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MINI-REVIEW

Mitochondrial cholesterol: mechanisms of import and effects on mitochondrial function

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Abstract Mitochondria require cholesterol for biogenesis and membrane maintenance, and for the synthesis of steroids, oxysterols and hepatic bile acids. Multiple pathways mediate the transport of cholesterol from different subcellular pools to mitochondria. In steroidogenic cells, the steroidogenic acute regulatory protein (StAR) interacts with a mitochondrial protein complex to mediate cholesterol delivery to the inner mitochondrial membrane for conversion to pregnenolone. In non-steroidogenic cells, several members of a protein family defined by the presence of a StAR-related lipid transfer (START) domain play key roles in the delivery of cholesterol to mitochondrial membranes. Subdomains of the endoplasmic reticulum (ER), termed mitochondria-associated ER membranes (MAM), form membrane contact sites with mitochondria and may contribute to the transport of ER cholesterol to mitochondria, either independently or in conjunction with lipid-transfer proteins. Model systems of mitochondria enriched with cholesterol in vitro and mitochondria isolated from cells with (patho)physiological mitochondrial cholesterol accumulation clearly demonstrate that mitochondrial cholesterol levels affect mitochondrial function. Increased mitochondrial cholesterol levels have been observed in several diseases, including cancer, ischemia, steatohepatitis and neurodegenerative diseases, and influence disease pathology. Hence, a deeper understanding of the mechanisms maintaining mitochondrial cholesterol homeostasis may reveal additional targets for therapeutic intervention. Here we give a brief overview of mitochondrial cholesterol import in steroidogenic cells, and then focus on cholesterol trafficking pathways that deliver cholesterol to mitochondrial membranes in non-steroidogenic cells. We also briefly discuss the consequences of increased mitochondrial cholesterol levels on mitochondrial function and their potential role in disease pathology.

Keywords Mitochondrial cholesterol · START proteins · Stard1 · Stard3 · MLN64 · MAM · Cholesterol trafficking · Mitochondrial function

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Introduction

Cholesterol levels in mitochondria are approximately 40-fold lower than in the plasma membrane, and 4.5-fold lower than in the endoplasmic reticulum (ER) (Horvath and Daum 2013). Because of its low abundance, relatively little attention has been paid to the role of mitochondrial cholesterol in mitochondrial lipid homeostasis and mitochondrial function. However, scarcity does not signify a lack of impact, and one could argue that the low levels of cholesterol make mitochondrial membranes highly sensitive to even small changes in their absolute cholesterol content. Mitochondria require cholesterol for biogenesis and maintenance of their membranes as well as for steroid, oxysterol and hepatic bile acid synthesis. Cholesterol levels in the mitochondrial membranes are determined by transport of cholesterol to the outer mitochondrial membrane (OMM), transfer from the outer to the inner mitochondrial membrane (IMM), and by the rate of cholesterol metabolism in the mitochondrial matrix. Multiple pathways exist to transport cholesterol from different sources to mitochondria, and their relative activity depends in part on the availability of cholesterol. Alterations in mitochondrial cholesterol import can change the cholesterol levels and biophysical properties of mitochondrial membranes and thus affect mitochondrial function. Increased mitochondrial cholesterol levels are associated with decreased antioxidant levels and changes in oxidative phosphorylation, which increases the

susceptibility to injury and cell death in several pathophysiological conditions. On the other hand, elevated mitochondrial cholesterol levels in cancer cells lower their chemosensitivity by preventing mitochondrial membrane permeabilization. Therefore, understanding the mechanisms of mitochondrial cholesterol homeostasis and their effects on mitochondrial and cellular function may reveal additional approaches of therapeutic intervention for a variety of pathological conditions.

We will briefly outline the mechanisms of mitochondrial cholesterol import for steroidogenesis, followed by a more detailed overview of potential transport pathways in non-steroidogenic cells and a discussion of functional consequences of alterations in mitochondrial cholesterol homeostasis.

