Delta/\Delta}$ cells stimulated with Bt_2cAMP in the presence of 10μM 19-atriol do not accumulate pregnenolone despite 3β-HSD inhibition. Treatment with 3β-HSD inhibitor trilostane (20μM) causes pregnenolone accumulation. **(B)** 19-atriol also blocked pregnenolone accumulation in MA-10:*Tspo*$^{+/+}$ and MA-10:*Tspo*$^{\Delta/\Delta}$ cells treated with 20μM 22(R)-hydroxycholesterol. **(C)** Both 19-atriol and 19-hydroxytestosterone treatment inhibit pregnenolone formation in Bt_2cAMP-treated MA-10:*Tspo*$^{+/+}$ cells. **(D)** Western blot demonstrates 19-atriol treatment (0-10μM) has no effect on STAR and CYP11A1 expression.

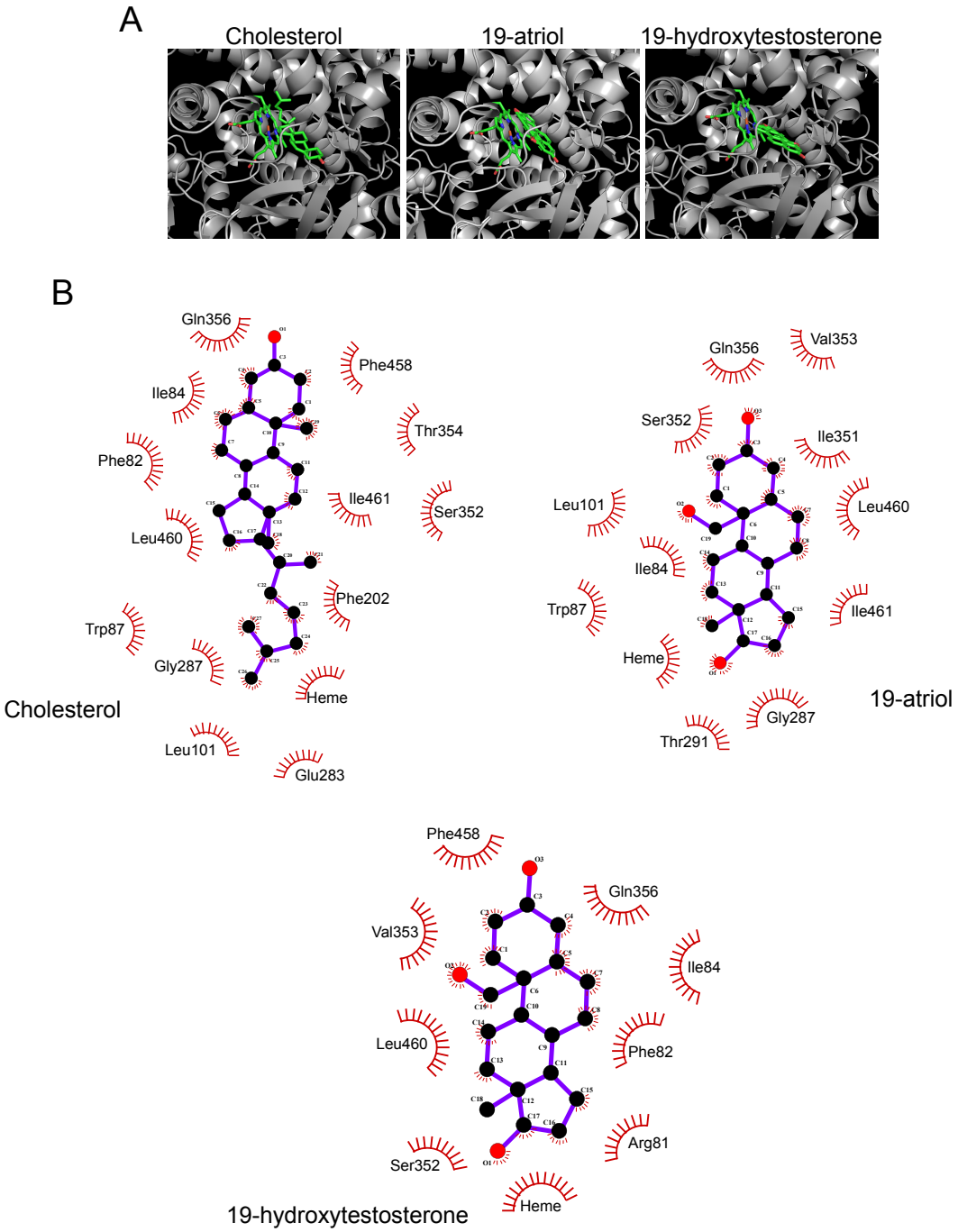

Figure 2.6 19-atriol and 19-hydroxytestosterone dock into CYP11A1 active site in a highly similar orientation to cholesterol. (A) Autodocking of 19-atriol and 19-hydroxytestosterone predict highly similar binding to CYP11A1 active site compared to cholesterol. **(B)** Ligplot predictions for cholesterol, 19-atriol, 19-hydroxytestoterone showing interacting amino acid residues.

respectively. LigPlot models predict several interacting resides in common between cholesterol, 19-atriol, and 19-hydroxytestosterone. The CYP11A1 binding site primarily interacts with its ligands via hydrophobic interactions and all three ligands share several interacting amino acids, including Ile84, Gln356, Ser352, and Leu460 (Figure 2.6B).

Discussion

Recent studies on TSPO function using *in vivo* and *in vitro* gene-deletion systems have demonstrated that TSPO is not required for steroidogenesis which calls for a reexamination of the identified pharmacological compounds such as 19-atriol and PK11195 that were considered to be key in linking the protein to steroidogenesis. Study of PK11195 action in *Tspo* gene-deleted cells demonstrates that while the compound is able to induce steroid hormone production, this effect is present regardless of TSPO protein expression and PK11195 action is not mediated through TSPO (34). In this study we examine and demonstrate that 19-atriol's inhibitory activity is also unrelated to TSPO expression.

19-atriol was demonstrated to be an inhibitor of progesterone production in a dose-dependent manner after stimulation with Bt_2cAMP and 22(R)-hydroxycholesterol treatment. While we observed a similar decrease in progesterone production, this effect is not different between MA-10:*Tspo*$^{+/+}$ and MA-10:*Tspo*$^{\Delta/\Delta}$ cells, indicating that the inhibitory effect of 19-atriol is not dependent upon TSPO protein expression.

Next, to determine if 19-atriol was a potential inhibitor of 3β-HSD, we provided MA-10:*Tspo*$^{+/+}$ and MA-10:*Tspo*$^{\Delta/\Delta}$ cells with pregnenolone, the immediate precursor to progesterone synthesis in the steroidogenic pathway. After pregnenolone treatment, we continued to see inhibition of 19-atriol on progesterone production in both MA-10:*Tspo*$^{+/+}$

and MA-10:*Tspo*$^{\Delta/\Delta}$ cells, strongly suggesting that the compound is inhibitory to the final step of progesterone synthesis, the conversion of pregnenolone to progesterone by 3β-HSD and again reiterating the lack of TSPO's role in 19-atriol action. This effect could be removed by increasing the relative amount of pregnenolone substrate. We were able to observe restoration of high levels of progesterone production at the highest dose of pregnenolone (50μM) despite the presence of 10μM 19-atriol, suggesting that 19-atriol likely inhibits 3β-HSD by competing for the active site.

To determine if 19-atriol was acting to inhibit the transport of cholesterol across the intermembrane space, MA-10:*Tspo*$^{+/+}$ and MA-10:*Tspo*$^{\Delta/\Delta}$ cells were treated with 19-atriol and 22(R)-hydroxycholesterol, a cholesterol derivative that can diffuse past the aqueous intermembrane space and be catalyzed to pregnenolone by CYP11A1 in the mitochondrial matrix without specialized protein transport machinery to induce steroid production, thus bypassing the obstacle of cholesterol transport to the matrix. Despite the fact that 22(R)-hydroxycholesterol can freely enter the mitochondrial matrix, we continued to observe this dose-dependent reduction in progesterone levels in both MA-10:*Tspo*$^{+/+}$ and MA-10:*Tspo*$^{\Delta/\Delta}$ cells. The presence of 19-atriol's inhibitory effect after 22(R)-hydroxycholesterol treatment demonstrates that cholesterol translocation to the mitochondrial matrix was not the part of the steroidogenic pathway 19-atriol acted upon and is highly suggestive of CYP11A1 inhibition. We also observed no change in STAR, CYP11A1, or TSPO expression with 19-atriol treatment.

19-atriol's structural similarity to pregnenolone also identifies it as a potential substrate for 3β-HSD. We hypothesized that 19-atriol would be catalyzed by 3β-HSD to

a novel product, 19-hydroxytestosterone, which could also be responsible for some of the inhibitory effects observed in prior experiments. Mass spectrometric analysis of cell culture supernatants identified that 19-atriol is catalyzed to 19-hydroxytestosterone and both molecules are able to prevent pregnenolone accumulation. Our study also indicates that 19-hydroxytestosterone is able to moderately inhibit 3β-HSD.

A possible explanation for 19-atriol and 19-hydroxytestosterone's ability to bind to CYP11A1 active site and prevent cholesterol from entering is the presence of the hydroxyl group on carbon 19. In the conversion of cholesterol to pregnenolone, two intermediates are formed, 22(R)-hydroxycholesterol, and 20(R), 22(R)-dihydroxycholesterol. Analysis of CYP11A1 shows that these intermediates bind 100 to 300 times tighter to the active site than does cholesterol, evidenced by the lack of accumulation of these intermediates during steroidogenesis (35). 22(R)-hydroxycholesterol forms hydrogen bonds with a water molecule, which, in turn, interacts with Thr291 and Gly287 as well as the iron present in the heme group and contributes to its retention in the active site over cholesterol, which lacks these hydroxyl groups in its side chain (36). Structural modelling of 19-atriol and 19-hydroxytestosterone also predicted that the compounds are able to dock into the active site in an orientation similar to cholesterol, orienting the 19-hydroxyl group towards the heme iron, and supports this explanation. We were able to make similar predictions for the 3β-HSD active site. 19-atriol and 19-hydroxytestosterone are predicted

to interact with all three of the same residues as pregnenolone and to form hydrogen bonds of similar lengths with Arg194.

Reports in the literature have demonstrated that TSPO-binding ligands are able to induce or inhibit steroidogenesis. Data from *ex vivo* models of glaucoma demonstrated that allopregnanolone synthesis in response to increased ocular pressure provided protective effects for neuronal cells. PK1195 treatment stimulated allopregnanolone synthesis, reducing signs of histological damage, while 19-atriol treatment provided exactly the opposite effect, inhibiting allopregnanolone production and resulting in severe histological damages (37). It was hypothesized that activation of TSPO by PK11195 in turn induced increased allopregnanolone synthesis, thus producing its protective effects. However, our group has demonstrated that PK11195, like 19-atriol, exerts its effects in a TSPO-independent manner (16) and, given our findings, 19-atriol's inhibitory effect on allopregnanolone synthesis likely results from it or 19-hydroxytestosterone's ability to inhibit the CYP11A1 enzyme.

In summary, our results strongly demonstrate that 19-atriol-mediated inhibition of steroidogenesis is a direct effect of it and 19-hydroxytestosterone's ability to compete for and inhibit CYP11A1 and 3β-HSD, both of which are completely unrelated to TSPO expression and function (Figure 2.7).

