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SNAREs and Cholesterol Movement for Steroidogenesis

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Abstract

Steroidogenesis is a complex process through which cholesterol traffics to mitochondria and is converted via a series of enzymatic steps to steroid hormones. Although the rate-limiting step in this process is the movement of cholesterol from the outer to the inner mitochondrial membrane via the actions of StAR, a continuous supply of cholesterol must be delivered to the outer mitochondrial membrane during active steroidogenesis and this is derived from multiple sources, including lipoprotein uptake, endogenous cholesterol synthesis and release from stores within cytoplasmic lipid droplets. A number of mechanisms have been suggested to contribute to cholesterol trafficking to mitochondria; however, there is no definitive consensus and this is particularly so in regards to trafficking from cytoplasmic lipid droplets. In this paper we review experiments in which we have surveyed the expression of SNARE proteins in steroidogenic tissue and cells and examined the role of SNAREs in mediating cholesterol movement from lipid droplets to the mitochondria based on multiple studies that identified SNAREs as components of cytoplasmic lipid droplets. We established and characterized an *in vitro* mitochondria reconstitution assay system that enabled us to examine the impact of adding recombinant SNARE proteins specifically on the movement of cholesterol from model lipid droplets to the outer mitochondrial membrane. Using this reconstitution assay system in combination with siRNA knockdown experiments in rat primary granulosa cells or in steroidogenic cell lines, we showed that several SNARE proteins are important components in the trafficking of cholesterol from lipid droplets to the mitochondria for steroidogenesis.

Keywords

lipid droplet; SNARE; cholesterol; mitochondria; steroidogenesis

1. Steroidogenesis

Steroidogenesis entails a complex interplay among several subcellular compartments, multiple enzymes, substrates and products whereby cholesterol is converted to steroid

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hormones. In contrast to peptide hormones, preformed steroids are not stored in any appreciable amounts, but synthesized and secreted in response to stimulation by tropic hormones, such as ACTH in the adrenal. Although there is evidence for multi-level regulation of steroidogenesis (1), the rate-limiting step in the process is the trafficking of cholesterol from the outer (OMM) to the inner mitochondrial membrane (IMM) where CYP11A1 (cholesterol side chain cleavage enzyme) is located (2). Steroidogenic acute regulatory protein (StAR), along with accessory proteins such as VDAC (voltage dependent anion channel), is responsible for mediating the rate-limiting step of the movement of cholesterol from the OMM to the IMM in the adrenal and gonads (3,4). CYP11A1 mediates the initial enzymatic reaction in the production of all steroid hormones, the conversion of cholesterol to pregnenolone via the removal of the 6 carbon side chain. The pregnenolone produced then diffuses to the endoplasmic reticulum (ER) where it is further metabolized by 3-β hydroxysteroid dehydrogenase (3βHSD) and/or CYP17 (17α-hydroxylase) to steroid products (androgens) and intermediates that are then transported back to the mitochondria for further conversion to glucocorticoids or mineralocorticoids (5).

2. Cholesterol sources for steroidogenesis

Since the mitochondrial membrane is relatively cholesterol poor (6), there is a need for a constant supply of cholesterol to be delivered to the mitochondria as substrate for conversion into steroids. There are multiple sources for supplying cholesterol for steroidogenesis (7–9). First, adrenals, like all nucleated cells, can synthesize cholesterol from acetate within the ER via the rate-limiting enzyme HMG CoA reductase (10). In steroidogenic cells newly synthesized cholesterol traffics from the ER to the plasma membrane, and then traffics from the plasma membrane to the OMM (11,12). Second, cholesterol contained within circulating LDL can be delivered to cells following binding and endocytosis via LDL receptors (13). The cholesteryl esters carried in endocytosed LDL are hydrolyzed within lysosomes by lysosomal acid lipase, releasing unesterified cholesterol that traffics to the ER, where it is either reesterified to cholesteryl esters and stored within cytoplasmic lipid droplets or transported from the ER to the plasma membrane or mitochondria. Third, cholesterol contained within HDL, as well as LDL, can be delivered to cells following the binding of the lipoproteins to the scavenger receptor, class B type 1 (SR-B1) on the cell surface, with the subsequent transfer of cholesteryl esters across the plasma membrane to the cell interior without endocytosis of the intact lipoprotein particle (14,15). The cholesteryl esters delivered in this fashion must be hydrolyzed by hormone-sensitive lipase to unesterified cholesterol before being delivered to mitochondria (16,17). Fourth, cholesteryl ester-rich lipid droplets are prominently found in steroidogenic cells and can supply cholesterol for steroidogenesis following the hydrolysis of cholesteryl esters to unesterified cholesterol via the actions of hormone-sensitive lipase (18,19), whose activity is regulated by cyclic AMP/PKA. Indeed, the fact that lipid droplets are rapidly depleted following stimulation of steroidogenesis by ACTH suggests that this constitutes the preferred source of cholesterol for steroid synthesis, at least initially; however, it is apparent that there are multiple and redundant sources for supplying cholesterol to the OMM. Moreover, it seems that the mechanisms underlying the trafficking of cholesterol to mitochondria might well differ depending on the source of the cholesterol. For instance, the involvement of sterol carrier

