# Chapter 4
# Congenital Lipoid Adrenal Hyperplasia

**Walter L. Miller**

**Abstract** All steroid hormones are made from cholesterol, which is primarily taken up by steroidogenic cells from circulating lipoproteins. The intracellular mechanisms by which cholesterol is delivered to cellular destinations remains under investigation. Steroidogenic cells must transport large amounts of cholesterol to, and then into, the mitochondria, where the cholesterol side-chain cleavage enzyme, P450scc, resides. P450scc is the first enzyme in steroidogenesis, converting insoluble cholesterol to soluble pregnenolone. The combination of genetic studies of a rare disease, congenital lipoid adrenal hyperplasia (lipoid CAH), and studies of the cell biology of mitochondrial cholesterol import led to the discovery of the steroidogenic acute regulatory protein (StAR). StAR acts exclusively on the outer mitochondrial membrane to trigger mitochondrial cholesterol import, but the precise mechanism of its action remains unclear. Lipoid CAH is caused by StAR mutations and is thus the StAR knockout experiment of nature. Recent work has shown that P450scc mutations cause a disease that is a phenocopy of lipoid CAH. The discovery of StAR led to the discovery of the broader family of structurally related StAR-related lipid transfer (START)-domain proteins. It appears that some of these START proteins may also be involved in intracellular cholesterol trafficking upstream from StAR.

**Abbreviations**

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| 3βHSD | 3β-hydroxysteroid dehydrogenase |
| ACAT | acyl-coenzyme A cholesteroacyl transferase |
| ACTH | adrenocorticotropic hormone |
| ANT | adenine nucleotide |
| CRAC | cholesterol recognition amino acid consensus domain |
| ER | endoplasmic reticulum |
| FAD | flavin adenine dinucleotide |
| HDL | high density lipoproteins |
| HSL | hormone-sensitive neutral lipase |
| HMGCoA | 3-hydroxy-3-methylglutaryl co-enzyme A |

W. L. Miller, M.D. (✉)
Department of Pediatrics, University of California San Francisco,
San Francisco, CA 94143-1346, USA
e-mail: wlmlab@ucsf.edu

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| IMM | inner mitochondrial membrane |
| IMS | intramembranous space |
| Km | Michaelis constant |
| LAL | lysosomal acid lipase |
| LDL | low-density lipoproteins |
| LH | luteinizing hormone |
| MENTAL | MLN64 N-terminal |
| MENTHO | MLN64 N-terminal domain homologue |
| MLN64 | metastatic lymph node clone 64 |
| NADPH | nicotinamide adenine dinucleotide phosphate |
| NPC | Niemann-Pick type C |
| OMM | outer mitochondrial membrane |
| PAP7 | TSPO-associated protein 7 (ACBD3) |
| PBR | peripheral benzodiazepine receptor |
| PCP | phosphate carrier protein |
| PKA | protein kinase A |
| PKAR1A | protein kinase A regulatory subunit 1α |
| PRAX1 | TSPO-associated protein 1 |
| P450scc | mitochondrial cytochrome P450 specific for cholesterol side-chain cleavage |
| SF1 | steroidogenic factor 1 |
| SOAT | sterol O-acetyltransferase |
| SR-B1 | scavenger receptor B1 |
| StAR | steroidogenic acute regulatory protein |
| START | StAR-related lipid transfer domain |
| SREBPs | sterol regulatory element binding proteins |
| TSPO | 18 kDa translocator protein, |
| VDAC1 | voltage-dependent anion channel |

## Introduction: A Personal Historical Perspective on Lipoid CAH and StAR

Congenital lipoid adrenal hyperplasia (lipoid CAH) provides a perfect example of how study of a rare disease combined with contemporary biochemistry and genetics can open the door to an important new area of biology: the StAR-related lipid transfer domain (START) proteins. Early autopsy reports [1–4; Fig. 4.1] presaged the description of lipoid CAH as an inherited endocrine disorder, characterized by grossly enlarged, lipid-filled adrenals, disordered sexual development, and an apparent lack of adrenal steroidogenesis [5–7]. At that time, the enzymology of the conversion of cholesterol to pregnenolone was thought to require three enzymes, a 20α-hydroxylase, a 22-hydroxylase, and a 20, 22 “desmolase” [8]. Hence, the first case reported in English postulated that the disorder involved such an enzyme and

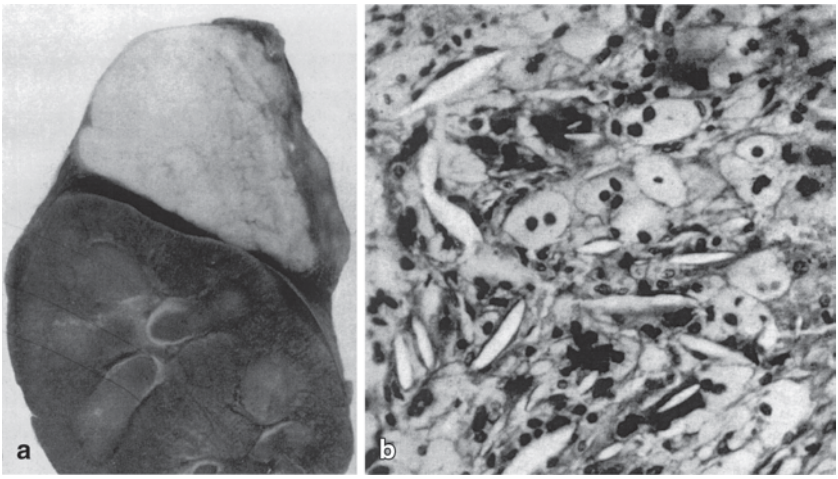


**Fig. 4.1** Congenital lipoid adrenal hyperplasia (Lipoid CAH) from the 1955 report by Sandison. *Left*: Section through the autopsied kidney and adrenal of the 3 month female with probable lipoid CAH. *Right*: Histologic section stained with Hematoxolin and Eosin at 280× magnification, showing "small rather spindle-shaped cholesterol crystal clefts" (*Reproduced from Sandison AT. 1955. A form of lipoidosis of the adrenal cortex in an infant. Arch Dis Childh 30: 538–541, with permission from BMJ Publishing Group, Ltd.*)

named it "20, 22 desmolase deficiency" [9]. The first study of lipoid CAH in vitro found that mitochondria from affected tissue could convert 20α-hydroxycholesterol to pregnenolone, but not cholesterol to pregnenolone, suggesting that the defect was in a specific cholesterol 20α-hydroxylase [10]. Although this conclusion was incorrect, the experimental design was prescient: 23 years later, we used a similar approach to show that lipoid CAH was due to a mutation in the steroidogenic acute regulatory protein, StAR [11].