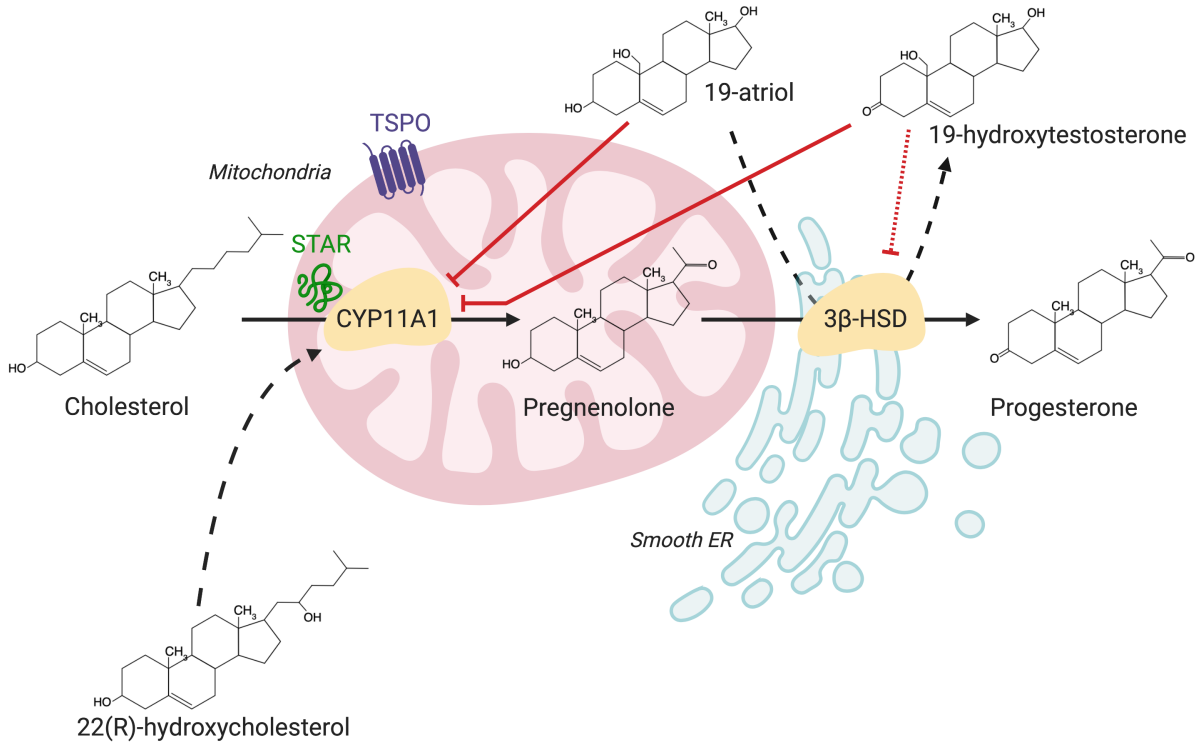

Figure 2.7 19-atriol inhibits steroidogenesis via inhibition of CYP11A1 and 3β-HSD. Upon stimulation, cholesterol is rapidly transported past the mitochondrial membranes by STAR and is converted by the CYP11A1 enzyme to 22(R)-hydroxycholesterol, an intermediate, before rapidly being converted to pregnenolone. Pregnenolone diffuses out of the mitochondria and is converted to progesterone by 3β-HSD in the endoplasmic reticulum. 19-atriol's structure enables it to act as a substrate of 3β-HSD and is converted to 19-hydroxytestosterone. Both ligands are able to inhibit CYP11A1 and 3β-HSD, although inhibition of 3β-HSD by 19-hydroxytestosterone is weaker than that of 19-atriol. Expression of TSPO is irrelevant to 19-atriol inhibition of steroidogenesis.

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CHAPTER 3

CRISPR/CAS9-MEDIATED DISRUPTION OF STAR IN MA-10 CELLS CONFIRMS ITS CRUCIAL ROLE IN MITOCHONDRIAL CHOLESTEROL IMPORT FOR STEROIDOGENESIS

Abstract

The steroidogenic acute regulatory protein (STAR) is a mitochondrial protein synthesized rapidly in response to stimulation by tropic hormones such as LH or ACTH. Although humans and mice with function-ablating mutations in the *STAR* gene show an almost complete inability to synthesize gonadal and adrenocortical steroids, the precise mechanism of mitochondrial cholesterol import has been shrouded in controversy. Recently, we demonstrated that the translocator protein (TSPO), thought to be an "indispensable" cholesterol channel in the outer mitochondrial membrane, is not involved in this process. Irrespective, STAR-independent steroidogenesis, although at a substantially lower rate, has been reported to occur in patients with completely inactivating *STAR* mutations that develop lipoid congenital adrenal hyperplasia. However, the extent of steroidogenesis possible in the absence of STAR has not been definitively evaluated in any steroidogenic cell type *in vitro*. In order to study this, we generated *Star*-disrupted MA-10 Leydig cell lines via gene editing using CRISPR/Cas9 and examined their ability to synthesize steroids. Our results indicate that *Star*-disrupted MA-10 cells show only a diminutive ability to synthesize steroids (~10% of control levels) underscoring the importance of STAR in this process. The *Star*-disrupted MA-10 cell clones maintain expression of TSPO, the steroidogenic enzymes CYP11A1 and 3β-HSD, and are capable of producing progesterone utilizing the water-soluble 22(R)-hydroxycholesterol. These findings corroborate that STAR function is essential to steroidogenesis and cannot be compensated by TSPO expression.

Introduction

Steroidogenesis is the synthesis of steroid hormones in various tissues of the body. These steroid hormones are essential to life and control several key metabolic and reproductive processes and include the glucocorticoids and mineralocorticoids of the adrenal cortex, ovarian and placental progestins and estrogens, and testicular androgens. Also included in this category are neurosteroids such as pregnenolone and allopregnanolone which are synthesized in the brain. All steroid hormones share a common precursor, cholesterol, which is converted to the first steroid, pregnenolone, by the CYP11A1 enzyme in the mitochondrial matrix[1,2]. Pregnenolone then diffuses out of the mitochondria and is converted to the various steroid hormones based on the availability of cell-type specific enzymes[3].

Due to its hydrophobic nature, cholesterol is unable to cross the aqueous intermembrane space between the mitochondrial membranes and gain access to the CYP11A1 enzyme without a specialized transport mechanism. In fact, the delivery of cholesterol from the outer to the inner mitochondrial membrane acts as the rate-limiting step in steroidogenesis[4]. Work to identify the exact mechanism and proteins involved in facilitating this transport between the mitochondrial membranes has determined that likely candidate proteins would be consistent with several pieces of key experimental evidence. First, experimental evidence demonstrated protein synthesis was essential for acute steroid production[5–9]. Second, these proteins were likely to be synthesized in response to stimulation by tropic hormones from the anterior pituitary such as ACTH[10]. Third, these proteins were likely located in or closely associated with the mitochondria[11]. Several protein candidates were identified, including sterol-carrier protein 2 (SCP2), which was

later ruled out because SCP2-null mice lacked specific defects in steroidogenesis[12], the peripheral benzodiazepine receptor (PBR), later renamed to translocator protein (TSPO), and the steroidogenic acute regulator protein (STAR).

Both TSPO and STAR have characteristics that fit with the existing experimental evidence that made them viable candidates. Both were highly expressed in mitochondria[13–17] and both had cholesterol-binding domains that could potentially play a key role in cholesterol trafficking[18,19]. TSPO, however, lacks several of the important characteristics of the key regulator of acute steroidogenesis. It is not exclusively expressed in steroidogenic tissues[20] and is constitutively expressed, showing no change in response to tropic hormones such as ACTH or LH. *In vitro* experiments demonstrated that TSPO ligands such as PK11195 could induce steroidogenesis[16] but later experiments in TSPO knockout cells show that these effects were independent of TSPO expression[21]. Deletion and knockdown of TSPO in MA-10 Leydig cells[21] as well as conditional and global deletion of TSPO in mice show no change in steroid hormone levels[22,23]. These findings lead us to question TSPO as a candidate for the key cholesterol transporter between the mitochondrial membranes.

STAR currently stands as the most promising candidate as the specialized protein transporter between the two mitochondrial membranes. STAR was first discovered as a mitochondrial protein that is newly synthesized in response to the tropic hormone LH[14,15] and also contains the cholesterol binding START domain[19], all characteristics consistent with the experimental evidence named above. In addition, STAR knockout animals have near complete abolished steroidogenesis and accumulation of intracellular lipid stores[24,25]

and exhibit a phenotype highly similar to human patients with loss-of-function mutations in the STAR gene[26–28].

Experimental evidence *in vitro* using COS-1 cells have supported the model that STAR is a key player in the regulation of acute steroidogenesis. COS-1 cells transfected with STAR as well as several downstream enzymes are able to produce steroids in low quantities and support the existing evidence that the full length STAR protein is localized to the mitochondria[29,30]. These models, though, remain difficult to use in the study of acute steroidogenesis because COS-1 cells are derived from kidney tissue and do not possess the enzymatic profile associated with steroidogenesis. The exact mechanism by which STAR acts to transport cholesterol also remains controversial. Previous work provided evidence that removal of the first 62 amino acids of STAR from the N terminus which contains the mitochondria targeting signal did not abolish steroidogenesis in CYP11A1-transfected COS-1 cells and has proposed that STAR acts on the outer mitochondrial membrane rather than in the intermembrane space[30]. This, however, is inconsistent with *in vivo* data published several years later in which reintroduction of a similar N-terminal truncated version of STAR into STAR global knockout mice was unable to rescue the original knockout phenotype[31], calling into question the functionality of using CYP11A1-transfected COS-1 models for the study of mitochondrial cholesterol transport.

In the human placenta, steroidogenesis occurs without the presence of STAR[32] and previous work has suggested STARD3, another START-domain containing protein constitutively expressed in placenta tissue, may be able to perform this function[33]. STARD3's N-terminal anchors the protein to the late endosome[34] and expression of the a truncated STARD3 containing only it's START domain has been shown to stimulate

steroidogenesis in isolated mitochondria[35] but whether this is true in intact steroidogenic cells has yet to be determined.

The lack of ideal *in vitro* models for the study of the STAR protein remains a major limiting factor in this field of study and in order to directly address this important need, our group has generated a series of STAR knockout cell lines from the MA-10 Leydig cell line using CRISPR/Cas9. These models confirm STAR's role as the key regulator of acute steroidogenesis in Leydig cells and create a more ideal system to study STAR and test the functionality of other START domain proteins in mitochondrial cholesterol transport.

Materials and Methods

Cell Culture

MA-10 cells were cultured in DMEM Hi-glucose (Sigma) supplemented with 10% fetal bovine serum (Invitrogen), 1% penicillin-streptomycin and 1% non-essential amino acids. To generate MA-10 subclones, cells were seeded in 96-well plates at a density of 1 cell/well and allowed to expand.

Chromosome Quantification

Cells were arrested in metaphase with KaryoMAX Colcemid for 8 hours, trypsinized, and resuspended in 0.075M KCl for 15 minutes to induce swelling. Cells were pelleted and fixed in 1:1 acetic acid:methanol for 1 hour on ice. Cell suspension was dropped onto slides and flamed to induce bursting. Slides were dried and stained with Giemsa for 5 minutes. Chromosomes of 15-20 cells were counted per clone.

Generation of STAR-deleted MA-10 cells

MA-10:*Star*Δ/Δ cells were generated by targeting exon 2 of the *Star* gene (NCBI: Gene ID: 20845; Reference Sequence: NM_011485.5) using the clustered regularly

interspaced short palindromic repeats (CRISPR) system. The guide RNA sequence 5'-GGATGGGTCAAGTTCGACGT-3' was cloned into the pX330-U6-Chimeric_BB-cBh-hSpCas9 plasmid. The plasmid was transfected into MA-10 cells using TransIT-X2 (Mirius Bio). After 48 hours, cells were seeded in 96-well plates at a density of 1 cell/well and allowed to expand for screening.