proteins, vesicular trafficking, components of the cytoskeleton and specific plasma membrane-mitochondrial or ER-mitochondrial interactions have been suggested to be responsible for facilitating the trafficking of cholesterol (12,20). The mechanisms whereby cholesterol is transported from cytoplasmic lipid droplets to mitochondria have not been well appreciated.

3. Lipid droplets

Although all cells can accumulate cytoplasmic lipid droplets under certain pathological conditions, only steroidogenic cells display the presence of cholesteryl-ester-rich lipid droplets under physiological conditions, whereas adipose cells physiologically accumulate triglyceride-rich lipid droplets (19,21). Following exposure to tropic hormones, there is a rapid movement of lipid droplets in steroidogenic cells so that they appear in close apposition to mitochondria (22), further supporting their importance as a source of cholesterol for steroid production. Lipid droplets are dynamic organelles that traffic throughout the cytoplasm and have been shown to interact with several different intracellular organelles such as ER and mitochondria (23). Proteomic studies primarily examining triglyceride-rich droplets and a smaller number examining cholesteryl-ester rich droplets in steroidogenic cells have documented the presence of a wide variety of proteins associated with lipid droplets, including structural proteins, lipid synthesizing enzymes, lipases and a number of proteins involved in vesicular transport (24–27), such as SNARE (soluble NSF attachment protein receptor) proteins (28). Indeed, experiments examining the interaction of triglyceride-rich lipid droplets with mitochondria have suggested that SNAP23 (synaptosomal-associated protein of 23 kDa) promotes the interaction of lipid droplets with mitochondria (29). Since the mechanisms regulating the movement of cholesterol from cholesteryl ester-rich lipid droplets to mitochondria for steroidogenesis had not been well studied, we recently sought to examine the potential roles of SNARE proteins in this process (30).

4. SNARE expression

In our initial studies we used RT-qPCR to examine the expression levels of various SNARE mRNAs in mouse adrenals before and after ACTH treatment, in primary rat granulosa cells, and in mouse Leydig tumor cell (MLTC) line with and without treatment with cAMP (30). Mouse adrenals expressed mRNA of each of the SNARE proteins examined (SNAP23, SNAP25, NSF, α-SNAP, syntaxin-2, syntaxin-3, syntaxin-4, syntaxin-5, syntaxin-6, syntaxin-7, syntaxin-8, syntaxin-11, syntaxin-12, syntaxin-16, syntaxin-17, syntaxin-18), although expression levels varied, with SNAP25 and α-SNAP showing increased expression following ACTH treatment (30). Each of the SNAREs was detected in rat primary granulosa cells with the exception of α-SNAP, whereas SNAP23 and syntaxin-7 expression levels were increased with cAMP (30). All of the SNAREs were expressed in MLTC cells except SNAP25 and syntaxin-3, with α-SNAP expression increased with cAMP treatment (30). Thus, SNAREs are expressed in steroidogenic cells, but there appears to be some cell specific expression and cell specific hormonal regulation.