Advances in protein chemistry and the rapidly developing understanding of cytochrome P450 enzymes permitted the demonstration that cholesterol was converted to pregnenolone by a single mitochondrial cytochrome P450 enzyme, termed P450scc (where scc denotes cholesterol side chain cleavage; [12–14]. Others confirmed that lipoid CAH mitochondria could not convert cholesterol to pregnenolone but could not explain the efficacy of 20α-hydroxycholesterol as a substrate as reported by Degenhart [10]. Semi-quantitative CO-induced difference spectra indicated that lipoid CAH mitochondria had about half as much total P450 as control mitochondria, but had normal 11-hydroxylase activity, which was known to be catalyzed by a mitochondrial P450. Thus, this report concluded that lipoid CAH was caused by an absence of cytochrome P450scc [15], which remained the accepted explanation for this disease until 1991.

I first became aware of lipoid CAH in 1977 during my clinical fellowship in pediatric endocrinology, while taking care of a child with this disorder; several years later, I helped Bert Hauffa prepare a clinical report of this UCSF patient, reviewing the 34 cases then reported [16]. At that time I was developing a lab devoted to

studying steroidogenesis, so I added lipoid CAH to the list of diseases we should try to "solve." Our cloning of human P450scc [17] permitted us to make the first, halting efforts at genetic studies of lipoid CAH with genomic deoxyribonucleic acid (DNA) from our UCSF patient [16]. We found no gene deletions [18], and with Ken Morohashi's cloning of the corresponding gene [19], we found normal P450scc exonic sequences. In collaboration with Paul Sanger, who was taking care of an affected family in New York, we obtained small amounts of affected testicular tissue from which we made ribonucleic acid (RNA), and found normal amounts of messenger RNA (mRNA) for P450scc and its electron-donating redox partners in affected gonadal tissue [20]. Thus, lipoid CAH was not due to a mutation in the enzyme system converting cholesterol to pregnenolone, suggesting a lesion in an upstream factor that delivered cholesterol to the mitochondria. The factors involved in this process were not understood at that time, and examination of then-contemporary candidates (sterol carrier protein 2, endozepine, GRP-78) did not yield an answer [20]. The work on endozapine by Hall and Shively [21] led us to the peripheral benzodiazepine receptor (PBR, now called mitochondrial transporter protein, TSPO), but in collaboration with Jerome F. Strauss 3rd (then at the University of Pennsylvania) we cloned this gene and found it was not mutated in lipoid CAH [22].

The factor that was missing in lipoid CAH had to have the three characteristics of the postulated "acute regulator" of steroidogenesis: (i) rapid inducibility by cyclic adenosine monophosphate (cAMP), (ii) short half-life evidenced by cycloheximide sensitivity, and (iii) capacity to induce steroidogenesis [23]. The work of Orme-Johnson [24–26] and Stocco [27] identified a family of peptides, termed pp30, pp32, and pp37 that appeared to have many of these properties. Barbara Clark in Doug Stocco's lab purified one of these, obtained a partial amino acid (AA) sequence, cloned it from mouse Leydig MA-10 cells, and found that its expression in MA-10 cells promoted steroidogenesis: the "steroidogenic acute regulatory protein," StAR, had been discovered [28].

Doug Stocco, Jerry Strauss, and my lab quickly joined forces. We had noted that placental steroidogenesis remained intact in lipoid CAH, indicating that the responsible gene had to be expressed in the adrenals and gonads, but not in the placenta [29]. I remember discussing this with Jerry on the phone in the summer of 1994 when he told me that Teruo Sugawara's northern blots showed that StAR was expressed in the adrenals and gonads but not in the placenta. We immediately concluded that StAR was the likely cause of lipoid CAH. Teruo and Jerry had cloned and were sequencing human StAR complementary DNA (cDNA) [30]. As soon as they had preliminary data from the 5′ and 3′ ends, we synthesized human StAR oligonucleotides and used polymerase chain reaction (PCR) to amplify the cDNA from the gonadal cDNA from Sanger's patient and from another patient from Alan Rogol in Virginia. Even before the full-length human cDNA sequence had been completed, we had found that StAR was mutated in lipoid CAH, thus establishing the basis of the disease. We then found mutations in the genomic DNA of these patients and our UCSF patient, thus firmly establishing the essential role of StAR by showing that lipoid CAH is a knockout experiment of nature [11]. Although it was clear that StAR mutations caused lipoid CAH, we needed an assay for StAR activity

to show how StAR dysfunction caused the phenotype. We first did this by transfecting StAR cDNA expression vectors into COS-1 cells that had been transfected with vectors for human P450scc and its redox partners, adrenodoxin and adrenodoxin reductase, and measuring pregnenolone production [11]. However, because the activity of P450scc can vary with the abundance of these redox partners, we wanted to fix the ratio of these two proteins to eliminate assay variability. We did this with a catalytically active fusion protein of P450scc, adrenodoxin, and adrenodoxin reductase, which we had built previously [31, 32]. As a positive control we used 22-hydroxycholesterol, as by then it was known that soluble hydroxysterols are freely accessible to mitochondrial P450scc, bypassing the mitochondrial cholesterol import system [33]. Thus we were quickly able to establish the quantitative assay for StAR's action that remains in use today [11]. This experiment essentially recapitulated Degenhart's 1972 experiment: The 20-OH cholesterol they used bypassed the absent action of the then-unknown StAR protein, explaining why they found that this hydroxysterol, but not cholesterol, was converted to pregnenolone by lipoid CAH mitochondria. We soon found a splicing mutation in a fourth patient [34], and, as is so often the case, as soon as we had reported the cause of a rare disease, samples came in from around the world, permitting a general description of the genetics of lipoid CAH [35]. Thus, these early experiments proved that StAR enhanced cholesterol access to P450scc and that lipoid CAH served as a gene knockout of nature, proving its essential role. These early experiments also showed that there was a low level of StAR-independent steroidogenesis. This crucial observation led to our "two-hit model" that explains the clinical findings in lipoid CAH [35].

## Delivery of Cholesterol to Mitochondria

The biosynthesis of all steroid hormones begins with the conversion of cholesterol to pregnenolone in mitochondria, followed by complex tissue-specific pathways leading to glucocorticoids and mineralocorticoids in the adrenal, estrogens and progestins in the placenta and ovary, and androgens in the testis [36]. Most steroidogenic tissues have specialized mechanisms to deliver cholesterol to mitochondrial P450scc. Lipoid CAH is one of several genetic disorders in these early steps in steroidogenesis, which have helped to elucidate this complex biology.