Hormone Assays

Cells were plated at $5.0x10^4$ cells/well in a 96-well plate and allowed to attach overnight. Cells were then stimulated with either 0.5mM N6,2′-O-Dibutyryladenosine 3′,5′-cyclic monophosphate sodium salt (Sigma) or 20μM of 22(R)-hydroxycholesterol for six hours (Sigma). Cell culture supernatant was collected and stored at -80C. Cell culture supernatant and I^{125}-labeled progesterone trace (MP Bio) were incubated overnight with Staigmiller anti-progesterone antibody. A charcoal and dextran solution was added to the samples and incubated for 10 minutes. Samples were centrifuged at 3000rpm for 10 minutes and resulting radioactivity in the supernatant was measured. Progesterone concentrations were calculated based on standard curve run with each assay. Results were normalized to the total protein content of each well.

Immunoblots

Cells collected in SDS buffer containing protease inhibitors (Sigma) were sonicated and protein concentration was quantified using bicinchoninic assay. Proteins were separated by SDS-PAGE, transferred to a PVDF membrane and immunoblotted using an affinity-purified rabbit polyclonal antibody against STAR amino acids 93-105, a rabbit monoclonal antibody against TSPO (Abcam) and β-Actin (LI-COR) as a loading control. TSPO and

STAR were detected using a Goat anti-rabbit Poly-HRP (Life Technologies) secondary antibody and IRDye800 (LiCor) labeled secondary antibodies.

Cloning and expression of fusion proteins

MA-10 cells were stimulated with 0.5mM Bt_2cAMP for six hours in DMEM. Cells were collected and RNA was extracted using Trizol. The resulting RNA was reverse transcribed using the High-capacity cDNA Reverse Transcription Kit (Applied Biosystems). The cDNA encoding the first 62 amino acids of the murine STAR protein (MFLATFKLCAGSSYRHMRNMKGLRHQAVLAIGQELNWRALGDSSPGWMGQVRRRS SLLGSQLEATLYS) were amplified and cloned into BglII and AgeI sites in pmApple-N1 vector using standard restriction cloning methods. The plasmid was transfected into MA-10 and Cos7 cells using TransIT-X2 and imaged 48 hours post-transfection.

Results

MA-10 cells are highly heterogeneous in chromosome number and near tetraploidy

MA-10 subclones are highly heterogenous in morphology and chromosome count. Chromosome counts from metaphase spreads revealed MA-10 subclones have 65-80 chromosomes on average, nearing tetraploidy (Figure 3.1A-B).

Progesterone production correlates to STAR expression but not to TSPO expression

MA-10 subclonal cell lines were stimulated with Bt_2cAMP for six hours to induce STAR expression and immunoblots revealed that STAR expression varied widely among MA-10 subclones. By comparison, TSPO expression was relatively consistent among subclones (Figure 3.2A). All clones produced progesterone in response to Bt_2cAMP stimulation but clones with lower STAR expression also had reduced progesterone synthesis compared to clones with abundant STAR expression.

A

	Clone 1	Clone 4	Clone 5
Median	69	73.5	79
Average	68 ± 2.5	70 ± 3.9	77 ± 3.0
Range	40 - 83	51 - 91	60 - 91

B

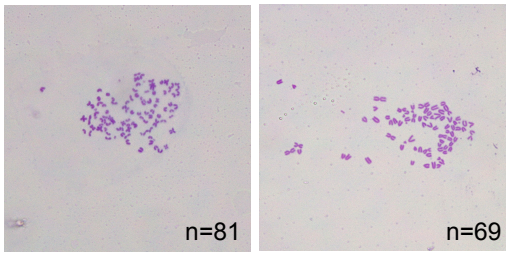

Figure 3.1 MA-10 subclones vary in chromosome number and near tetraploidy. (A) Chromosome numbers from three MA-10 clones indicate near tetraploidy. **(B)** Representative images of metaphase spreads from Clone 1.

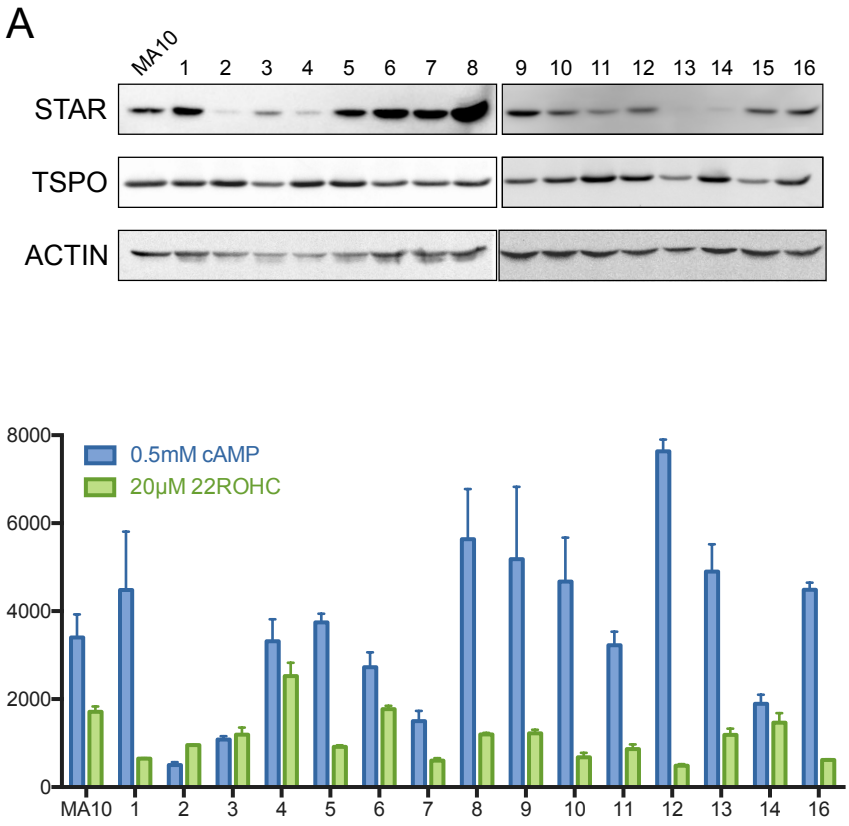

Figure 3.2 Progesterone production in MA-10 clones correlate to StAR expression but not to TSPO expression. (A) Western blot for StAR and TSPO in eight selected MA-10 clones stimulated with Bt_2cAMP. β-Actin (ACTIN) was used as control for protein loading. (B) Corresponding progesterone production in these stimulated MA-10 clones normalized to total protein content indicated a faithful correlation to StAR expression.

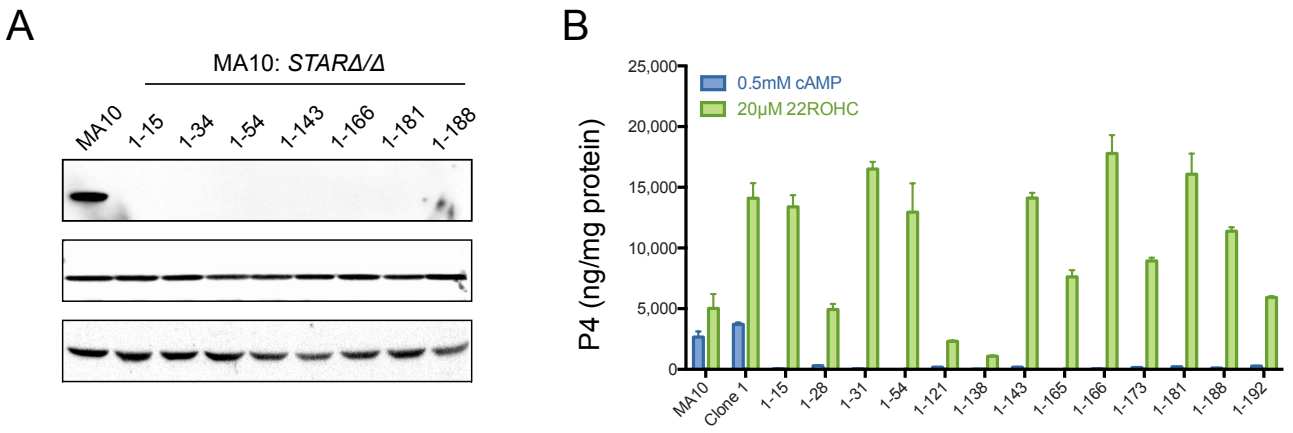

Figure 3.3 StAR deletion dramatically reduces progesterone production in MA10:*StARΔ/Δ* sub-clones. (A) Western blot for StAR and TSPO in eight MA10:*StARΔ/Δ* sub-clones treated with Bt_2cAMP. MA10:*StARΔ/Δ* sub-clones do not express StAR, but express TSPO at levels similar to the parent clone. β-Actin (ACTB) was used as control for protein loading. (B) Progesterone production by MA10:*StARΔ/Δ* clones treated with Bt_2cAMP and 22(R)-hydroxycholesterol. Bt_2cAMP induces less than 1% of progesterone production compared to the parent MA-10 sub-clone (Clone 1) used to derive MA10:*StARΔ/Δ* sub-clones. Progesterone values were normalized to total protein content.

By comparison, TSPO expression was relatively constant among MA-10 subclones and did not correlate with levels of progesterone synthesis (Figure 3.2B).

STAR deletion dramatically reduces progesterone synthesis despite TSPO expression

The *Star* gene has seven exons. The translation start codon is located in exon 1 and stop codon in exon 7. Using the CRISPR/Cas9 system, a guide RNA was designed to target Cas9 to the beginning of exon 2. Frame shift or premature stop codon formation at this locus would result in a truncated version of the protein containing only the first 52 amino acids. The antibody against murine STAR was generated against amino acids 93-105, coded by exons 3-4.

MA-10 Clone 1 was transfected with px330 encoding the selected guide RNA targeting exon 2. After selection, several clones were identified with a complete lack of

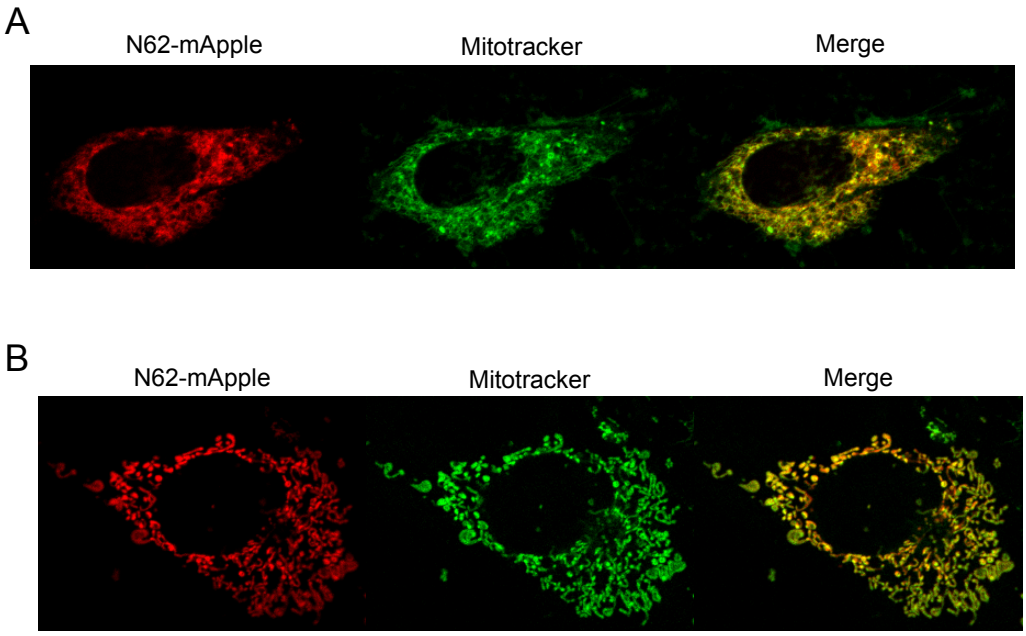

Figure 3.4 N-terminal of STAR localizes proteins to the mitochondria. Confocal imaging of **(A)** MA-10 and **(B)** COS7 cells expressing a fusion protein encoding the first 62 amino acids of STAR the fluorescent protein mApple localizes to the mitochondria, colocalizing with Mitotracker Green staining.