5. Mitochondria reconstitution assay

In order to examine the role of SNAREs in cholesterol movement to mitochondria for use in steroidogenesis, we developed a mitochondria reconstitution assay that was based on a system previously established and utilized almost 40 years ago (31). The major components of the assay are shown in Figure 1. First, mitochondria to be used in the assay were freshly isolated from rat adrenals by sucrose density centrifugation. Examination by electron microscopy revealed that the mitochondria preparations were relatively pure, but with some scattered membranous segments likely representing ER and plasma membranes (30). Cytochrome C oxidase activity measurement showed a 6.6-fold enrichment of mitochondria and NADPH-dependent cytochrome C reductase measurement showed only a 3% ER contamination (30). Second, we generated and purified recombinant StAR and seven SNARE proteins (SNAP23, SNAP25, NSF, α-SNAP, syntaxin-5, syntaxin-7 and syntaxin-17) that were produced in *E. coli*. Unfortunately, syntaxin-5 and syntaxin-7 were insoluble in the bacterial expression system and could not be further studied in the reconstitution assay. Third, we made a cholesterol phospholipid emulsion to act as a model lipid droplet. Fourth, we included succinate to the assay system in order to provide energy. Fifth, since preliminary experiments using pregnenolone as a read-out for cholesterol movement to mitochondria showed first an increase and then a decline with time, we added inhibitors of 17α-hydroxylase (abiraterone) and 3-βHSD (trilostane) to prevent further conversion of pregnenolone to other steroid products. In this manner the *in vitro* reconstitution assay could evaluate the impact of adding recombinant SNAREs to a system composed of lipid droplets (cholesterol emulsion), recombinant StAR to mediate cholesterol movement from the OMM to the IMM, and mitochondria to supply CYP11A1 for production of pregnenolone, without other interfering components. Initial experiments showed that the addition of StAR to mitochondria and the cholesterol emulsion led to an increase in pregnenolone production in a time dependent fashion, consistent with the effects of StAR to mediate cholesterol transport into the mitochondria (30). The addition of a cocktail containing all of the recombinant SNAREs to the assay significantly increased pregnenolone further, supporting a role for SNAREs in the process (30). In addition to measuring pregnenolone as evidence for the movement of cholesterol into mitochondria, by adding aminoglutethimide to the assay to inhibit CYP11A1 we could document a significant increase of cholesterol content in mitochondria incubated with cholesterol emulsion, StAR and the cocktail of recombinant SNAREs using fluorescent microscopy or by re-isolating the mitochondria and directly measuring mitochondrial cholesterol content (30). Furthermore, since SNARE proteins are GTP-binding proteins and GTP hydrolysis is important for trafficking, we examined the effects of GTP and GTPγs (a nonhydrolyzable GTP analogue) in the reconstitution assay. Consistent with their known effects on trafficking, addition of GTP significantly stimulated pregnenolone production in the presence of the SNARE cocktail, the cholesterol emulsion, StAR and mitochondria, whereas GTPγs decreased pregnenolone production (30).

6. Impact of SNAREs on steroidogenesis *in vitro*

Having characterized the reliability and functionality of the mitochondria reconstitution system, we next examined the effects of the recombinant SNARE proteins individually and

in combination on facilitating cholesterol movement to mitochondria by adding them to an incubation containing mitochondria, cholesterol emulsion and recombinant StAR. Addition of α-SNAP significantly increased pregnenolone production, whereas omitting α-SNAP from a cocktail containing all of the SNARE proteins reduced pregnenolone production (30), highlighting the importance of α-SNAP. Addition of either SNAP25 or syntaxin-17 to the system significantly increased pregnenolone production; however, omitting either from the cocktail of SNARE proteins had no significant effect on pregnenolone production (30), suggesting a role for each, but highlighting redundant or overlapping functions. In contrast, neither the addition of NSF or SNAP23 or omission of either from the cocktail of SNARE proteins had an effect on pregnenolone production (30). This is particularly noteworthy in view of the reported importance of SNAP23 in mediating the interaction of triglyceride-rich lipid droplets with mitochondria , highlighting another important difference in the metabolism of triglyceride-rich versus cholesterol-rich lipid droplets.

7. Impact of SNAREs on cellular steroidogenesis

Whereas the *in vitro* mitochondria reconstitution assay documented the impact of several SNARE proteins on cholesterol movement to mitochondria, we next sought to test the impact of each of the recombinant SNARE proteins tested, along with syntaxin-5 and syntaxin-7, which could not be examined in the reconstitution assay due to their insolubility, on cellular steroidogenesis. For these studies we used siRNA to knockdown the expression of each of the SNAREs in rat primary granulosa cells (MLTC cells were used for studying α-SNAP since granulosa cells do not express α-SNAP) and measured progesterone production following cAMP stimulation (30). After confirming the successful knockdown of the expression of each of the target SNARE proteins by immunoblotting, we observed that knockdown of SNAP25, α-SNAP, syntaxin-5 and syntaxin-17 all resulted in a significant reduction in progesterone production, whereas neither knockdown of SNAP23 or syntaxin-7 influenced steroid synthesis (30). It is important to note that the knockdown of SNAREs under these conditions in steroidogenic cells is quite different than the conditions in the mitochondria reconstitution assay system since the SNAREs could affect multiple steps involved in progesterone production in addition to the trafficking of cholesterol to the OMM as evaluated in the reconstitution system, such as ER and mitochondrial movement.