Intracellular cholesterol trafficking before cholesterol reaches steroidogenic mitochondria is essentially the same in steroidogenic and non-steroidogenic cells [37]. Cholesterol may be produced *de novo* from acetate via a complex pathway primarily found in the endoplasmic reticulum (ER) [38], but most steroidogenic cholesterol is imported from circulating lipoproteins. In rodents, the primary source is circulating high-density lipoproteins (HDL), which are imported into the cell by scavenger receptor B1 (SR-B1); in humans, the main source of steroidogenic cholesterol is low-density lipoproteins (LDL) (primarily derived from the diet), imported by receptor-mediated endocytosis via LDL receptors. However, patients with congenital abetalipoproteinemia, who have low LDL cholesterol, have normal

basal cortisol concentrations and have only mildly impaired cortisol responses to adrenocorticotropic hormone (ACTH) [39]. Furthermore, treatment with high doses of statins does not impair cortisol secretion [40], hence endogenously produced cholesterol appears to be sufficient. LDL can suppress the rate-limiting enzyme in cholesterol synthesis, 3-hydroxy-3-methylglutaryl co-enzyme A (HMGCoA) reductase. A family of basic helix-loop-helix transcription factors called the sterol regulatory element binding proteins (SREBPs) regulates cholesterol uptake and its intracellular transport and utilization.

After an LDL particle is internalized by receptor-mediated endocytosis, the resulting endocytic vesicles fuse with lysosomes; the LDL proteins undergo proteolysis, and the liberated cholesteryl esters are hydrolyzed to "free" cholesterol by lysosomal acid lipase (LAL). However, the term "free cholesterol" is misleading as the solubility of cholesterol is only about 20 micromoles per liter; thus, "free cholesterol" refers to cholesterol that is bound to proteins or membranes, but lacks an ester covalently bound at carbon 3. Free cholesterol may be used by the cell or stored in lipid droplets following re-esterification by acyl-coenzyme A cholesterol-acyl-transferase (ACAT) also known as sterol O-acetyltransferase (SOAT1). HDL cholesteryl esters that enter the cell via SR-B1 are acted on by hormone-sensitive neutral lipase (HSL), following which the free cholesterol may similarly be used or re-esterified for storage. ACTH (in the adrenal) and luteinizing hormone (LH; in the gonad) increase intracellular cAMP, which stimulates HSL and inhibits ACAT, thus increasing the pool of free cholesterol available for steroidogenesis. Increasing cAMP stimulates HMGCoA reductase and LDL uptake. When intracellular cholesterol concentrations are high, transcription of the genes for LDL receptor, HMGCoA reductase, and LAL is repressed while ACAT is induced, thereby decreasing cholesterol uptake, synthesis, and de-esterification. Conversely, when intracellular cholesterol concentrations are low, this process is reversed [Fig. 4.2] [reviewed in 37].

Mutations in LAL cause Wolman disease and its milder variant, cholesterol ester storage disease [41]. Wolman disease is a lethal disorder characterized by visceral accumulation of cholesteryl esters and triglycerides, with secondary adrenal insufficiency. Affected infants develop hepatosplenomegaly, malabsorptive malnutrition, and developmental delay; adrenal insufficiency may be treated with hormonal-replacement therapy, but this has little impact on the relentless course of the disease, which is usually fatal in about a year. The diagnosis is suggested by calcifications that outline the adrenals (which is radiographically distinct from the gross adrenal hyperplasia of lipoid CAH) and is confirmed by finding deficient lysosomal acid lipase activity in leukocytes or fibroblasts. Bone marrow transplantation may ameliorate the disease by unclear mechanisms. In contrast to LAL, there is no known human HSL deficiency.

Endosomal/lysosomal cholesterol transport requires the NPC1 and NPC2 proteins that are mutated in Niemann-Pick type C (NPC) disease, characterized by endosomal accumulation of LDL-cholesterol and glycosphingolipids. Patients develop ataxia, dementia, speech loss, and spasticity at 2–4 years, and typically die at 10–20 years [42]. Cholesterol and other lipids accumulate in neurons, and there is robust glial infiltration. The diagnosis is made by finding characteristic foamy Niemann-Pick cells and "sea-blue" histiocytes in bone marrow. NPC2, a soluble

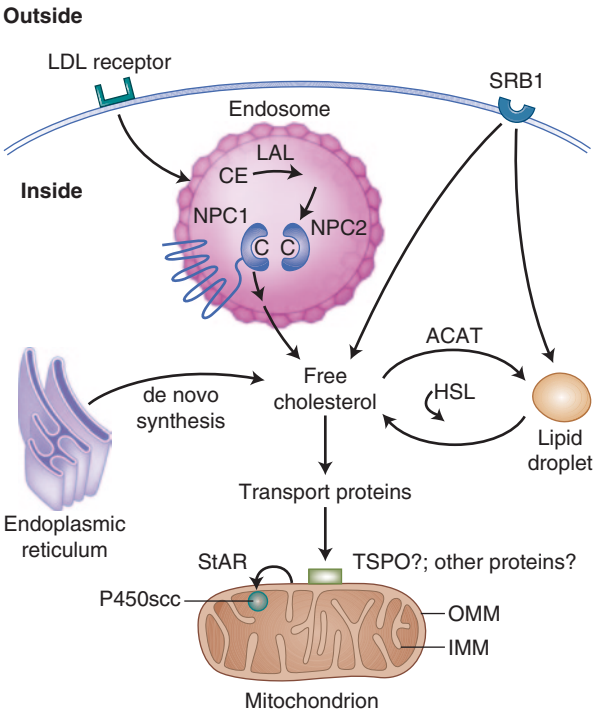


**Fig. 4.2** Intracellular cholesterol trafficking. Human steroidogenic cells take up circulating low-density lipoproteins (LDL) by receptor-mediated endocytosis, directing the cholesterol to endosomes; rodent cells utilize cholesterol from high-density lipoproteins (HDL) via scavenger receptor B1 (SRB1). Cholesterol may also be synthesized from acetate in the endoplasmic reticulum (ER). Cholesteryl esters are cleaved by lysosomal acid lipase (LAL); free cholesterol is then bound by NPC2, transferred to NPC1, and exported. The metastatic lymph node clone 64/terminal domain homologue (MLN64/MENTHO) system resides in the same endosomes as the Niemann-Pick type C (NPC) system, but its role in cholesterol trafficking remains uncertain. Cholesterol may be re-esterified by acyl-CoA: cholesterol transferase (ACAT) and stored in lipid droplets as cholesteryl esters. Free cholesterol may be produced by hormone-sensitive lipase (HSL). Cholesterol can reach the outer mitochondrial membrane (OMM) by non-vesicular means by utilizing START-domain proteins or other cholesterol transport proteins. Movement of cholesterol from the OMM to the inner mitochondrial membrane (IMM) requires a multi-protein complex on the OMM. In the adrenals and gonads, the steroidogenic acute regulatory protein (StAR) is responsible for the rapid movement of cholesterol from the OMM to the IMM, where it can be converted to pregnenolone by P450scc (© *W.L. Miller*)

151 AA glycoprotein in the lysosomal lumen [43], binds cholesteryl esters with the cholesterol side chain oriented in a hydrophobic pocket and the polar 3βOH group exposed, allowing LAL to cleave the cholesteryl ester bond while bound to NPC2. Free cholesterol is then transferred to the N-terminal domain of NPC1, a 1278 AA glycoprotein with 13 transmembrane domains that span the endo-lysosomal membrane [44]. NPC1 binds cholesterol with the 3βOH group buried in the protein and

the side chain partially exposed; then inserts the cholesterol into the lysosomal membrane with the hydrophobic side chain going in first.