Mitochondrial cholesterol import in steroidogenic cells

The steroidogenic acute regulatory protein Stard1

The acute steroidogenic response in adrenal and gonadal endocrine tissues is determined by the rapid synthesis, phosphorylation and degradation of the steroidogenic acute regulatory protein (StAR), now called Stard1, which is predominantly expressed in steroidogenic tissues and to some extent in liver cells (Manna et al. 2009; Hall et al. 2005). Loss-of-function mutations in Stard1 cause congenital lipoid adrenal hyperplasia, a disease characterized by impaired steroid hormone synthesis. Transcription of Stard1 is acutely upregulated in response to adrenocorticotropic and luteinizing hormones, and phosphorylation of Stard1 at the OMM further stimulates cholesterol transport and steroidogenesis (Arakane et al. 1997). Stard1 is expressed as a 37 kD protein with an OMM-targeted mitochondrial leader sequence and a lipid binding domain that accommodates one cholesterol molecule (reviewed in: (Miller 2007; Lavigne et al. 2010; Strauss et al. 2003)). Stard1 acts at the OMM, and mediates the transport of cholesterol from the OMM to the IMM (Bose et al. 2002; Arakane et al. 1998), which in turn regulates steroidogenesis by controlling the rate of cholesterol conversion to pregnenolone by the CYP11A1 enzyme in the mitochondrial matrix (Strauss et al. 2003; Miller and Bose 2011; Issop et al. 2012; Papadopoulos et al. 2007; Rone et al. 2009). Cholesterol delivery to the OMM does not rely on Stard1, but is mediated by other transport mechanisms. In steroidogenic cells, cholesterol used for steroidogenesis is largely derived from cholesteryl esters stored in lipid droplets (Kraemer et al. 2013), and could be transported from lipid droplets to mitochondria by lipid carrier proteins or be transferred across contact sites between the two organelles (Issop et al. 2012). Although the mechanisms of Stard1-mediated mitochondrial cholesterol import have been described in detail, some aspects still require further clarification, including the observation that depletion of Stard1 decreases total mitochondrial cholesterol levels despite its minor role in cholesterol transport to the OMM (Montero et al. 2008).

The mitochondrial cholesterol import complex in steroidogenic cells

To mediate cholesterol transport to the IMM, Stard1 interacts with the voltage dependent anion channel 1 (VDAC1) and the translocator protein (TSPO), formerly known as the peripheral benzodiazepine receptor (Bose et al. 2008a; Liu et al. 2006; West et al. 2001; Rone et al. 2012). VDAC1 and TSPO associate in the OMM and interact with the ATPase family AAA domain-containing 3A (ATAD3a) protein in the IMM, forming the core of a larger protein complex that mediates mitochondrial cholesterol import (Rone et al. 2012). Previous studies have suggested that the adenine nucleotide transporter (ANT), which interacts with VDAC1 to mediate ATP/ADP transport, plays a role in cholesterol import (McEnery et al. 1992); however, recent data clearly indicate that ANT is not part of the cholesterol import complex (Rone et al. 2012). Additional proteins associate with the complex on the cytosolic or the matrix side (Rone et al. 2012). For example, a protein kinase A anchoring protein (PAP7, also termed acyl-CoA binding domain-containing 3) binds to TSPO on the cytosolic side and recruits the protein kinase A regulatory subunit RI-alpha thus ensuring efficient phosphorylation of complexed Stard1 (Fan et al. 2010; Liu et al. 2003). CYP11A1 is associated with the complex on the matrix side (Rone et al. 2012).

The mechanism whereby VDAC, TSPO and ATAD3a collectively mediate cholesterol transport to the IMM is not fully known. Both VDAC1 and TSPO interact with cholesterol, but whether ATAD3a binds cholesterol is not yet known (Bay et al. 2008; Popp et al. 1995; Campbell and Chan 2007; Campbell and Chan 2007; Hiller et al. 2008; Lacapere and Papadopoulos 2003). VDAC spans the OMM in an open barrel structure, forming a hydrophilic diffusion pore for the exchange of small ions and metabolites across the OMM (Hiller et al. 2008; Bayrhuber et al. 2008; Hiller et al. 2010; Choudhary et al. 2014; Colombini 2012). Given the hydrophilicity of the diffusion pore, it is highly unlikely that cholesterol is transported through VDAC to the IMM. Instead, NMR analysis of VDAC in sterol-containing vesicles revealed two binding sites for cholesterol on the outside of the barrel structure (Hiller et al. 2008). TSPO has 5 transmembrane helices in the OMM, forming a pore-like arrangement that contains a cholesterol binding cholesterol-recognition amino acid consensus (CRAC) motif (Jamin et al. 2005; Li et al. 2001). It has been proposed that cholesterol bound to TSPO is released when Stard1, or other endogenous or synthetic ligands, activate TSPO, leading to cholesterol transfer to the