STAR expression compared to Clone 1. All clones, however, retained identical TSPO expression to the Clone 1 (Figure 3.3A). Stimulation of the MA-10:STARΔ/Δ clones with Bt_2cAMP revealed dramatically reduced progesterone production compared to MA-10 and Clone 1 despite presence of TSPO protein. Treatment of the MA-10:STARΔ/Δ clones with freely soluble 22(R)-hydroxycholesterol showed high levels of progesterone synthesis, indicating intact CYP11A1 and 3β-HSD in MA-10:STARΔ/Δ clones (Figure 3.3B).

N-terminal of STAR contains mitochondrial targeting sequence

Expression of a fusion protein encoding the first 62 amino acids of STAR (N62) followed by the fluorescent protein mApple results in highly specific mitochondrial localization in both MA-10 and Cos7 cells (Figure 3.4A-B).

Discussion

Cholesterol's extreme hydrophobicity combined with its essential role in cellular metabolism poses a unique trafficking challenge for the steroidogenic cell. Studies over the past several decades have made attempts to identify the proteins responsible for transport of cholesterol across different cellular compartments and controversy has continued over the relative importance of TSPO and STAR in mitochondrial cholesterol trafficking. Early studies indicated that TSPO-binding pharmacological compounds were able to induce steroidogenesis[16,17] but more recent work has demonstrated these compounds, in fact, act to induce steroidogenesis by TSPO-independent effects[21]. In addition, deletion of TSPO in steroidogenic cells as well as global TSPO deletion in mice results in no change in steroid hormone levels[21–23]. The results of this study supports the conclusion that TSPO is not an integral part of the steroidogenic machinery. Progesterone synthesis does not correlate well with TSPO expression and TSPO expression cannot support steroidogenesis in the absence of STAR.

Since its identification, STAR has been a prime candidate as the key mitochondrial cholesterol transporter. STAR is a unique protein comprised of two parts—a mitochondria targeting signal and a cholesterol-binding START domain[19]. Expression of the STAR mitochondrial targeting signal fused to a fluorescent protein shows strong localization to the mitochondria in both MA-10 and COS-7 cells, supporting previous work that STAR is

a mitochondrial protein[36]. STAR is newly synthesized in response to stimulation with tropic hormones or Bt_2cAMP, consistent with previous evidence where addition of cycloheximide or puromycin could inhibit acute steroidogenesis[5,6,9], likely by preventing the translation of STAR at the ribosomal level. In addition, mice deficient in STAR protein[25] as well as human patients with inactivating mutation[29] show an inability to synthesize steroid hormones, consistent with the data we present in this study. STAR protein deletion results in a near complete ablation of steroid hormone synthesis in MA-10 Leydig cells despite intact enzymatic machinery, strongly suggesting that STAR is an indispensable part of the mitochondrial steroidogenic machinery.

This work also provides a novel model for the study of mitochondrial cholesterol transport proteins. Human placenta progesterone production remains a mystery due to its complete lack of STAR expression[32]. STARD3 has been proposed as a potential candidate to perform the function of STAR in this tissue[35,37] and the MA-10:STARΔ/Δ clones generated here provide a novel tool for the identification of other proteins that can perform this function.

In conclusion, our study provides concrete evidence that STAR, not TSPO, is the essential mitochondrial cholesterol transporter during acute steroidogenesis.

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CHAPTER 4

BALANCE OF EXTRACELLULAR AND INTRACELLULAR CHOLESTEROL SOURCING IN STEROIDOGENESIS

Abstract

Steroid hormones are essential hormones derived from cholesterol, a unique cellular substrate mobilized in large quantities during acute induction of steroidogenesis. Over the past several decades, multiple factors have pointed away from the use of serum when inducing steroidogenesis during *in vitro* experimentation. Studies have indicated an inability for some cells to respond to tropic hormones in the presence of serum and others sought to eliminate confounding factors potentially provided by supplementation of serum in culture media. As a result, studies of steroidogenic cells have primarily used serum-free culture conditions and soluble intermediates such as dibutyryl-cyclic adenosine monophosphate (Bt_2cAMP) for the study of cholesterol trafficking. While this has not had adverse effects on the identification of mitochondrial cholesterol transporters, these conditions fail to replicate physiological conditions and prevent cells from accessing serum lipoproteins, an important source of cholesterol and cholesterol esters available to cells in the body. In this study we generate and characterize several hCG-responsive subclones of the MA-10 Leydig cell line and demonstrate that availability of serum lipoproteins increases progesterone output both in response to stimulation with Bt_2cAMP as well as hCG. These observations highlight the importance of providing adequate culture conditions for steroidogenic cells for the study of cholesterol sources and trafficking mechanisms.

Introduction

Steroid biosynthesis, an essential life function, starts with cholesterol as a substrate. Bioconversion of cholesterol to the first steroid precursor, pregnenolone (1-3), by CYP11A1 in the mitochondria is the first step in steroidogenesis (4,5). Subsequent enzymatic conversions of pregnenolone to generate different steroids occur on the basis of necessary steroidogenic tissue-specific hormonal outputs (6,7). Studies on steroidogenic cell function over the past six decades have uncovered cholesterol regulatory and transport systems (8), and signaling pathways associated with triggering a steroidogenic response in granulosa, luteal, adrenocortical and Leydig cells (9-17). Across all steroidogenic model systems that have been pivotal to elucidating many of the mechanisms, *in vitro* studies continue to present contextual limitations to refining specific functional descriptions.

One such example can be found in the progression of *in vitro* studies performed to define regulation of cholesterol homeostasis in steroidogenic cells. As steroids are only synthesized on demand, there is sudden escalation in need for acquiring and/or mobilizing cholesterol in steroidogenic cells (18-21). However, stimulation of steroidogenic cells in culture has always been performed under serum-free conditions that provide no extracellular cholesterol source (22). Although this had no adverse ramifications in studies that examine mitochondrial cholesterol import that were predominant in the 1990s (23), it distorts physiological relevance in studies investigating upstream mechanisms that lead to mitochondrial cholesterol delivery.

Existence of cholesterol-enriched lipid stores as droplets is a core characteristic consistent across steroidogenic cells of the adrenals, ovaries and testes (24-27). The

hormone sensitive lipase (HSL), a broad lipid hydrolase that also possesses cholesterol esterase activity (28,29), has been demonstrated to be critical for cholesterol mobilization from lipid droplets in steroidogenic cells (30,31). Although relevance of this core mechanism is undisputable, these studies have been performed by stimulating steroidogenic cells under serum-free conditions that force cells to either synthesize cholesterol *de novo* or use stored forms (32). Moreover, demonstration that free cholesterol used for steroid hormone biosynthesis resides within the plasma membrane, and that this pool can be replenished by extracellular low-density lipoproteins (LDL) (20), suggest use of medium containing serum could be substantial for sustaining a physiological balance of cholesterol sourcing for steroidogenesis.

We uncovered three possible explanations for the widespread use of serum-free media conditions. First, evidence was presented that stimulation of cultured primary bovine luteal cells using luteinizing hormone (LH) only yielded receptor-mediated signaling and progesterone production in serum-free growth conditions (33,34), suggesting that the presence of serum inhibits responsiveness to trophic hormones. Second, there was rationale that avoiding serum eliminates the possibility of unknown factors including steroids and trophic hormones in culture medium that could introduce variability and artifacts (22). Third, removing serum prevented pseudosubstrate effects that may occur due to high accumulating concentration of steroids (35-37), perhaps by ensuring a moderate rate of steroidogenesis. Although the first explanation could have been misconceived (38), it is also possible that cell type specific responses supporting growth versus differentiation could be a core factor. Similar effects have not been reported

for adrenocortical or testicular steroidogenic cells. For the second explanation, despite lack of direct experimental evidence, horse serum originally used in steroidogenic cell culture (39,40) contains variable levels of steroids and circulating trophic factors (FSH and LH) (41). Use of fetal bovine serum (FBS) for culture of steroidogenic cells has been used only in select recent studies (42,43). For the third explanation, the main consideration of containing excess steroid production is something that could be avoided by selecting optimal early timepoints. Collectively, emphasis in the above explanations are context-specific without direct relevance to cholesterol homeostasis in steroidogenic cells.

Addressing the role of serum in cholesterol sourcing for steroidogenesis, the present study shows that availability of extracellular cholesterol substantially increases the steroidogenic response with no negative impacts to studying steroidogenesis in MA-10 Leydig cells. This robust effect was mediated by the presence of serum lipoproteins, which was consistent in both stimulations using hCG and Bt_2cAMP. These findings indicate that use of serum-containing cell culture medium in studies that examine intracellular cholesterol trafficking is vital to understanding the cellular physiology of steroidogenesis.

Materials and Methods

MA-10 cells and culture

MA-10 cells (44) were cultured in DMEM and 10% fetal bovine serum and 1% penicillin-streptomycin and 1% non-essential amino acids as previously described.

Selection of LH-responsive MA-10 subclones

MA-10 cells were plated at a density of 1 cell/well in 96-well plates and allowed to expand for two weeks to form colonies. Resulting subclonal lines were then plated at 5 x 10^4 cells per well in a 0.1% gelatin-coated 96-well plates and allowed to attach overnight. The next day the subclones were stimulated with 1.5IU/mL hCG or 0.5mM Bt_2cAMP for six hours in DMEM supplemented with 10% FBS.

Progesterone assay

For all experiments, MA-10 cells were plated at 5 x 10^4 cells per well in a 0.1% gelatin-coated 96-well plate. Cells were allowed to attach overnight before stimulation for progesterone production. Cells were stimulated in DMEM containing relevant supplements using 0.5mM Bt_2cAMP (Sigma) or 1.5IU/mL hCG. Cell culture supernatant was collected and progesterone was quantified via radioimmunoassay as described in chapter 3. Progesterone produced was normalized to total protein content in each well.

Immunoblots

Stimulated cells were collected in SDS buffer with protease inhibitor. Proteins were separated by SDS-PAGE, transferred to a PVDF membrane and immunoblotted using rabbit polyclonal antibody against STAR or hCG receptor (BosterBio). Membranes were also blotted using a monoclonal mouse antibody for B-Actin (Santa Cruz) as a loading control. Detection of STAR and hCG receptor and B-Actin was performed using (Poly-HRP) secondary antibody and IRDye800 (Licor) labeled secondary antibodies using Azure c600 imager.

Oil Red O Staining and Quantification

LH-responsive subclones were plated at 80-90% confluence and allowed to attach overnight. Cells were fixed in 4% paraformaldehyde for 15 minutes. Cells were stained in working Oil Red O solution (2:3 Oil red O stock to distilled water) for 30 minutes. After staining, cells were washed five times with distilled water and imaged. For quantification, 100% isopropanol was added to the plate and incubated for 10 minutes shaking at 200rpm on an orbital shaker at room temperature. Absorbance of eluate was measured at 492nm.

Results

Serum lipoproteins increase progesterone production in MA-10 Leydig cells

MA-10 cells stimulated with Bt_2cAMP in the presence of 10% fetal bovine serum produced significantly more progesterone compared to cells stimulated in serum-free medium. Supplementation of treatment medium with 10% lipoprotein-depleted fetal bovine serum showed a much more modest increase in progesterone production (Figure 4.1A).