Two late endosomal proteins, metastatic lymph node clone 64 (MLN64) and MLN64 N-terminal domain homologue (MENTHO) may also participate in cholesterol trafficking [45]. MLN64 can bind cholesterol and co-localizes with NPC1 in late endosomes [46]. The N-terminal "MLN64 N-TerminAL" ("MENTAL") domain is structurally related to MENTHO [47], contains 4 transmembrane domains, and targets MLN64 to late endosomal membranes. The C-terminal domain of MLN64 is the START domain that is very similar to the lipid-binding domain of StAR [48, 49]. The MENTAL domains of MENTHO and MLN64 can interact to form homo- and heterodimers and to bind cholesterol, suggesting a role in endosomal cholesterol transport. MLN64 lacking the MENTAL domain (N-234 MLN64) has ~50–60% of StAR-like activity to stimulate mitochondrial cholesterol uptake [50, 51]. The START domain of MLN64 may interact with cytoplasmic HSP60 to stimulate steroidogenesis in placental mitochondria [52]. An essential function for MLN64 is not established as knockout of the START domain of MLN64 yields viable, neurologically intact, fertile mice with normal plasma and hepatic lipids [53]. Human genetic disorders of MLN64 or MENTHO are not known. Accumulation of cholesterol in NPC1-deficient cells increases MLN64-mediated cholesterol transport to mitochondria and accumulation of cholesterol in the outer mitochondiral membrane (OMM), suggesting a role in cholesterol transport from endosomes to mitochondria [54].

Intracellular cholesterol transport may be "vesicular" (mediated by membrane fusion) or "non-vesicular" (bound to proteins). Both vesicular and non-vesicular cholesterol transport occur in steroidogenic cells, but non-vesicular transport involving high-affinity cholesterol-binding START-domain proteins appears to be the principal means for cholesterol transport to mitochondria [37]. START domain proteins are found in all eukaryotes; the 15 mammalian START proteins are termed STARD1–15 (StAR itself is also known as STARD1, but is designated in this chapter by its classical name, StAR) [49]. STARD4, D5, and D6, the START proteins most closely related to StAR, bind cholesterol, are induced by SREBP, and lack N-terminal signal sequences, suggesting they are cytosolic sterol transport proteins [37]. StAR, STARD3 (MLN64), D4, and D5 bind cholesterol with high-affinity and specificity, facilitate cholesterol transport, and appear to play roles in cellular cholesterol homeostasis [55]. STARD5 is predominantly expressed in Kupffer cells, macrophages, and proximal renal tubules and appears to act primarily as a bile-acid-binding protein [56, 57]. StAR and STARD6 stimulate the movement of cholesterol from the OMM to the inner mitochondrial membrane (IMM), but STARD4 and STARD5 do not [58]. STARD4 and/or D5 may bring cholesterol to the OMM; however, STARD4 knockout mice have no changes in steroidogenesis and minimal changes in weight and serum lipids, hence an essential function of STARD4 is not apparent [59]. Thus, it appears that the principal mechanism for getting cholesterol to the mitochondria is by non-vesicular transport involving proteins that are structurally related to StAR, but that StAR itself plays a minor role in this step. The OMM of adrenal mitochondria contains abundant cholesterol, whereas the IMM

contains relatively little cholesterol. Whether all OMM cholesterol is available for steroidogenesis is unclear. Early studies identified a distinct pool of "steroidogenic" OMM cholesterol that was distinct from the structural membrane cholesterol and could be mobilized by cAMP [60]. Whether there are two kinetically distinct pools of cholesterol in the OMM, and how cholesterol is transferred to the IMM remain under investigation.

## Entry of Cholesterol into Steroidogenic Mitochondria: Action of the Steroidogenic Acute Regulatory Protein, StAR

Unlike cells that produce polypeptide hormones, which store mature hormone in secretory vessicles for rapid release, steroidogenic cells store very little steroid, so that steroid secretion requires more steroid synthesis, which can be induced by several mechanisms [36, 37]. In the adrenal, ACTH promotes steroidogenesis at three distinct levels. First, over the course of months, ACTH stimulates adrenal growth via several growth factors; second, over the course of days, ACTH, in the adrenal zona fasciculata and angiotensin II in the zona glomerulosa stimulate transcription of steroidogenic enzyme genes, especially the *CYP11A1* gene encoding P450scc, thus increasing the amount of steroidogenic machinery; third, within an hour, ACTH stimulates the activation of preexisting StAR (by its phosphorylation at Ser195) and the synthesis of new StAR. StAR then appears to interact with a macromolecular complex on the OMM to increase cholesterol flux from the OMM to the IMM, where it becomes the substrate for P450scc. The first two modes of steroid regulation comprise the chronic steroidogenic response and the action of StAR comprises the acute steroidogenic response [36, 37].

Co-expression of StAR and P450scc in nonsteroidogenic cells increases conversion of cholesterol to pregnenolone, suggesting that StAR triggers the acute steroidogenic response [23]. The indispensible role of StAR was established by finding that StAR mutations cause lipoid CAH [11, 34, 35]. However, some steroidogenesis can take place without StAR: The human placenta synthesizes steroids via P450scc but expresses little or no StAR [30], and cells expressing P450scc but not StAR can convert cholesterol to pregnenolone [31, 32] at ~14 % of the maximal StAR-induced rate [11, 35]. The mechanisms underlying this StAR-independent steroidogenesis remain unclear. The placenta produces a cleavage product of MLN64 that appears to have StAR-like activity [51], or placental StAR-independent activity might happen without a transport protein, especially if soluble oxysterols are the substrate. The crystal structure of the START domain of MLN64 [61], computational models of StAR [62, 63], and low-resolution crystallography of StAR [64] all show a globular protein with an α/β helix-grip fold and an elongated hydrophobic pocket that accommodates one cholesterol molecule with its 3β-OH group coordinated by the two polar residues. These structures and the crystal structure of STARD4 [65] are characterized by long α-helixes at the N- and C-termini, two short α-helixes, and nine antiparallel β sheets that form a helix-grip fold.