IMM (Rone et al. 2012; Lacapere and Papadopoulos 2003). Another model describes TSPO as an exchanger, transporting cholesterol into and pregnenolone or other sterols out of mitochondria (Lacapere and Papadopoulos 2003), but evidence for such export activity is still lacking. The third protein of the core import complex, ATAD3a, is a recently identified AAA-ATPase (ATPases Associated with diverse cellular Activities) that spans the IMM and has an N-terminal extension into the intermembrane space (Rone et al. 2012). ATAD3a is essential for mitochondrial morphology and for the formation of functional contact sites between mitochondria and endoplasmic reticulum (ER), possibly through interaction of its N-terminal domain with OMM proteins (Gilquin et al. 2010; Li et al. 2014), but its exact function is not yet fully characterized (Li and Rousseau 2012). In view of the ubiquitous expression of VDAC1, TSPO and ATAD3a, a similar core complex could mediate cholesterol transport from the OMM to the IMM in non-steroidogenic cells as well. Moreover, given that these proteins play key roles in several aspects of mitochondrial function, including contact site formation, mitochondrial dynamics, ion homeostasis, and bioenergetics, cholesterol transport may be co-regulated with these processes, and vice versa, cholesterol might influence mitochondrial function through interaction with the import complex proteins.

Mitochondrial cholesterol import in non-steroidogenic cells

The START domain protein family

Several potential pathways for cholesterol transport to the OMM and from the OMM to the IMM have been described in non-steroidogenic cells. Generally, cholesterol can move between subcellular membranes by vesicular transport, cytosolic lipid transfer proteins, or protein-mediated transfer across membrane contact sites (Mesmin and Maxfield 2009; Ikonen 2008; Lev 2010). Mitochondria are well known to form membrane contact sites with the ER (Raturi and Simmen 2013; van Vliet et al. 2014; Vance 2014; Hayashi et al. 2009), and can interact with lipid droplets (Murphy et al. 2009; Helle et al. 2013), the plasma membrane (Fridolfsson et al. 2012), and possibly with endosomes (Zhang et al. 2002; Sheftel et al. 2007; Calore et al. 2010). While vesicular transport to mitochondria has not been observed, both carrier-mediated transport and movement across membrane contact sites contribute to mitochondrial cholesterol import. START proteins are characterized by the presence of a StAR-related lipid transfer (START) domain with high structural homology to Stard1 (reviewed in: (Lavigne et al. 2010; Strauss et al. 2003; Calderon-Dominguez et al. 2014; Clark 2012; Alpy et al. 2009; Alpy and Tomasetto 2005; Alpy and Tomasetto 2014)). START domains consist of a conserved sequence of approximately 210 amino acids, folded in an α/β helix-grip structure to form a hydrophobic lipid binding pocket (Lavigne et al. 2010; Alpy and Tomasetto 2005; Ponting and Aravind 1999; Iyer et al. 2001). The mammalian START protein family has 15 members grouped into 6 subfamilies with different lipid binding specificities and/or additional functional domains (reviewed in: (Strauss et al. 2003; Clark 2012; Alpy and Tomasetto 2005; Alpy and Tomasetto 2014)). Five START proteins bind cholesterol, namely StAR itself (Stard1), Stard3, Stard4, Stard5 and Stard6. While Stard1 is targeted to mitochondria and Stard3 localizes to late endosomes, Stard4, Stard5 and Stard6 are cytosolic proteins that consist only of a START domain. Based on their structural similarity to Stard1 and the fact that they are not restricted to the OMM, these other cholesterol-binding START proteins could potentially transport cholesterol to the OMM, and in addition mediate cholesterol transfer from the OMM to the IMM in a mechanism comparable to Stard1. The mechanisms involved in these transport steps have not yet been fully elucidated.