MA-10 cells respond poorly to hCG despite LH receptor expression

MA-10 cells produced significantly less progesterone in response to hCG compared to stimulation with Bt_2cAMP (Figure 4.1B) despite consistent presence of LH receptor in cell lysates (Figure 4.1C).

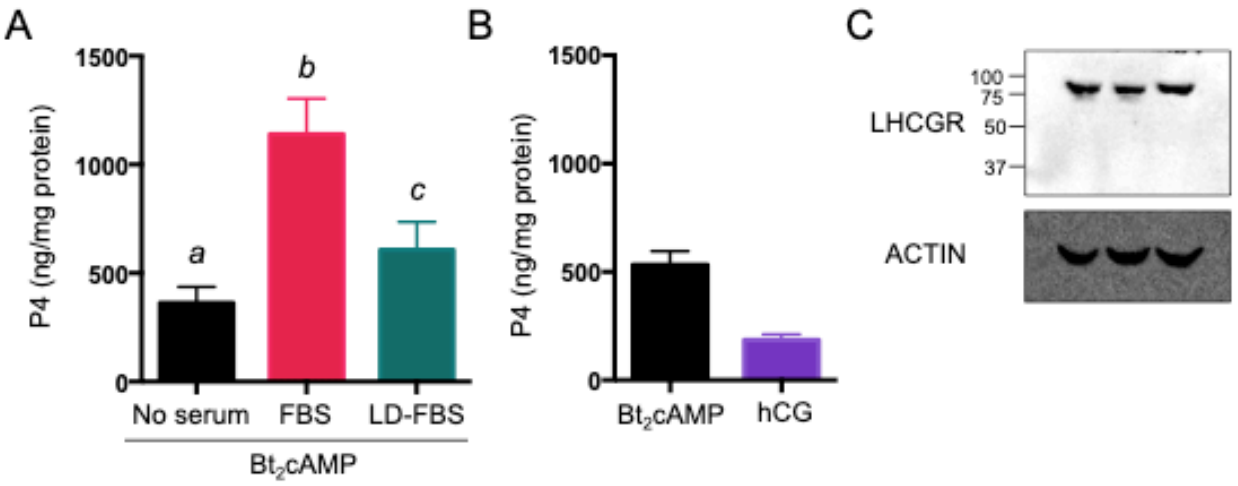

Figure 4.1 Serum lipoproteins enhance progesterone production in MA-10 cells (A) Stimulation of MA-10 cells with 0.5mM Bt_2cAMP for three hours in medium supplemented with 10% fetal bovine serum show increased progesterone production compared to cells stimulated in serum-free or lipoprotein-depleted fetal bovine serum (LD-FBS)-supplemented medium. **(B)** MA-10 cells stimulated with hCG produce significantly less progesterone compared to Bt_2cAMP-stimulated cells. **(C)** MA-10 cells maintain expression of LH receptor protein despite low response to hCG stimulation.

A subpopulation of MA-10 cells are able to respond to LH

Single-cell MA-10 subclones produced progesterone in response to Bt_2cAMP but only a subset were able to synthesize progesterone when stimulated with hCG (Figure 4.2A-B). Five healthy MA-10 subclones morphologically consistent with MA-10 cells were chosen for further characterization (Figure 4.2B).

MA-10 subclones vary in hCG-responsiveness, STAR expression, and chromosome number

Stimulation of MA-10 cells using Bt_2cAMP induces expression of STAR, the essential cholesterol transporter of the mitochondria. Bt_2cAMP treatment of MA-10 subclones also induces STAR expression, consistent with their ability to produce progesterone in

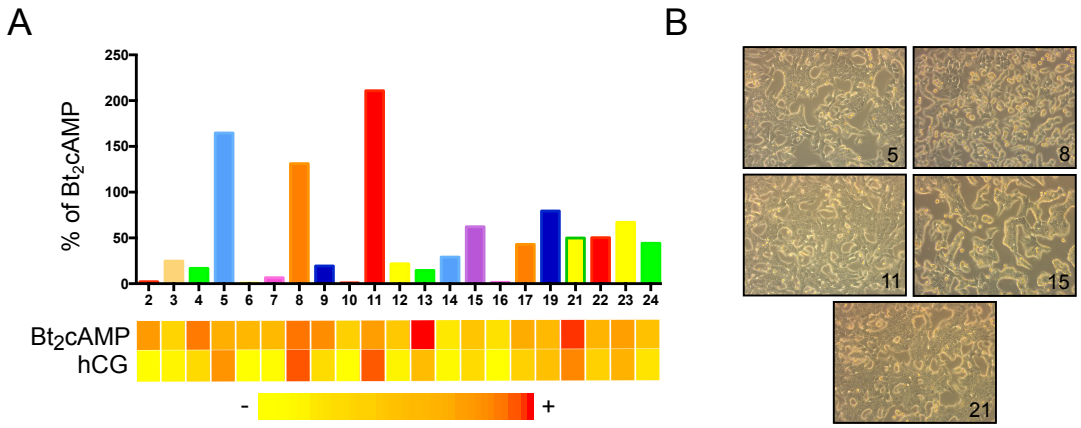

Figure 4.2 Some MA-10 subclones produce progesterone in response to hCG. (A) Progesterone production of MA-10 subclones with hCG production expressed as a percentage of progesterone production with Bt_2cAMP stimulation. Heatmap comparison of progesterone production by individual MA-10 subclones after stimulation with either Bt_2cAMP or hCG for six hours in DMEM-FBS. **(C)** Representative images of MA-10 subclone cultures.

A

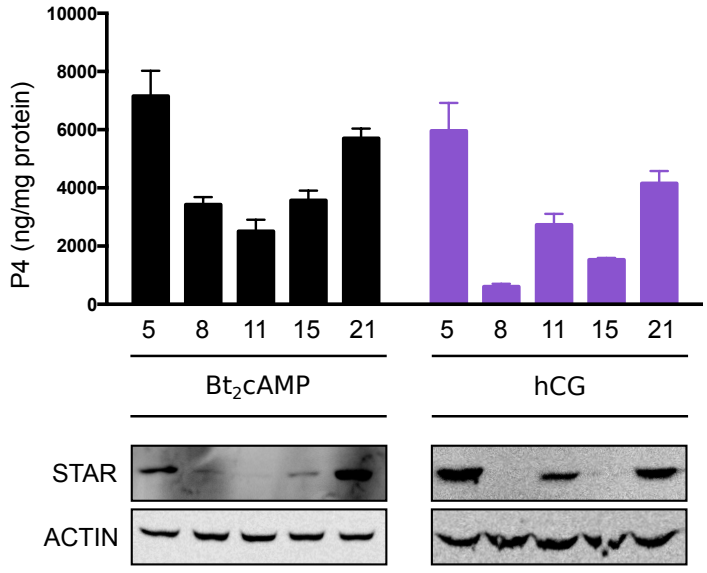

Figure 4.3 hCG treatment induces STAR expression in MA-10 subclones. (A) MA-10 subclones are heterogeneous in progesterone production and STAR expression in response to Bt_2cAMP and hCG stimulation for six hours in DMEM-FBS. STAR expression correlates with progesterone production. Progesterone is normalized to total protein content. **(B)** Chromosome count of MA-10 subclones vary in number and average between tetraploid and pentaploid.

B

	5	8	11	15	21
Median	84	84	101	90.5	86.5
Average	85.9	84.2	100.3	90.1	85.2
Range	76-99	73-95	95-109	82-96	74-96

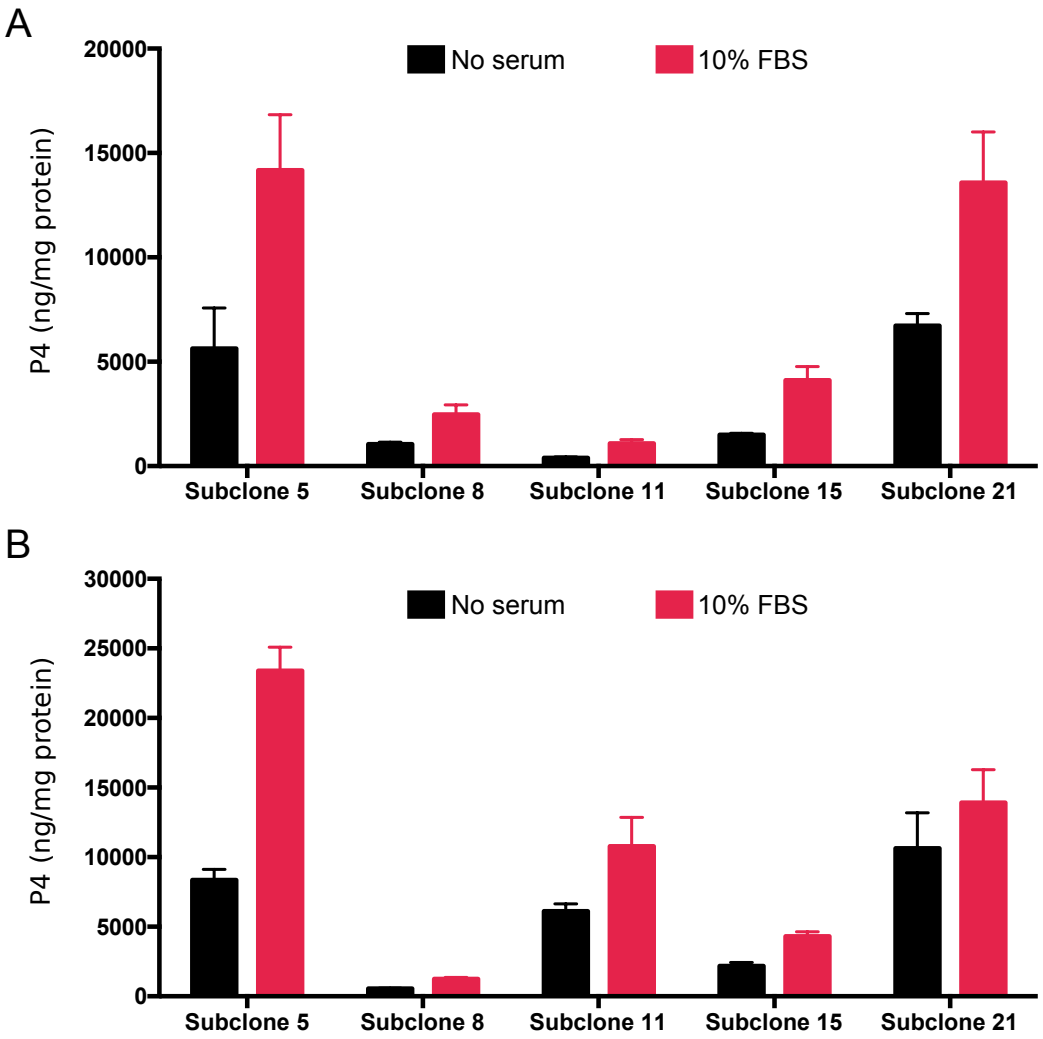

Figure 4.4 Addition of serum increases progesterone production MA-10 subclones in response to both Bt_2cAMP and hCG. (A) MA-10 subclones stimulated with Bt_2cAMP and **(B)** hCG for six hours show increased progesterone production when treatment medium is supplemented with 10% FBS.

response to stimulation. Stimulation with hCG was also able to induce both STAR expression and progesterone production in MA-10 subclones (Figure 4.3A). MA-10 subclones are also highly heterogeneous in chromosome number and range from 73-109 chromosomes per cell and average 84.2-100.3 chromosomes per cell (Figure 4.3B).

FBS enhances progesterone production in MA-10 subclones

Addition of 10% fetal bovine serum to treatment media enhanced progesterone production in MA-10 subclones both in response to stimulation with Bt_2cAMP (Figure 4.4A) as well as in response to hCG (Figure 4.4B).