How StAR induces the import of cholesterol into mitochondria remains unclear. StAR is synthesized as a 37-kDa protein with an N-terminal mitochondrial leader sequence that is cleaved during mitochondrial import to yield 30-kDa intramitochondrial StAR. The 37 kDa cytoplasmic form is often termed a "precursor" and the 30 kDa intramitochondrial protein its "mature form," but these terms do not describe the biology of StAR [66]. Moreover, deletion of the leader peptide has no effect on its activity [67]. Mitochondrial localization experiments show that StAR is active on the OMM but not in the IMS or on the matrix side of the IMM, and experiments manipulating the speed of StAR's mitochondrial entry show that faster import decreases activity and slower import increased activity [68]. Thus, StAR acts exclusively on the OMM and its activity is proportional to how long it remains on the OMM, so that it is the OMM localization of StAR, and not its cleavage from the 37 kDa form to the 30 kDa form, that determines its activity.

## Mechanism of StAR's Action—An Ongoing Study

The mechanism by which StAR triggers cholesterol flux from the OMM to the IMM requires pH-induced conformational changes in StAR that are needed for StAR to accept and discharge cholesterol [69, 70]. Cholesterol is blocked from reaching StAR's binding pocket by a set of hydrogen bonds that immobilize the C-helix but are disrupted when the surface residues of StAR are protonated as happens when StAR interacts with charged phospholipids on the OMM, thus eliciting a conformational change that permits cholesterol access [70]. Thus, the activity of StAR on the OMM requires an acid-induced disruption of hydrogen bonds and a consequent conformational change in StAR to permit it to bind and release cholesterol. StAR can transfer cholesterol between synthetic, protein-free membranes in vitro, but with non-physiologic stoichiometry [71], and the biologically inactive StAR mutant R182L can transfer cholesterol to membranes in vitro [72], indicating that cholesterol binding is necessary but not sufficient for StAR activity. The phosphorylation of StAR on Ser 195 doubles its activity [73]. It appears that the protein kinase A (PKA) anchor protein AKAP121 recruits the type II PKA regulatory subunit α (PKAR2A) to the OMM, which phosphorylates StAR, whereas the type I kinase drives StAR transcription [74]. Interaction between the 37 kDa StAR and HSL has also been reported [75]. StAR is recycled as each molecule moves hundreds of molecules of cholesterol into the mitochondria before it is inactivated by mitochondrial import [68, 76]. Low levels of steroidogenesis will persist in the absence of StAR at about 14% of the StAR-induced rate [11, 35], accounting for the steroidogenic capacity of tissues that lack StAR, such as the human placenta.

StAR appears to interact with a multi-protein complex on the OMM. In addition to AKAP121 and PKAR2A mentioned above, several other proteins have been implicated, but what role each plays in the mitochondrial importation of cholesterol

remains unclear [77–79]. The first identified and most extensively studied of these proteins is the PBR, now also called TSPO [80]. PBR/TSPO is a ubiquitously expressed 18 kDa mitochondrial protein; based on the binding of drug ligands, it is estimated that it comprises about 2 % of adrenal OMM protein [81, 82]. PBR/TSPO lacks a mitochondrial targeting sequence, but its C-terminal half targets it to the OMM [83]. Ligands of PBR/TSPO appear to stimulate cholesterol movement from OMM to IMM and stimulate steroidogenesis [84]. PBR/TSPO is a component of a 140–200 kDa complex consisting of PBR/TSPO, the 34-kDa voltage-dependent anion channel (VDAC1), the 30-kDa the adenine nucleotide transporter (ANT), the 10-kDa diazepam-binding inhibitor (acyl-CoA-binding domain 1, ACBD1), the TSPO-associated protein-1 (PRAX-1), and the PKA regulatory subunit RIα-associated protein 7 (PAP7) [78]. PAP7, also known as acyl-CoA binding domain-containing protein 3 (ACBD3), appears to bind both TSPO and regulatory subunit RIα (and RII to a lesser extent) of cAMP-dependent protein kinase A (PKA) [85]. PBR/TSPO interacts with VDAC on the OMM, possibly anchoring the multi-protein complex to the OMM and assisting the binding and import of StAR [86]; VDAC1 also interacts with ANT on the IMM [87]. While StAR and PBR/TSPO appear to interact functionally [88], no physical interaction has been found; protein cross-linking experiments identify contacts between StAR and VDAC1 and phosphate carrier protein (PCP), but not PBR/TSPO [77]. VDAC1 interacts with PBR/TSPO [83, 86], but how VDAC-1 might participate in cholesterol import is unclear as VDAC1 has a cylindrical structure with a hydrophilic interior for anion transport but is ill-suited as a channel for hydrophobic cholesterol [88, 89]. VDAC is found at contact sites between the OMM and the IMM [90] where it may complex with hexokinase, ANT, creatine kinase, and proteins of the Bcl-2 family [91]. PBR/TSPO has five transmembrane α-helices spanning the OMM [92], suggesting it functions as a cholesterol channel that would act downstream from StAR. PBR/TSPO has a cytoplasmic domain containing a “cholesterol recognition amino acid consensus” (CRAC) domain that binds cholesterol, suggesting that this domain participates in transferring cholesterol from the OMM to the IMM [93]. Mutagenesis of the CRAC domain interferes with cholesterol binding and cholesterol transfer to the IMM, and blocking the binding of cholesterol to CRAC prevents steroidogenesis [94]. PBR/TSPO knockdown in Leydig cells disrupts cholesterol transport and steroidogenesis [95]. It has been reported that disruption of the TSPO gene in Leydig cells blocked cholesterol transport into the mitochondria and reduced steroid production [96], and that PBR/TSPO knockout mice experience embryonic lethality [84], suggesting an indispensible role in development. However, in a recent report, tissue-specific knockout of PBR/TSPO in mouse Leydig cells yielded no reproductive or steroidogenic phenotype [97]. Thus, PBR/TSPO joins MLN64 [53] and StARD4 [59] on a growing list of proteins for which there is good biochemical evidence for a role in steroidogenesis, but for which the mouse knockout data do not confirm a role, and for which no definitive answer is known from a human genetic disease. Thus, the role of PBR/TSPO in steroidogenesis remains controversial.

## Mutations in StAR—Congenital Lipoid Adrenal Hyperplasia (Lipoid CAH)

As reviewed in the Introduction, and in greater detail elsewhere [98], lipoid CAH is a rare disorder that has played a central role in driving contemporary understanding of the regulation of steroidogenesis and the discovery of the START-domain proteins. Lipoid CAH is the most severe genetic disorder of steroid hormone synthesis, characterized by absent or very low serum concentrations of all steroids, high basal ACTH and plasma renin activity, an absent steroidal response to long-term treatment with high doses of ACTH or hCG, and grossly enlarged adrenal glands filled with cholesterol and cholesteryl esters. Although these findings initially suggested a lesion in the conversion of cholesterol to pregnenolone, the *CYP11A1* gene for P450scc is not mutated in these patients, and the mRNAs for adrenodoxin reductase and adrenodoxin are intact [20]. Furthermore, the placenta (a fetal tissue) continues to produce progesterone in lipoid CAH, proving that the cholesterol side chain cleavage system remains intact, and permitting normal term gestation [29]. The normal P450scc system plus the accumulation of cholesterol esters in the affected adrenal suggested that the lesion lay in a factor upstream from P450scc, such as in a factor involved in mitochondrial cholesterol transport [20]; mutations in PBR/TSPO were sought and excluded [22]. Thus, the responsible factor appeared to be expressed in the adrenal and gonad, but not in the placenta, and the discovery that StAR expression had this tissue distribution helped to identify it as the disordered step in lipoid CAH [11].