Stard3/MLN64

Stard3 was initially identified in a breast cancer-derived metastatic lymph node cDNA library, and therefore termed MLN64 (Tomasetto et al. 1995). Unlike the other cholesterol-binding START domain proteins, the Stard3 protein consists of two functional domains, each comprising about half the protein (Alpy et al. 2001; Moog-Lutz et al. 1997; Watari et al. 1997). The C-terminus of Stard3 forms a cytosolic, cholesterol-binding START domain, with approximately 35 % sequence identity to Stard1 (Moog-Lutz et al. 1997; Watari et al. 1997; Bose et al. 2000a), while the N-terminal domain is comprised of four membrane-spanning helices that anchor Stard3 in the late endosomal perimeter membrane and also bind cholesterol (Alpy et al. 2005). Structural studies and molecular modeling have confirmed that the START-domains of Stard1 and Stard3 bind cholesterol in a similar manner (Lavigne et al. 2010; Bose et al. 2000a; Bose et al. 2000b; Tsujishita and Hurley 2000; Reitz et al. 2008; Murcia et al. 2006), suggesting that Stard3, like Stard1, mediates mitochondrial cholesterol import. Indeed, the addition of a bacterially expressed soluble Stard3-START domain increases steroidogenesis in isolated placental mitochondria (Zhang et al. 2002; Tuckey et al. 2004), indicating that Stard3-START can mediate the transport of cholesterol from the OMM to the IMM similar to Stard1. Ectopic expression of only the Stard3-START domain in steroidogenic cells or in cells co-transfected with CYP11A1 stimulates pregnenolone synthesis nearly to the same extent as expression of Stard1 (Watari et al. 1997; Bose et al. 2000a; Tuckey et al. 2004;

Soccio et al. 2005). Full-length Stard3 might, therefore, be expected to mediate the transfer of endosomal cholesterol to the OMM and IMM. Several studies have found increased mitochondrial pregnenolone or bile acid formation in cells transfected with full-length Stard3 (Zhang et al. 2002; Watari et al. 1997; Ren et al. 2004). The increase in pregnenolone formation due to Stard3 expression was more pronounced, when specifically endosomal cholesterol was followed using radiolabeled cholesteryl esters incorporated into lipoproteins (Zhang et al. 2002), consistent with Stard3-mediated transport of endosomal cholesterol to mitochondria. However, full-length Stard3 stimulates mitochondrial cholesterol import to a much lesser extent than Stard1 or soluble Stard3-START (Watari et al. 1997). The difference in the activity of Stard3-START and full-length Stard3 can be ascribed to the presence of the N-terminal domain, which exerts additional effects on endosomal cholesterol homeostasis. Thus, ectopic expression of high levels of full-length Stard3 or the Stard3-N-terminal domain causes the formation of enlarged, cholesterol-filled late endosomes (Alpy et al. 2005; Alpy et al. 2002; Holtta-Vuori et al. 2005; Liapis et al. 2012), although endosomal morphology in cells expressing low levels of Stard3 appears normal (Holtta-Vuori et al. 2005). The expression of only the Stard3-N-terminal domain also caused a nearly 3-fold decrease in progesterone synthesis from lipoprotein-derived, endosomal cholesterol in COS cells transfected with CYP11A1 and 3β-hydroxysteroid reductase (Zhang et al. 2002). These findings suggest that the Stard3-N-terminal domain, when present above certain threshold levels, sequesters cholesterol in the endosomal perimeter membrane, possibly due to its oligomerization (Alpy et al. 2005; Alpy et al. 2002; Alpy and Tomasetto 2006), and can inhibit the import of endosomal cholesterol into mitochondria. In addition, a recently identified FFAT (two phenylalanines in an acidic tract) motif in the Stard3-N-terminal domain may lead to Stard3 interaction with the ER protein VAP-A (Alpy et al. 2013), and thus counteract Stard3-mediated cholesterol transfer to mitochondria. Based on findings of decreased cholesterol synthesis and esterification in macrophages overexpressing Stard3, it has been proposed that Stard3 stimulates cholesterol transport to distinct pools in the ER that are accessible to sterol-regulatory element binding proteins (SREBP), but not to acetyl-CoA acyltransferase (Borthwick et al. 2010).