Stimulation with hCG depletes neutral lipid stores in MA-10 subclones

A key characteristic of steroidogenic cells are their many lipid stores, a characteristic also observed via oil red O staining in the MA-10 subclones. Stimulation with hCG for three hours, inducing progesterone production, depletes intracellular neutral lipid stores. This reduction is much more dramatic in cells stimulated in the absence of serum but is still present in cells with access to serum (Figure 4.5A). Representative images show the reduction in oil red O staining in hCG-treated cells (Figure 4.5B).

Discussion

Studies of cholesterol trafficking mechanisms have traditionally placed steroidogenic cells in serum-free conditions, eliminating the possibility of extracellular lipoprotein particles as a source of cholesterol substrate of steroidogenesis. In this study, we demonstrate that addition of serum, and thus lipoproteins, to treatment media can and does provide a substantial source of cholesterol for steroidogenesis and suggests that the presence of these proteins may be important to uncovering the physiological role of extracellular cholesterol in steroidogenesis.

One of the reasons that serum-free medium was favored in earlier studies was due to the inability for the LH to stimulate steroidogenesis in primary bovine luteal cells cultured in serum-supplemented medium (34). A later publication by the same group,

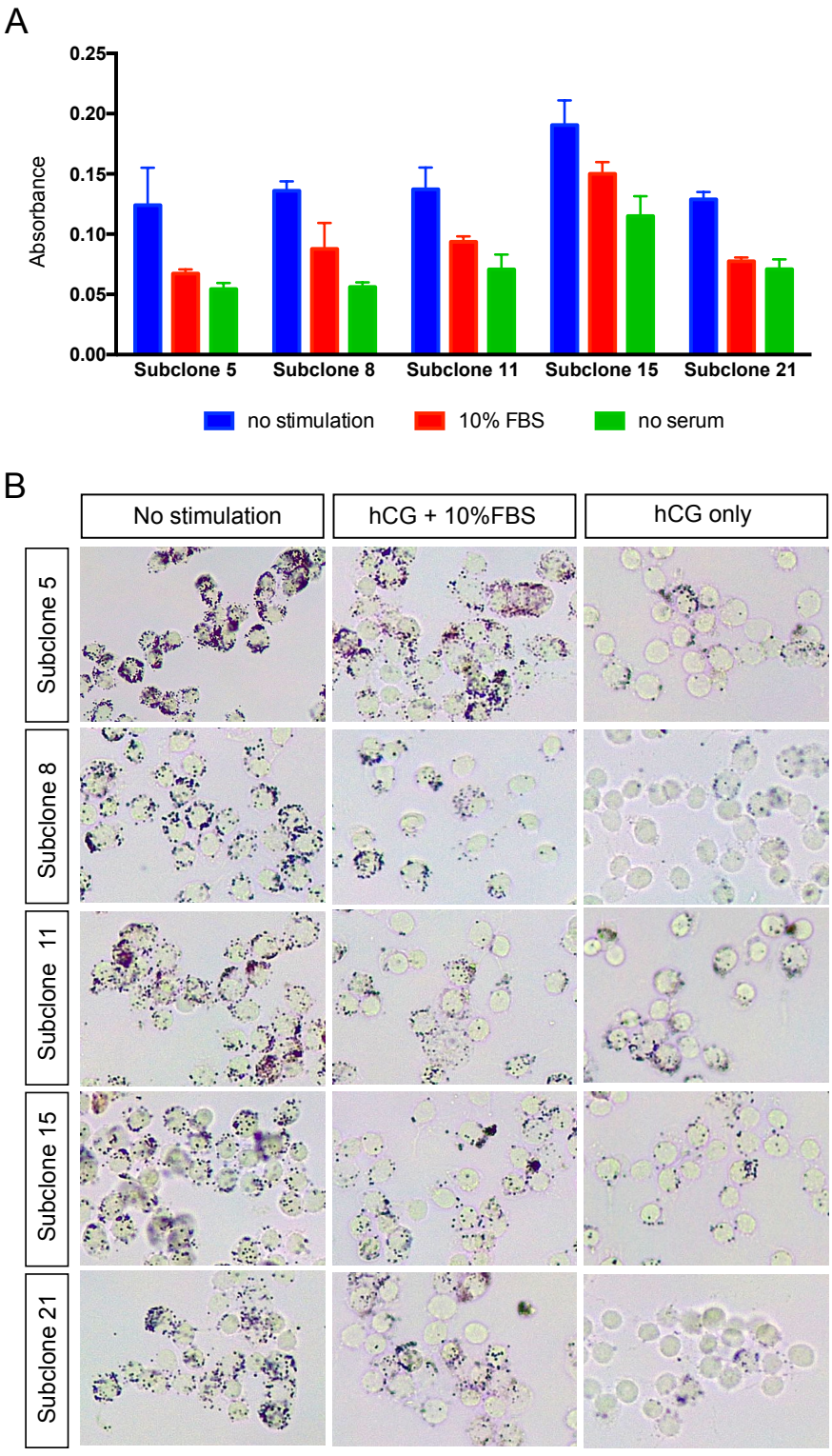

Figure 4.5 Steroidogenesis depletes neutral lipid stores in MA-10 subclones. (A) Oil Red O staining of MA-10 subclones stimulated with hCG for three hours reveal higher intracellular neutral lipid content in cells in serum-supplemented media compared to those in serum-free media. **(B)** Representative images of Oil red O staining in unstimulated and hCG stimulated cells in serum-free or serum-supplemented media.

however, showed that this inhibition was due to the presence of amphotericin-B as a supplement in the growth medium,

which inhibited steroid synthesis due to its cholesterol-binding activity. In addition to the removal of amphotericin-B which restored LH-stimulated steroidogenesis, addition of insulin to the culture medium provided even further increases in progesterone production (38). This highlights the importance of designing an ideal cell culture system for steroidogenic cultures in order to obtain reliable, reproducible results. These culture systems, however, can lack physiological conditions, leading to conflicting results and lack of reproducibility and consistency among similar experiments (38).

Standard cell culture conditions include 10% fetal bovine serum and supplements such as penicillin and streptomycin. While these are standard growth conditions, serum is typically removed upon stimulation using LH or cell-permeable pathway intermediates such as Bt_2cAMP (42,45). While culture conditions for primary granulosa or luteal cells have been more carefully examined as mentioned above, this has not yet been done for other steroidogenic cell types such as the Leydig cell.

The use of testicular interstitial tumor derived MA-10 cells (40,44,46), a popular steroidogenesis model, was key in identifying the existence and role of steroidogenic acute regulatory protein in steroidogenesis (14,45). While MA-10 cells are maintained long term in 10% fetal bovine serum, varying amounts of progesterone in serum provided an unwanted variable in measurements of steroid output, leading to the current standard practice of stimulating acute steroidogenesis in serum-free conditions, rather than in the standard growth medium (42,45,47). In addition to the use of serum-free treatment media,

declining expression of LH receptor in MA-10 cells (48) has resulted in the use of Bt_2cAMP as the primary mode of stimulation. While Bt_2cAMP is highly efficient in stimulating steroidogenesis in a variety of cell types, it is also a signal for many other cellular processes, including lipolysis in adipocytes (49).

While studies on mitochondrial cholesterol import did not consider the relevance of upstream events, experimental evidence solidifies STAR's place as the key cholesterol transporter of the mitochondria (23,50). As we continue to search beyond the mitochondria, however, these considerations may become crucial. Recent studies have proposed that mitochondria and endoplasmic reticulum (ER) interactions by a physical association between the two organelles called MAMs (mitochondria-associated membranes) could assemble a conduit for cholesterol (51,52). While associations between ER and mitochondria are well-documented, lack of serum lipoproteins during stimulation eliminates a key source of steroidogenic cholesterol and could potentially result in an overestimation of contributions made by *de novo* synthesized cholesterol from the ER.

Evidence that hormone sensitive lipase (HSL) that mediates mobilization of cholesterol from lipid droplets has been demonstrated in several studies and deletion of HSL in mice results in a dramatic reduction in steroid production by adrenal cells (30) (31), suggesting that HSL hydrolysis of cholesterol esters may play a role in providing cholesterol for steroidogenesis. Consistent with this idea, we observed that hCG stimulation reduced neutral lipid stores in MA-10 cells regardless of treatment medium but cells treated in the presence of serum were significantly less depleted compared to

cells stimulated in serum-free conditions. This data indicates that steroidogenesis depletes cellular cholesterol stores and presence of serum can modulate this depletion, potentially either by directly providing cholesterol for steroidogenesis or by replenishing lipid droplet stores as they become depleted.

In summary, previous literature and culture conditions have allowed for the elucidation of mitochondrial transport mechanisms but going forward, careful considerations need to be taken in order to create appropriate conditions for the study of cholesterol trafficking and sourcing for the steroidogenic process.