Lipoid CAH is the StAR gene knockout experiment of nature, revealing the complex physiology of the StAR protein [98]. StAR promotes steroidogenesis by increasing the movement of cholesterol into mitochondria, but in the absence of StAR, steroidogenic cells still make small amounts of steroids by StAR-independent steroidogenesis [35, 37, 68]. This observation led to the two-hit model of lipoid CAH [35] [Fig. 4.3]. The first hit is the loss of StAR itself, leading to a loss of most, but not all steroidogenesis, leading to a compensatory rise in ACTH and LH. These increased tropic hormones increase production of intracellular cAMP, which increases biosynthesis of LDL receptors, their consequent uptake of LDL cholesterol, and *de novo* synthesis of cholesterol. In the absence of StAR, this increased intracellular cholesterol accumulates as in a storage disease, causing the second hit, which is the mitochondrial and cellular damage caused by the accumulated cholesterol, cholesterol esters, and their autooxidation products [35, 37].

The two-hit model explains the unusual clinical findings in lipoid CAH. In response to tropic stimulation by hCG, fetal Leydig cells normally make large amounts of testosterone; in lipoid CAH, this stimulation leads to accumulation of cholesterol and cellular destruction early in gestation. This eliminates the testosterone biosynthesis needed for development of male external genitalia, so that an affected 46, XY fetus does not undergo normal virilization and is born with female-appearing external genitalia and a blind vaginal pouch. However, Wolffian duct derivatives (seminal vesicle, ejaculatory ducts) are well developed [99], indicating the presence of some testosterone synthesis in early fetal life as predicted by the two-hit

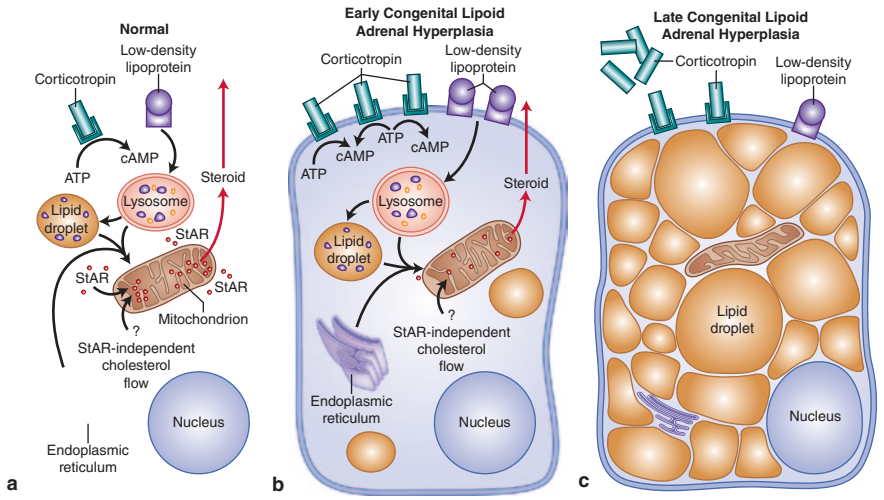


**Fig. 4.3** Two-hit model of congenital lipoid adrenal hyperplasia (lipoid CAH). **a** In normal adrenal cells, cholesterol is primarily derived from low-density lipoproteins, and the rate-limiting step in steroidogenesis is movement of cholesterol from the outer mitochondrial membrane (OMM) to the inner mitochondrial membrane (IMM). **b** Early in lipoid CAH, StAR independent steroidogenesis moves small amounts of cholesterol into mitochondria, yielding sub-normal steroidogenesis; adrenocorticotropic hormone (ACTH) secretion increases, stimulating further accumulation of cholesteryl esters in lipid droplets. **c** As lipids accumulate, they damage the cell through physical engorgement and by the action of cholesterol auto-oxidation products; steroidogenic capacity is destroyed, but tropic stimulation continues. Ovarian follicular cells remain unstimulated and undamaged until puberty, when small amounts of estradiol are produced, as in **b**, causing phenotypic feminization, with infertility and hypergonadotropic hypogonadism. Modified from [35]

model. The undamaged Sertoli cells function normally and produce Müllerian inhibitory hormone, so that the phenotypically female 46, XY fetus with lipoid CAH has no cervix, uterus, or fallopian tubes. The steroidogenically active fetal zone of the adrenal is similarly affected, eliminating most dehydroepiandrosterone (DHEA) biosynthesis, thus eliminating the feto-placental production of estriol, so that mid-gestation maternal and fetal estriol levels are very low [29]. The definitive zone of the fetal adrenal, which differentiates into the zonae glomerulosa and fasciculata, normally produces very little aldosterone. Because fetal salt and water homeostasis is maintained by the placenta, stimulation of the glomerulosa by angiotensin II generally does not begin until birth, so that the glomerulosa does not necessarily experience the "second hit" during fetal life. Consequently, many newborns with lipoid CAH do not have a salt-wasting crisis until after several weeks of life when chronic stimulation then leads to cellular damage [35, 100].

The two-hit model also explains the spontaneous feminization of affected 46, XX females who receive glucocorticoid and mineralocorticoid replacement therapy in infancy and hence reach adolescence [35, 101, 102]. The fetal ovary makes little or no steroids and contains no steroidogenic enzymes after the first trimester [103]; consequently, the ovary remains largely undamaged until it is stimulated by go-

nadotropins at the time of puberty when it then produces small amounts of estrogen by StAR-independent steroidogenesis. Continued stimulation results in cholesterol accumulation and cellular damage, so that there is minimal biosynthesis of progesterone in response to the LH surge in the latter part of the cycle. Because gonadotropin stimulation only recruits individual follicles and does not promote steroidogenesis in the whole ovary, most follicles remain undamaged and available for future cycles. Cyclicity is determined by the hypothalamic-pituitary axis, and remains normal. With each new cycle, a new follicle is recruited and more estradiol is produced by StAR-independent steroidogenesis. Although net ovarian steroidogenesis is impaired, enough estrogen is produced (especially in the absence of androgens) to induce breast development, general feminization, monthly estrogen withdrawal and cyclic vaginal bleeding [35, 101]. However, progesterone synthesis in the latter half of the cycle is disturbed by the accumulating cholesterol esters so that the cycles are anovulatory. Measurements of estradiol, progesterone, and gonadotropins throughout the cycle in affected adult females with lipoid CAH confirm this model [102]. Similarly, examination of StAR-knockout mice confirms the two-hit model [104]. Thus, examination of patients with lipoid CAH has elucidated the physiology of the StAR protein in each steroidogenic tissue.