The endosomal cholesterol sequestration by high levels of the Stard3-N-terminal domain makes it difficult to assess Stard3-mediated mitochondrial cholesterol import by ectopic expression of Stard3. Work from our laboratory shows that depletion of Stard3 decreases pregnenolone formation in CHO cells expressing a CYP11A1 fusion construct (Charman et al. 2010; Kennedy et al. 2012), suggesting that endogenous Stard3 transports cholesterol to mitochondria. The correlation between mitochondrial cholesterol levels and Stard3 expression in cells exposed to anthrax lethal toxin is also consistent with Stard3-mediated mitochondrial cholesterol import: cells resistant to the toxin had low Stard3 and mitochondrial cholesterol levels, while cells susceptible to the toxin had high Stard3 and mitochondrial cholesterol levels (Ha et al. 2012). Mitochondrial cholesterol import is impaired in embryonic fibroblasts and primary granulosa cells from transgenic mice expressing a Stard3 protein with a loss-of-function mutation in the START domain (Kishida et al. 2004). However, the same transgenic mice lacking Stard3-START function have only minor alterations in cholesterol metabolism in vivo (Kishida et al. 2004), indicating high redundancy in mitochondrial cholesterol import pathways, or that Stard3 mediates transient changes in cholesterol trafficking, which are masked by chronic adjustments of lipid metabolism in the animals. In addition, Stard3-mediated cholesterol transport to mitochondria may mostly play a role under (patho)physiological conditions like Niemann-Pick Type C1 (NPC1)-deficiency, where endosomal cholesterol levels are increased. The late endosomal NPC1 protein mediates the transport of endosomal cholesterol to the ER and the plasma membrane (Ory 2004; Karten et al. 2009; Rosenbaum and Maxfield 2011). When NPC1 is absent or dysfunctional, unesterified cholesterol accumulates in late endosomes, and cellular cholesterol homeostasis is disturbed due to impaired cholesterol trafficking to the ER. NPC1-deficient cells and tissue have elevated mitochondrial cholesterol levels (Charman et al. 2010; Yu et al. 2005; Fernandez et al. 2009), which are lowered by depletion of Stard3, suggesting that more cholesterol is transported by Stard3 to mitochondria when endosomal cholesterol levels are high (Charman et al. 2010). Despite the increased mitochondrial cholesterol levels in NPC1-deficient cells, cholesterol arrival at the IMM was the same as in wildtype cells (Charman et al. 2010), indicating that cholesterol transfer from the OMM to the IMM became limiting under these conditions.

In view of the similarities of Stard3 and Stard1, it is conceivable that Stard3 interacts with a similar cholesterol import complex of VDAC1, TSPO and ATAD3a. Depletion of VDAC1 decreases cholesterol arrival at the IMM in CHO cells (Kennedy et al. 2012), indicating a role for VDAC1 in cholesterol import in non-steroidogenic cells, but whether this occurs through interaction with Stard3 is unknown. For full-length Stard3, such a mechanism would require at least transient contact between endosomes and mitochondria. Close apposition between mitochondria and endosomes has been described (Zhang et al. 2002), but contact site formation has been reported only under special conditions (Sheftel et al. 2007; Calore et al. 2010). Alternatively, the Stard3 START domain could be cleaved from the transmembrane domain and translocate to mitochondria. This mechanism has been proposed to mediate placental steroidogenesis (Bose et al. 2000a; Bose et al. 2000b; Tuckey et al. 2004), but there is no evidence for Stard3 cleavage in other cells. In addition, incubation with

low-density lipoproteins increases pregnenolone formation in CHO cells expressing CYP11A1 even when the cells are semi-permeabilized to remove cytosolic proteins (Charman et al. 2010), which speaks against the need for soluble factors in the transport of endosomal cholesterol to mitochondria. Indirect movement of endosomal cholesterol through the ER to mitochondria is possible, but seems unlikely in view of the accumulation of mitochondrial cholesterol in NPC1-deficiency. Clearly, further studies are required to elucidate the role of Stard3 in cholesterol trafficking. Given that endosomal cholesterol accumulates in NPC1-deficient cells but not in Stard3-depleted cells, Stard3-mediated cholesterol transport likely represents only a small fraction of total endosomal cholesterol transport, which can, however, still be of functional relevance. Stard3 also has effects not directly related to cholesterol transfer: a recent study showed that Stard3 influences endosome positioning through local sterol enrichment of the endosomal perimeter membrane, which in turn regulates endosomal association with the actin cytoskeleton (Holtta-Vuori et al. 2005).

Cytosolic START domain proteins of the Stard4 subfamily

The three members of the Stard4 subfamily that consist only of the cholesterol-binding START domain (Stard4, Stard5, and Stard6), have cholesterol transfer activity in vitro and in vivo (reviewed in: (Calderon-Dominguez et al. 2014)), and could therefore transport cholesterol to the OMM, as well as act through a mechanism similar to Stard1 to mediate cholesterol transfer to the IMM.