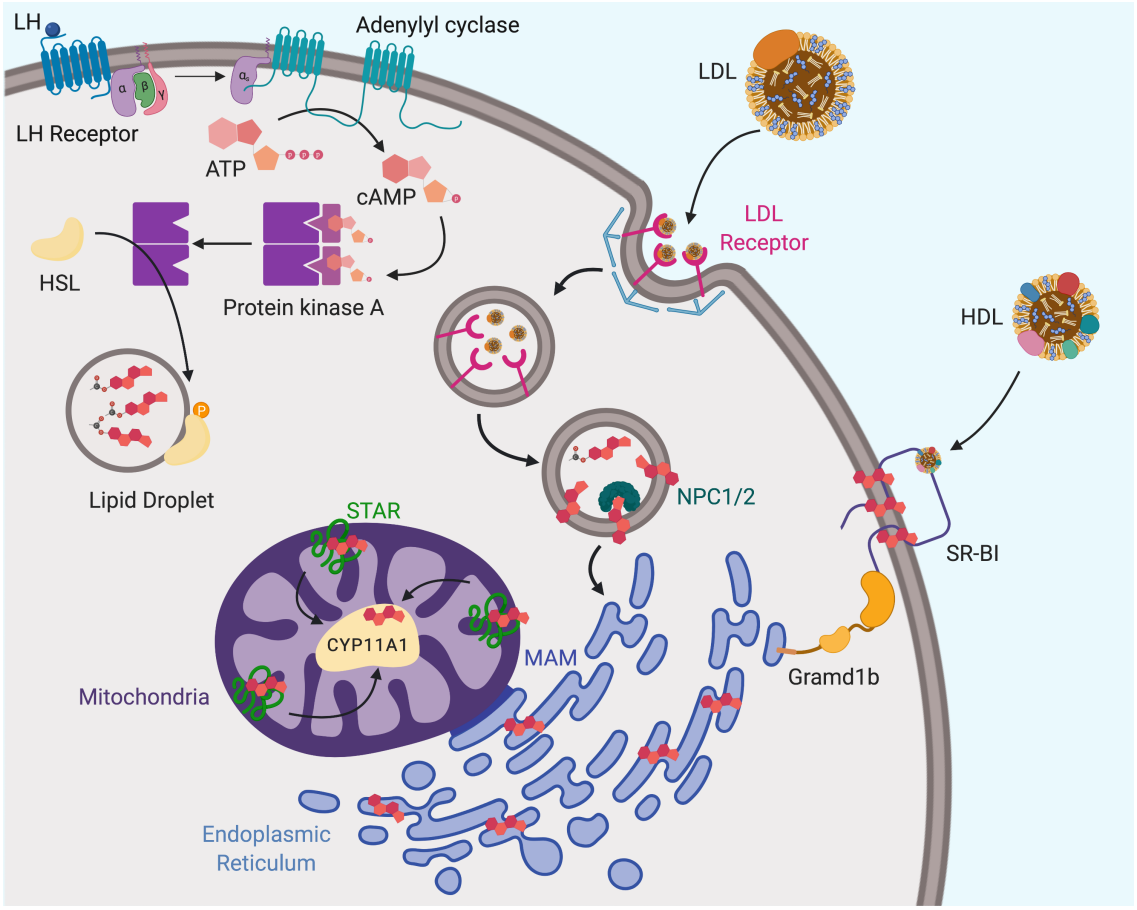

Figure 4.6 Current model of cholesterol acquisition pathways in steroidogenesis. Several distinct sources of cholesterol have been demonstrated to contribute to the cholesterol substrate pool for steroidogenesis—intracellular stores, such as lipid droplets or plasma membrane cholesterol, *de novo* cholesterol synthesis in the ER, and extracellular sources, such as serum lipoproteins. LH/hCG binding to the LH G-protein coupled receptor (top left) activates adenylyl cyclase, prompting the synthesis of cyclic-AMP (cAMP) from ATP. cAMP binds to regulatory subunits of protein kinase A (PKA), releasing its catalytic subunit. Literature has shown that PKA is capable of phosphorylating hormone sensitive lipase (HSL), cleave cholesterol esters, releasing free cholesterol (31,53). Steroidogenic cells also have access to serum lipoproteins, low density lipoprotein (LDL) and high density lipoprotein (HDL). LDL binds to LDL receptor on the plasma membrane and is internalized via clathrin-mediated endocytosis (54). In the lysosome, LDL particles are released from the receptor, cholesterol esters are cleaved by lysosomal acid lipase (55), and trafficked out of the endosomal compartment by NPC1/NPC2 (56). HDL binds to the scavenger receptor-BI (SR-BI) on the plasma membrane and deposits cholesterol at the membrane which can be internalized by ER-tethered ASTER proteins such as Gramd1b (57). *De novo* synthesized cholesterol can be transported to the mitochondria via mitochondria-associated membranes (MAMs) (51,52), specific ER-mitochondria contact sites that form a conduit for the trafficking of free cholesterol. Finally, at the level of the mitochondria, STAR binds the incoming cholesterol and transports it to the CYP11A1 enzyme where it is converted to pregnenolone, the first steroid precursor of the steroidogenic pathway (58).

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CHAPTER 5

EFFECTS OF ENDOPLASMIC RETICULUM CHOLESTEROL CONTENT ON STEROIDOGENIC ACUTE REGULATORY PROTEIN EXPRESSION IN STEROIDOGENIC CELLS

Abstract

Acute steroidogenesis in response to stimulation by tropic hormones requires rapid changes in cholesterol trafficking and homeostasis within steroidogenic cells, such as Leydig and adrenocortical cells. One of the major regulators of whole cell cholesterol homeostasis are the sterol regulatory element-binding proteins (SREBPs), located in the endoplasmic reticulum. When the ER is cholesterol-depleted, the SREBPs translocate to the nucleus, binding sterol regulatory elements (SREs), a consensus sequence found in the promoters of lipid synthesis and acquisition genes. Previous work identified five SRE binding sites on the promoter of the steroidogenic acute regulatory protein, which facilitates the essential transport of cholesterol to the inner mitochondrial membrane. We hypothesized that STAR may be a candidate for regulation by the members of the SREBP transcription factor family. We observed that addition of statins, inhibitors of HMG coA reductase, to MA-10 cells stimulated with dibutyryl-cyclic AMP resulted in a dramatic increase in progesterone production and a simultaneous increase in the expression of the steroidogenic acute regulatory protein (STAR). This increased STAR expression was also observed in human adrenocortical H295R cells.

Introduction

Steroid hormones are essential endocrine signals produced from cholesterol in the mitochondria in response to acute stimulation by tropic hormones such as ACTH or LH. The rate-limiting step of the steroidogenic process is the transfer of cholesterol across the two mitochondrial membranes to the CYP11A1 enzyme, which converts cholesterol to pregnenolone and commits it to the steroidogenic pathway. This process is regulated by the steroidogenic acute regulatory protein (STAR), an essential 30kDa mitochondrial protein that is newly synthesized in response to acute stimulation by tropic hormones or artificial pathway intermediates such as dibutyryl-cyclic AMP (Bt_2cAMP) (1,2). STAR is essential to maintain steroidogenic function but the exact factors that regulate its transcription remain unclear.

Studies focused on the factors regulating the STAR promoter have uncovered several transcription factors and their binding sites on the STAR promoter, including cAMP response element binding protein (CREB), GATA-4, JunB-Fos, AP1, SF-1, and others (3). Among the factors proposed to bind to the STAR promoter and facilitate transcription are the sterol-response element binding proteins (SREBPs). The SREBP proteins consist of two genes, *SREBP1* and *SREBP2*, which in turn produce three proteins, SREBP-1a and SREBP-1c, which are isoforms of the *SREBP1* gene, and SREBP2, encoded by the *SREBP2* gene (4,5). The SREBP proteins are ~120kDa proteins and consist of an N-terminal transcription factor domain and, at its C-terminal, a regulatory domain that interacts with the C-terminal of the SREBP cleavage activating protein (SCAP) (6,7). SCAP's N-terminal interacts with Insig proteins, which, when bound

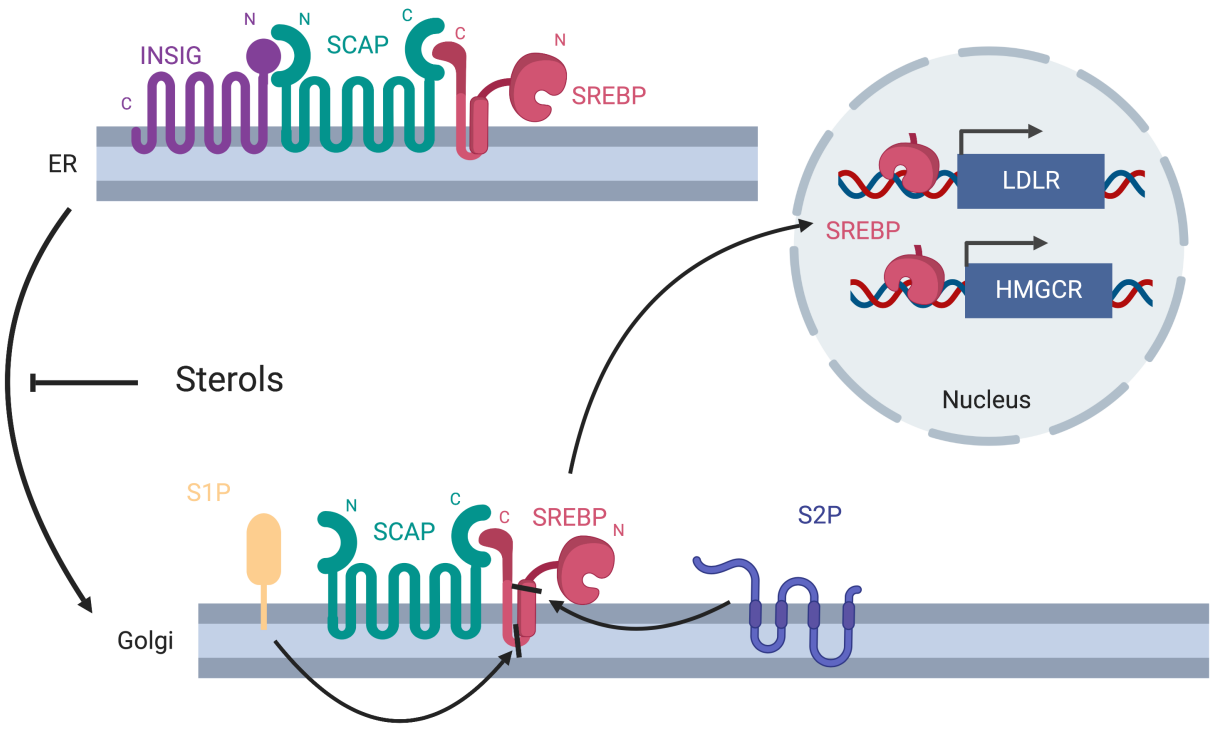


Figure 5.1 Mechanism of SREBP control of cellular cholesterol homeostasis. The sterol-regulatory binding proteins (SREBPs) are ER-bound, transmembrane proteins that contain a basic helix-loop-helix transcription factor domain at its N-terminus and interact with the SREBP cleavage activating protein (SCAP) at its C-terminus. SCAP interacts with Insig1/2, which, in high sterol conditions act to retain the SCAP/SREBP complex in the ER. In low sterol conditions, SCAP undergoes conformational changes which causes it to dissociate from Insig1/2 and be sorted, along with its bound SREBP, to the Golgi, where SREBP is cleavage by the Site 1 Protease (S1P) and Site 2 Protease (S2P), releasing the N-terminal transcription factor domain into the cytosol. This domain travels to the nucleus and binds to cholesterol synthesis and acquisition gene promoters, such as LDL receptor and HMG co-A reductase, facilitating their transcription and increased protein expression. This, in turn, raises the total cholesterol content of the cell.

to sterols, anchor the SREBP proteins in the ER. When the ER becomes sterol depleted, the SREBP-SCAP complex is released from Insig and is trafficked to the Golgi where the SREBP protein is cleaved by site 1 protease (S1P) and site 2 protease (S2P) (8,9). This releases its 60kDa N-terminus, a member of the basic helix-loop-helix leucine zipper transcription factor family, which translocates to the nucleus and binds to sterol regulatory elements (SRE) found in the promoters of lipid synthesis of acquisition genes, promoting their transcription. SREBP-1c primarily promotes transcription of fatty acid synthesis genes and SREBP-2 primarily works on cholesterol synthesis and acquisition proteins, the enzymes of the cholesterol synthesis pathway and LDL receptor. SREBP-1a promotes transcription of both fatty acid and cholesterol synthesis genes (10). A schematic of these mechanisms can be found in Figure 5.1.

Previous work has suggested that the promoter of STAR contains up to five SRE sites and luciferase assays demonstrate increased STAR promoter activity in cells overexpressing SREBP. In addition, treatment of MA-10 cells with statins, a class of HMG-coA reductase inhibitors that inhibits cholesterol synthesis, thus lowering the ER cholesterol content, also resulted in increased STAR promoter activity in these assays (11,12). We hypothesized that the SREBP/SCAP system may play a role in the acute regulation of STAR expression and regulate whole cell homeostasis in response to a sudden increase in demand for cholesterol substrate.

Materials and Methods

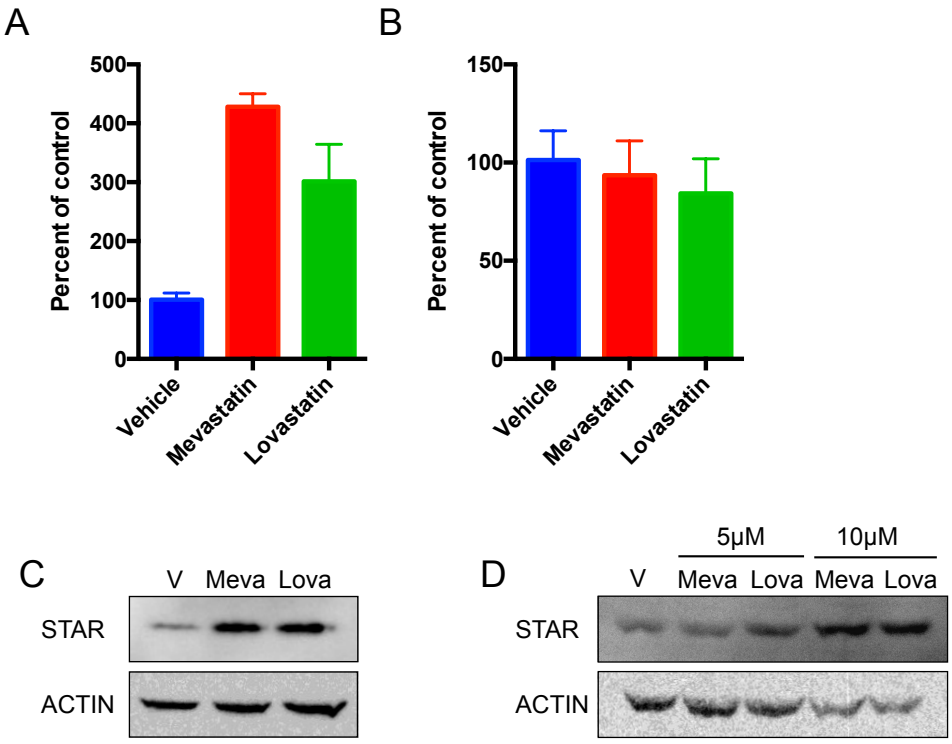
Cell culture

MA-10 cells were cultured in DMEM Hi-glucose (Sigma) supplemented with 10% fetal bovine serum (Invitrogen) and 1% penicillin-streptomycin.