Genetic analysis of patients with lipoid CAH has revealed numerous mutations in the StAR gene [35, 37, 66]. Lipoid CAH is relatively common in Japan and Korea, where the carrier frequency is approximately one in 300, so that one in every 250,000 to 300,000 newborns in these countries is affected for a total of about 500 patients in Japan and Korea. About 65–70% of affected Japanese alleles and about 90% of affected Korean alleles carry the mutation Q258X [35, 105, 106], which was identified in the first patients studied [11]. Other genetic clusters are found among Palestinian Arabs, most of whom carry the mutation R182L [35], in eastern Saudi Arabia, carrying R188C [100], and in Switzerland, carrying the mutation L260P [107]. Deletion of only 10 carboxy-terminal residues reduces StAR activity by half, and deletion of 28 carboxy-terminal residues by the common Q258X mutation eliminates all activity. By contrast, deletion of the first 62 amino-terminal residues has no effect on StAR activity, even though this deletes the entire mitochondrial leader sequence and forces StAR to remain in the cytoplasm [67]. Physical studies and partial proteolysis indicate that residues 63–193 of StAR (i.e., the domain that lacks most of the crucial residues identified by missense mutations) are protease-resistant and constitute a "pause-transfer" sequence, which permits the bioactive loosely folded carboxy-terminal molten globule domain to have increased interaction with the outer mitochondrial membrane [69].

The clinical findings in most patients with lipoid CAH are quite similar: An infant with normal-appearing female genitalia experiences failure to thrive and salt loss in the first weeks or months of life and is diagnosed with adrenal insufficiency because of hyponatremia and hyperkalemia and possibly also because of hyperpigmentation [35–37]. However, other clinical presentations are possible, including apparent sudden infant death syndrome (SIDS) [108] and late initial presentation of salt loss at about one year of age [100]. We also described an attenuated form called "non-classic lipoid CAH," which is caused by mutations that retain about 20–25% of normal StAR activity [109]. These are usually children who first experience

symptoms of adrenal insufficiency after several years, and the 46, XY patients have normal-appearing male external genitalia. These patients typically have minimal impairments in mineralocorticoid synthesis, evidenced by elevated plasma renin activity with normal serum sodium and potassium, and may also have mild hypergonadotropic hypogonadism. Some patients have been diagnosed in adulthood and have been mistaken for having "familial glucocorticoid deficiency," a blanket term referring to disorders of ACTH action, but elevated plasma renin, hypergonadotropic hypogonadism, and azospermia are not features of familial glucocorticoid deficiency. Most of these patients carry the StAR mutation R188C [110, 111]. Thus, the spectrum of clinical presentations of StAR defects is substantially broader than was initially appreciated from studies of classic lipoid CAH.

Treatment of lipoid CAH consists of hormonal replacement therapy, and can be thought of in three phases. First, at time of diagnosis, most infants are hypovolemic, hyponatremic, hyperkalemic, and acidotic as a consequence of both mineralocorticoid and glucocorticoid deficiency. Initial treatment typically consists of fluid recussitation, salt replacementation to replace lost sodium, mineralocorticoid therapy (fludrocortisol) to replace lost mineralocorticoid synthesis, and initial stress-dosing with glucocorticoids to deal with physiologic stress and to suppress the hypothalamic-pituitary-adrenal axis [16, 112]. Second, once the life-threatening peri-diagnostic period has been dealt with and the intravascular volume and salt deficit have been repaired, physiologic replacement doses of glucocorticoids and mineralocorticoids will permit normal growth and development to adulthood. The glucocorticoid requirement in lipoid CAH is analagous to that in Addison disease and is less than in the virilizing forms of congenital adrenal hyperplasias due to 21-hydroxylase or 11-hydroxylase deficiency because adrenal over-suppression of excess adrenal androgen production is not needed. With glucocorticoid supplementation confined to physiologic oral replacement doses of 6–8 mg $m^2$/d [113–115], growth in these patients should be normal, although ACTH may remain elevated even when supraphysiologic doses of glucocorticoids are given [16]. Severely affected 46, XY newborns have normal female external genitalia and should undergo orchiectomy later in life and be raised as females, with estrogen replacement therapy started at the usual age of puberty to permit feminization of the body habitus and prevent bone loss. Third, affected 46, XX females, typically have spontaneous pubertal feminization but anovulatory cycles and early secondary amenorrhea, also requiring sex hormone replacement therapy. Successful pregnancy has been achieved in an adult female with lipoid CAH by clomiphene citrate stimulation followed by progesterone supplementation to mimic the maternally produced first trimester progesterone that the affected mother could not produce [116].

## P450scc: Cholesterol's Mitochondrial Destination

Mutations in other genes can produce a clinical phenotype that is essentially the same as that caused by StAR mutations. Because these disorders derive from different molecular lesions, they should not be called lipoid CAH. Although the initial

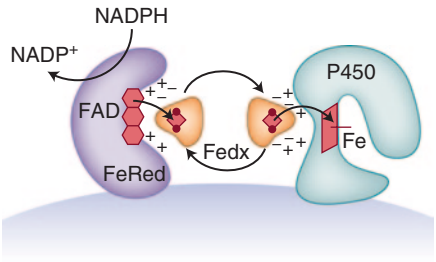


**Fig. 4.4** Organization of mitochondrial P450 enzyme systems. Nicotinamide adenine dinucleotide phosphate (NADPH) first donates electrons to the flavin adenine dinucleotide (FAD) moiety of ferredoxin reductase (FeRed); ferredoxin reductase then interacts with ferredoxin (Fedx) by charge-charge attraction, permitting electron transfer of the Fedx to the $Fe_2S_2$center (ball and stick diagram). Ferredoxin then dissociates from ferredoxin reductase and diffuses through the mitochrondrial matrix. The same surface of ferredoxin that received the electrons from ferredoxin reductase then interacts with the redox-partner binding-site of a mitochondrial P450, such as P450scc, and the electrons then travel to the heme ring of the P450. The heme iron then mediates catalysis with substrate bound to the P450. © W.L. Miller

genetic characterizations of patients with apparent lipoid CAH found no patients with mutations in the *CYP11A1* gene encoding P450scc, the first such patient was described in 2001 [117]. The physiology of these patients requires knowledge of the biochemistry of P450scc.