8. Conclusion

We established, characterized and confirmed the functionality of an *in vitro* mitochondria reconstitution assay system that consists of isolated rat adrenal mitochondria, a cholesterol emulsion as model lipid droplets, recombinant StAR protein to allow cholesterol transport from the OMM to the IMM where CYP11A is located, and enzyme inhibitors to prevent the further conversion of pregnenolone to other steroid products, all in the presence of an energy regenerating system. This enabled us to examine the impact of adding recombinant SNARE proteins specifically on the movement of cholesterol from the model lipid droplets to the OMM by measuring pregnenolone production as a read out. Using this reconstitution assay system in combination with siRNA knockdown experiments in rat primary granulosa cells or in the MLTC cell line, we showed that several SNARE proteins, SNAP25, α-SNAP, syntaxin-5 and syntaxin-17, are important components in the trafficking of cholesterol to the

OMM for steroidogenesis (see Figure 2), whereas other SNAREs, NSF, SNAP23 and syntaxin-7, are not.

As depicted in the cartoon in Figure 2, there are multiple sources for supplying cholesterol for steroidogenesis, including *de novo* synthesis within the ER, delivery from circulating LDL following binding and endocytosis via LDL receptors with subsequent lysosomal hydrolysis releasing unesterified cholesterol, delivery from HDL following the binding to SR-B1 with the ensuing transfer of cholesteryl esters across the plasma membrane and subsequent cytosolic hydrolysis by HSL, and finally following the hydrolysis of cholesteryl ester-rich lipid droplets by HSL. Whether the mechanisms underlying the trafficking of cholesterol to mitochondria differ depending on the source of the cholesterol is unknown, but the work reviewed here has focused on the role of SNAREs in facilitating the movement of lipid droplet cholesterol to mitochondria. The mechanisms how SNAREs facilitate this pathway of cholesterol trafficking have not yet been fully elucidated; however, several possibilities exist. First, SNAREs are known to be important components of membrane fusion and vesicular transport, and SNARE function and localization are influenced by cholesterol (32,33). Therefore, the active SNAREs might aid the movement of lipid droplets so they are in close physical proximity to mitochondria, thus indirectly facilitating the transfer of cholesterol from the lipid droplet to the OMM. Second, several SNAREs have been shown to interact directly with cholesterol (32,34). This raises the possibility that SNAREs directly interact with cholesterol and facilitate its trafficking to the OMM. These mechanisms are not mutually exclusive, and additional mechanisms might also be involved. Future studies will be directed at determining the function of SNAREs in this process as well as assessing the relative contribution and molecular mechanisms trafficking the various sources of cholesterol for steroidogenesis.

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Abbreviations

CYP11A1	cholesterol side chain cleavage enzyme
CYP17	17α-hydroxylase
ER	endoplasmic reticulum
3βHSD	3-β hydroxysteroid dehydrogenase
IMM	inner mitochondrial membrane
MLTC	mouse Leydig tumor cell
NSF	N-ethylmaleimide-sensitive factor
OMM	outer mitochondrial membrane

SR-B1	scavenger receptor class B type 1
SNAP23	synaptosomal-associated protein of 23 kDa
SNARE	soluble NSF attachment protein receptor
StAR	steroidogenic acute regulatory protein

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Highlights

- Description of a mitochondria reconstitution assay measuring cholesterol movement.
- Several SNARE proteins are involved in cholesterol trafficking for steroidogenesis.
- Cholesterol trafficking for steroidogenesis is reviewed.