Treatments and hormone assays. For all experiments, MA-10 cells were plated at 5 x 10^4 cells per well in a 0.1% gelatin-coated 96-well plate and allowed to attach overnight. Steroidogenesis was induced for 6 hours for experiments using 0.5mM Bt2cAMP or 1.5IU/mL hCG. Statins were added treatment medium (DMEM) at 5μM simultaneously with stimulation. Cell culture supernatant was collected for quantification of progesterone by radioimmunoassay as previously described in Chapter 3. Progesterone values were normalized to total protein content in each well. Experiments were repeated as four independent trials.

Immunoblots

Cells were collected in SDS buffer containing protease inhibitors (Sigma). Proteins were separated by SDS-PAGE, transferred to a PVDF membrane and immunoblotted using an affinity purified rabbit polyclonal antibody against STAR amino acids 93-105, a rabbit polyclonal antibody for SREBP-2 (Abclonal) and mouse monoclonal antibody against FLAG (Sigma). Membranes were also blotted using a monoclonal mouse antibody for β-Actin (Santa Cruz) as a loading control. Detection of TSPO and STAR was using a (Poly-HRP) secondary antibody and Actin using IRDye800 (LiCor) labeled secondary antibodies using quantitative imager (C600, Azure Biosystems).



Results

Figure 5.2 Statins increase progesterone production and STAR expression in MA-10 and H295R cells. (A) MA-10 cells treated with Bt_2cAMP for three hours in DMEM show significant increases in progesterone production in response to both mevastatin and lovastatin. **(B)** These effects were not observed with 22(R)-hydroxycholesterol treatment, suggesting that cholesterol transport into the mitochondria may play a role. **(C)** Treatment with both mevastatin (Meva) and lovastatin (Lova) result in increased STAR expression compared to vehicle control (V) in MA-10 cells stimulated with Bt_2cAMP. **(D)** Similar effects were observed in the H295R human adrenocortical cell line.

Statins increase steroidogenesis production by increasing STAR expression

MA-10 cells stimulated with Bt_2cAMP in the presence of 5μM mevastatin or lovastatin show dramatic increase in progesterone production compared to vehicle only controls, an effect that was not observed in 22(R)-hydroxycholesterol-treated cells (Figure 5.2A). Addition of mevastatin and lovastatin to treatment media of MA-10 cells stimulated with

Bt_2cAMP results in increased STAR expression in MA-10 and H295R cells (Figure 5.2B-C)

Discussion

In this study, we hypothesized that the SREBP proteins may act as transcription factors to control STAR transcription in response to changes in cholesterol homeostasis. We observed a dramatic increase in progesterone production by MA-10 stimulated with Bt_2cAMP in the presence of either mevastatin or lovastatin. This presence was not present when, instead of Bt_2cAMP, 22(R)-hydroxycholesterol, a freely soluble form of cholesterol, was used, suggesting that the addition of statin increased the availability of cholesterol to the CYP11A1 enzyme, rather than changes in enzyme activity or availability. The rate-limiting step of steroidogenesis is the transport of cholesterol to the inner mitochondrial membrane (13), facilitate by the steroidogenic acute regulatory protein (STAR). Western blots show that STAR is upregulated in MA-10 cells that are treated with statins compared to those that only receive the Bt_2cAMP stimulation. This is also observed in statin-treated H295R cells, a human adrenocortical cell line, suggesting these observed changes may be amongst several different types of steroidogenic cells.

Statins act to reduce cholesterol synthesis by competitively inhibiting HMG-coA reductase, resulting in a lowering of ER cholesterol content and activation of SREBP-2 (14). Previous literature identifies the presence of five SRE sites on the STAR promoter within 1000bp upstream of the start codon (12), suggesting regulation of STAR expression by SREBP may be possible.

It is worth noting that in patients, some experimental evidence has shown that statin use in male patients results in mild reductions in testosterone levels (15,16). While

it seems counterintuitive, this is not entirely unexpected in the patient context. Although all cells in the body make cholesterol, statins primarily work to prevent coronary heart disease and atherosclerosis by inhibiting synthesis by the liver, thus activating the SREBP/SCAP system and increasing the number of LDL receptors on the plasma membrane. This increases uptake of LDL by the liver, lowering the total plasma cholesterol (17). Given extensive evidence from other groups and from the previous chapter of this dissertation, we hypothesize this mild reduction may be due to the presence of statins combined with the lower availability of circulating serum lipoproteins which provides cholesterol as substrate for steroidogenesis. This condition is not recreated in our culture conditions as statins and Bt_2cAMP stimulation are provided in medium containing 10% FBS.

Overall, the data show that statin treatment in MA-10 cells results in increased progesterone production and that this can be explained by increases in STAR expression, which we also were able to observe in the H295R adrenocortical cell line.

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CHAPTER 6

CONCLUSIONS AND FUTURE DIRECTIONS

CONCLUSIONS AND FUTURE DIRECTIONS

Cholesterol is a unique substance, both essential to cellular function and yet difficult to transport due to its extreme hydrophobicity. It is a key component of cellular membranes and also acts as a substrate in multiple key physiological processes. In this work, we aimed to define the key players in mitochondrial cholesterol trafficking in the context of steroidogenesis, the various sources of cholesterol that act as substrate for steroidogenesis, and the intracellular factors that influence steroidogenesis.

19-atriol inhibits steroidogenesis through 3β-HSD and CYP11A1

19-atriol inhibition of steroidogenesis was proposed to act through TSPO, an outer mitochondrial membrane protein implicated as a key player in transport of cholesterol into the mitochondria. Chapter 2 of my study conclusively demonstrates that 19-atriol inhibition of steroidogenesis is completely independent of TSPO expression but is instead mediated by 19-atriols action on 3β-HSD and CYP11A1. In addition, my study identifies 19-atriol as a substrate for 3β-HSD and shows that it is converted to 19-hydroxytestosterone. Both compounds, 19-atriol and 19-hydroxytestosterone, prevent pregnenolone synthesis, suggesting that they can act to inhibit CYP11A1. This study provides additional evidence removeing TSPO from consideration as a key player in mitochondrial cholesterol transport and identifies 19-atriol as a novel pharmacological ligand for inhibition of steroidogenic enzymes.

Implication of 19-atriol as a research tool to study the role of allopregnanolone synthesis in glaucoma is currently of interest and elucidation of its mechanisms of action points allows for the continued examination of the role of steroids in glaucoma without the

consideration of TSPO inhibition as a potential mechanism. In addition, more experimentation can be performed to examine TSPO's true function as a mitochondrial protein. TSPO has been shown to bind porphyrins and continued study of the heme pathway in relation to TSPO may be able to identify its true function.

STAR is the essential mitochondrial cholesterol transporter in steroidogenesis

Chapter 3 defines the characterization of the first STAR-deleted steroidogenic cell line and demonstrates that STAR is essential to the steroidogenic process. MA-10 cells are highly heterogeneous, and so in order to create this cell line, sixteen subclones were generated and characterized before one was chosen for STAR deletion via CRISPR/Cas9. This study demonstrates that STAR deletion results in a complete loss of steroidogenesis despite intact downstream steroidogenic enzymes. In addition, the abundant production of progesterone with 22(R)-hydroxycholesterol treatment demonstrates that the inability of STAR-deleted cells to synthesize progesterone is due to the inability for cholesterol to cross the intermembrane space.

Using these models, we are now able to determine the exact location and mechanism of action of the STAR protein. Previous records have indicated that STAR acts at the outer membrane and can also maintain full function without its mitochondrial targeting signal but this is in conflict with data from *in vivo* models[1,2]. Using the STAR-deleted cells I generated, we are able to express various mutant forms of STAR in the proper cell type without interference from endogenous STAR and examine the functionality of these various mutants.

In addition, this model can be used to search for other proteins that may be redundant in function. This area is of particular interest in human medicine due to the lack

of STAR expression in the human placenta despite its ability to synthesize progesterone. Previous reports have suggested STARD3/MLN64 as a candidate protein and this model can be used to test if STARD3 can, in fact, perform this function.

Various sources of cholesterol contribute to pool of steroidogenic substrate

I looked to identify the extramitochondrial factors that influence steroidogenesis and the origin of that cholesterol substrate. In this study, I demonstrate that extracellular sources of cholesterol, such as serum lipoproteins, are able to contribute to the pool of cholesterol that ultimately becomes progesterone in MA-10 cells. In order to more closely study the physiological processes of cholesterol trafficking and avoid usage of cyclic AMP for stimulation, I characterized several MA-10 cell subclones that are able to respond to hCG stimulation and these also showed increased steroidogenic output in response to supplementation with lipoprotein containing serum, consistent with data from Bt_2cAMP-stimulated MA-10 cells. I also provide evidence that intracellular lipid pools such as cholesterol-ester-rich lipid droplets, may also contribute to steroidogenic cholesterol substrate, suggesting a complex variety of sources that steroidogenic cells drawn from in response to stimulation.

Inhibition of ER cholesterol synthesis increases progesterone output

In Chapter 5, I reported that treatment of MA-10 cells with HMG co-A reductase inhibiting statins resulted in the unexpected increase in progesterone synthesis, a result that is seemingly contradictory to our current knowledge about the role of *de novo* cholesterol synthesis in steroidogenesis. My data indicate that this increase is only present in Bt_2cAMP-stimulated cells and not in 22(R)-hydroxycholesterol-treated cells, suggesting that the ability to cross the mitochondrial membranes is key to this observed

increase. We know from data in CHAPTER 3 that this role is facilitated by the STAR protein and consistent with the importance of cholesterol transport for increased steroidogenesis. I observed an increase in STAR expression in statin-treated MA-10 cells. I was also able to observe this increase in STAR expression in the H295R human adrenocortical cell line, suggesting that the mechanisms controlling this change in protein expression may be conserved across species and cell types.

From the literature, we know that statins lower ER cholesterol levels, resulting in the migration and cleavage of the SREBP transcription factors which promote the transcription of cholesterol acquisition and synthesis genes. STAR has five predicted-SRE sites within 1000 base pairs upstream of its transcription start site and I hypothesize that STAR may be among the downstream genes regulated by SREBP. Moving forward, we can express the transcription factor domains of SREBP proteins and characterize the resulting STAR expression and progesterone synthesis in response to hCG-stimulation.

In addition to SREBP, there is also a potential role for SCAP as a regulator of steroidogenesis. SCAP has an identified phosphorylation site at serine821 and given the abundant knowledge of the role of protein kinase A activation in response to hCG, I would like to examine if SCAP phosphorylation plays a role in STAR expression by expressing a dominant negative S821A mutation of SCAP, eliminating the phosphorylation site.

References

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