Once cholesterol reaches the IMM, it may be converted to pregnenolone by the cholesterol side chain cleavage enzyme, P450scc, to initiate steroidogenesis. Most steroidogenic enzymes are cytochrome P450 enzymes, all of which have approximately 500 residues, contain a single heme group, and absorb light at 450 nm when reduced with carbon monoxide. The human genome contains 57 *CYP* genes encoding cytochrome P450 enzymes. Seven human cytochrome P450 enzymes are targeted to the mitochondria, with the other 50 being targeted to the ER; the roles of the human P450 enzymes have been reviewed recently [118]. P450 enzymes use their heme iron to activate molecular oxygen using electrons donated by nicotinamide adenine dinucleotide phosphate (NADPH). Mitochondrial P450 enzymes such as P450scc are designated as Type 1 P450 enzymes, and receive electrons from NADPH via an electron transfer chain consisting of a flavoprotein termed ferredoxin reductase and a small iron-sulfur protein termed ferredoxin [119] [Fig. 4.4]. The type 2 P450 enzymes in the ER receive electrons via the single 2-flavin protein P450 oxidoreductase [119]. All P450 enzymes can catalyze multiple chemical reactions, often with very different substrates.

Conversion of cholesterol to pregnenolone by P450scc is the first, rate-limiting and hormonally regulated step in the synthesis of all steroid hormones. P450scc catalyzes the 22-hydroxylation of cholesterol, 20-hydroxylation of 22(R)-hydroxycholesterol, and oxidative scission of the C20–22 bond of 20(R), 22(R)-dihydroxycholesterol (the side chain cleavage event), yielding pregnenolone and isocaproaldehyde. The binding of cholesterol and the initial 22-hydroxylation are rate-limiting as the efficiencies ($k_{cat}$/Km ratios) are much higher for the subsequent reactions,

and the high $K_D$ of ~3000 nM drives the dissociation of pregnenolone from P450scc [36]. Alternatively, soluble hydroxysterol substrates such as 22(R)-hydroxycholesterol can enter the mitochondrion without the action of StAR. The conversion of cholesterol to pregnenolone is slow with a net turnover number of about 6 to 20 molecules of cholesterol per molecule of P450scc per second [14, 120]. Because 20-hydroxycholesterol, 22-hydroxycholesterol, and 20, 22-hydroxycholesterol can all be isolated from bovine adrenals, and because 3 moles of NADPH are required per mole of cholesterol converted to pregnenolone, it was initially thought that three separate enzymes were involved. However, protein purification and reconstitution of enzymatic activity in vitro showed that a single protein, P450scc, converts cholesterol to pregnenolone [reviewed by 37]. The crystal structures of bovine [121] and human [122] P450scc, the latter in complex with ferredoxin show that the single active site of P450scc is in contact with the IMM. It is the expression of the *CYP11A*1 gene that renders a cell "steroidogenic." Pregnenolone appears to exit the mitochondrion unaided; no transport protein has been found, and physiologic evidence does not suggest the presence of such a transporter.

Catalysis by P450scc requires two electron-transfer intermediates, ferredoxin reductase and ferredoxin [reviewed in 119]. Ferredoxin reductase receives electrons from NADPH then forms a 1:1 complex with ferredoxin which then dissociates and forms an analogous 1:1 complex with P450scc or other mitochondrial P450 enzymes, thus functioning as an indiscriminate, diffusible electron shuttle for all mitochondrial P450s (Fig. 4.4). In addition to the inherent properties of P450scc, the abundances of ferredoxin reductase and ferredoxin determine catalytic activity [31]. Genetic disorders of human ferredoxin reductase and ferredoxin have not been described, and mouse knockouts have not been reported. Mutation of the Drosophila ferredoxin reductase homologue *dare* causes developmental arrest and degeneration of the adult nervous system secondary to disrupted ecdysone production [123]. In vitro, the requirement for ferredoxin reductase and ferredoxin is not absolute as P450scc fused to microsomal P450 oxidoreductase remains active when targeted to the mitochondria, but is inactive when targeted to the ER even when supplied with the 22(R)-hydroxycholesterol substrate that bypasses the StAR system [32]. Thus, the mitochondrial localization is essential for the enzymatic activity of P450scc.

## P450scc Deficiency

The suppression of uterine contractility by progesterone prevents spontaneous abortion and is essential for the maintenance of mammalian pregnancy. However, different species use different strategies for the production of this progesterone: In rodents and ungulates, the source is maternal as the maternal corpus luteum of pregnancy continues to produce progesterone to term, whereas in primates, the source is a fetal tissue, the placenta. Thus, the available models of defective P450scc function, a spontaneously occurring *CYP11A1* deletion in the rabbit [124] and knockout of the *CYP11A1* gene in the mouse [125] are not informative about the potential

consequences of disorders in human P450scc. Once StAR mutations were found to cause lipoid CAH [11, 34, 35], it seemed logical that the dependence of human pregnancy on placental progesterone would be incompatible with P450scc-deficient fetuses surviving to term [126]. Nevertheless, beginning in 2001 [117], patients were reported who had clinical and hormonal findings that were indistinguishable from those of patients with StAR mutations, but who had mutations in P450scc. To date, 19 such patients have been described, but no patient with a P450scc mutation has had the adrenal hyperplasia typically seen in lipoid CAH [127]. Most of these patients had mutations that ablated all P450scc activity. It is not clear how these fetuses with P450scc mutations reached term gestation; one possibility is that these pregnancies were carried to term because of unusually protracted maintenance of the maternal corpus luteum of pregnancy, which normally involutes in the second trimester, but this has not been investigated directly. These patients may be clinically indistinguishable from those with lipoid CAH, and are treated in exactly the same fashion. The 46, XY genetic males fail to produce testosterone during fetal life, and are born with female external genitalia although their internal reproductive structures are male as their testes produced anti-Müllerian hormone. Following birth, these patients require steroid hormone replacement therapy and may have long-term survival. As with non-classical lipoid CAH, a milder "non-classical" form of P450scc deficiency that is clinically and hormonally indistinguishable from non-classic lipoid CAH has been reported in patients with P450scc mutations that retain 10–20% of wild-type activity [128, 129]. No hormonal test distinguishes lipoid CAH from P450scc deficiency, but the grossly enlarged adrenals that give lipoid CAH its name have not been described in patients with P450scc mutations, sometimes permitting radiologic distinction, but the only definitive test to distinguish these disorders is DNA sequencing [130].

Another disorder occasionally mistaken for lipoid CAH is deficiency of steroidogenic factor 1 (SF1), a transcription factor required for adrenal and gonadal, but not for placental, expression of genes for the steroidogenic enzymes [131]. More than 50 patients have been described carrying SF1 mutations [132, 133]. The phenotype of SF1-deficient patients is variable; some are 46, XY with a female phenotype and adrenal failure, thus resembling lipoid CAH, but in most cases, the gonadal phenotype predominates and there is little if any impairment of adrenal steroidogenesis. The Leydig cells may have lipid accumulation and progressive degeneration similar to the findings in lipoid CAH [133].

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