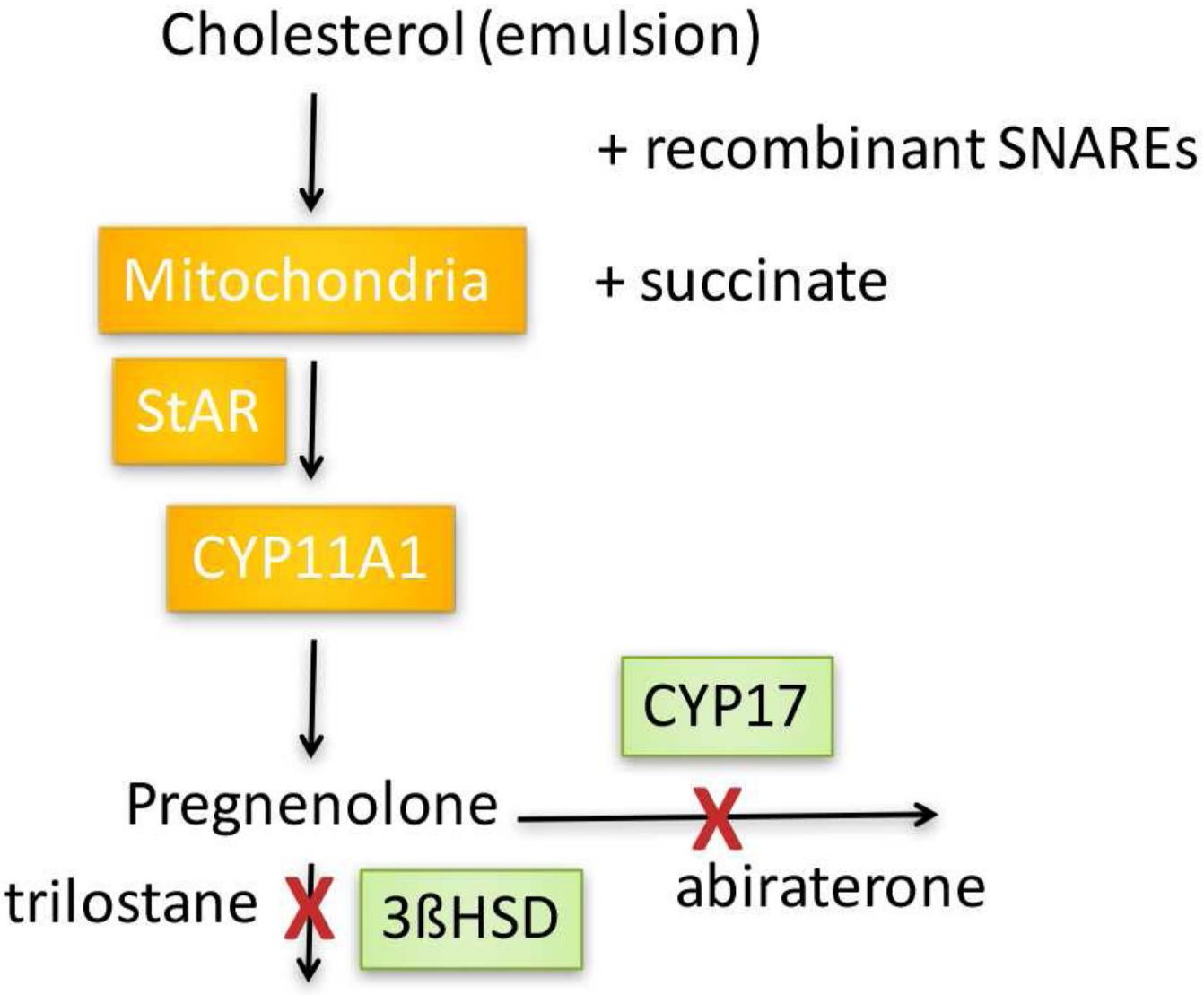

Figure 1.

Schematic composition and metabolic pathways contained within the mitochondria reconstitution system. The reconstitution assay is a buffered system that contains a cholesterol:phospholipid emulsion as model lipid droplets, freshly isolated rat adrenal mitochondria, recombinant StAR protein to mediate cholesterol movement from the outer to inner mitochondrial membrane, recombinant SNARE proteins, and succinate for energy, along with trilostane to inhibit 3βHSD and abiraterone to inhibit CYP17. Pregnenolone is then measured to determine the efficiency of cholesterol movement and metabolism by CYP11A1 located on the inner mitochondrial membrane.

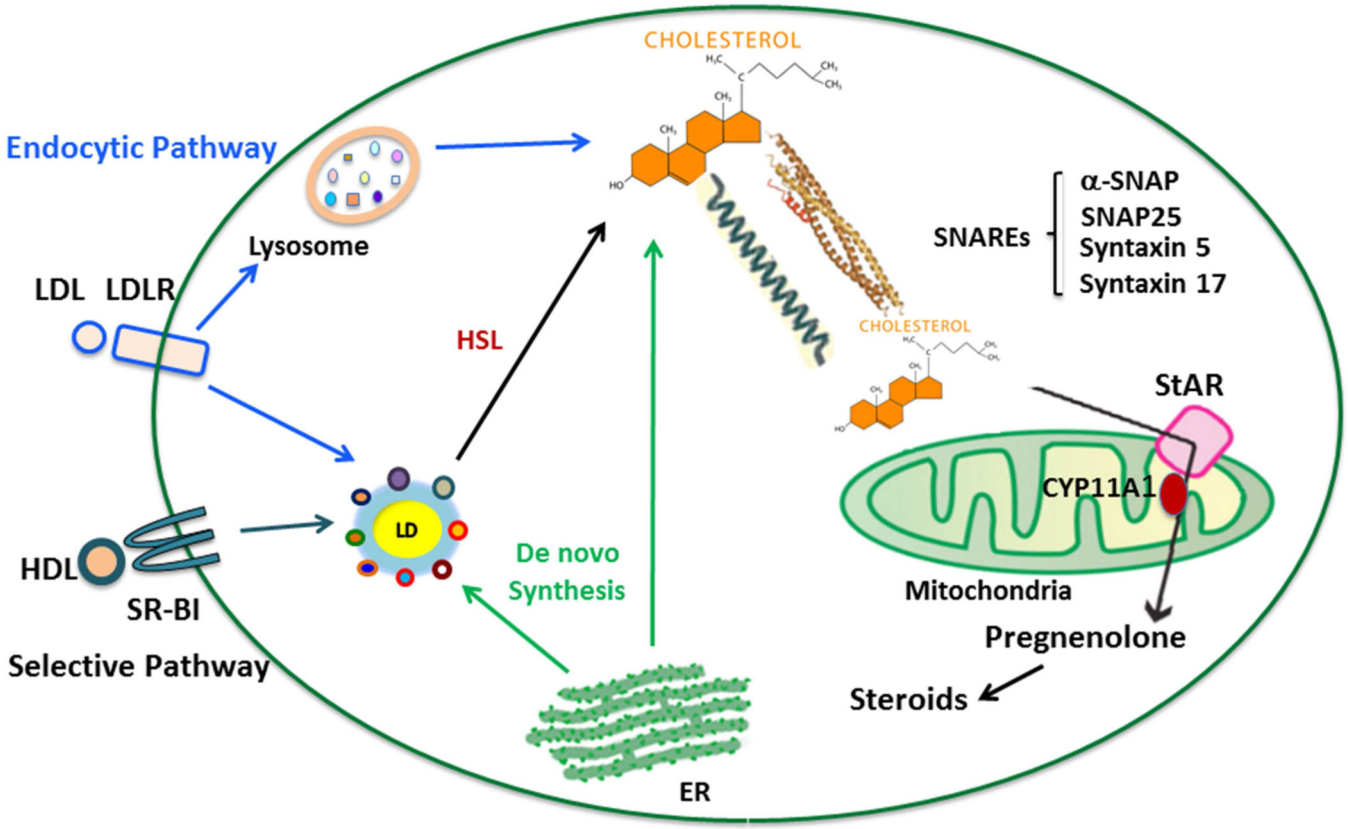

Figure 2.

Cartoon depicting the trafficking of cholesterol to mitochondria for steroidogenesis. Cholesterol for steroidogenesis can be supplied by *de novo* synthesis in the ER, via LDL receptor-mediated endocytic uptake and lysosomal hydrolysis, via SR-B1-mediated selective uptake of cholesteryl esters or via hydrolysis of cholesteryl ester-rich lipid droplets by hormone sensitive lipase. SNARE proteins such as α-SNAP, SNAP25, syntaxin-5 and syntaxin-17 facilitate the movement of free cholesterol to the outer mitochondrial membrane. StAR then mediates the movement of cholesterol from the outer to the inner mitochondrial membrane where CYP11A1 converts cholesterol to pregnenolone, the first steroid product. Abbreviations: ER, endoplasmic reticulum; HDL, high density lipoprotein; HSL, hormone-sensitive lipase; LD, lipid droplet; LDL, low density lipoprotein; LDLR, LDL receptor; SNARE, soluble NSF attachment protein receptor; StAR, steroidogenic acute regulatory protein.