Stard4 is ubiquitously expressed. Low cellular cholesterol levels and early ER stress upregulate Stard4 expression through the transcription factors SREBP2 and ATF, respectively (Soccio et al. 2005; Yamada et al. 2006). Overexpression studies of Stard4 confirm its ability to transfer cholesterol to mitochondria and/or the ER. Thus, Stard4 increases progesterone formation in COS7 cells expressing CYP11A1 and 3β-hydroxysteroid reductase (Soccio et al. 2005), stimulates bile acid and cholesteryl ester synthesis in hepatocytes (Rodriguez-Agudo et al. 2008), and increases cholesterol esterification in a human osteosarcoma cell line (Mesmin et al. 2011). Depletion of Stard4 in HepG2 hepatoma cells leads to a retention of cholesterol at the plasma membrane, reduced cholesterol esterification, and lower cholesterol levels in the ER (Garbarino et al. 2012). In one study, the impaired cholesterol transport to the ER in Stard4-depleted cells was restored by microinjection of methyl-β-cyclodextrin, a synthetic cyclic oligosaccharide that complexes cholesterol much like a carrier protein (Mesmin et al. 2011). This similarity further indicates that Stard4 acts to equilibrate cholesterol content among cellular membranes according to their biophysical properties, preferentially targeting the ER and mitochondria. However, similar to the observations in mice with a loss-of-function mutation in the Stard3-START domain, mice lacking Stard4 show only minor alterations in lipid metabolism (Riegelhaupt et al. 2010), highlighting the redundancy in mitochondrial cholesterol import pathways, and that chronic alterations in lipid metabolism may mask the absence of Stard4-mediated transient movements of cholesterol (Calderon-Dominguez et al. 2014).

The roles of Stard5 and Stard6 are less well characterized than that of Stard4. Stard5 binds not only cholesterol and hydroxycholesterols, but also primary bile acids, raising the possibility that Stard5 acts as a bile acid transporter rather than a sterol carrier (Letourneau et al. 2013a; Letourneau et al. 2013b; Letourneau et al. 2012). Stard6 is highly efficient in transporting cholesterol to mitochondria for pregnenolone formation (Bose et al. 2008b). However, Stard6 is mostly expressed in male germ cells (Bose et al. 2008b; Soccio et al. 2002; Gomes et al. 2005), with some expression in the ovary (LaVoie et al. 2014), and therefore does not seem to represent a general mechanism for cholesterol trafficking to mitochondria.

Cholesterol transport from the ER to mitochondria

There are two main potential pathways for the transport of ER-derived cholesterol to mitochondria, namely transport via the plasma membrane and transport across ER-mitochondria membrane contact sites. Most endogenously-synthesized cholesterol is first transported to the plasma membrane and could reach mitochondria from there by cytosolic carrier proteins (Mesmin and Maxfield 2009; Ikonen 2008; Lev 2010). However, cholesterol transport from the plasma membrane to mitochondria is most active when plasma membrane cholesterol levels are above a certain threshold (Lange et al. 2009), which makes it less likely to contribute to mitochondrial cholesterol homeostasis when cellular cholesterol levels are low. The second potential pathway, the transfer of cholesterol across ER-mitochondria membrane contact sites, is supported by several lines of evidence, but still requires direct experimental proof. ER-mitochondria contact sites are formed by protein-protein interaction (but not membrane fusion) between the OMM and specialized, highly dynamic subdomains of the ER, termed mitochondria-associated ER membranes (MAM). MAM play a role in processes including phospholipid synthesis and transport, calcium homeostasis, mitochondrial morphology, autophagy and cell survival (reviewed in: (Raturi and Simmen 2013; van Vliet et al. 2014; Vance 2014; Hayashi et al. 2009)). Up to 20 % of the surface of the mitochondrial network is in close apposition with MAM (Rizzuto et al. 1998). Compared to the bulk of the ER and to mitochondria, MAM are enriched in cholesterol (Area-Gomez et al. 2012; Fujimoto et al. 2012; Hayashi and Fujimoto 2010; Williamson et al. 2011). The presence of acetyl-CoA acyltransferase and HMG-CoA reductase in MAM (Vance 2014;

Rusinol et al. 1994) points to a role for MAM in cholesterol metabolism and possibly in mitochondrial cholesterol import. This hypothesis is supported by observations that depletion of mitofusin-2, a key component of ER-mitochondria contact sites, reduces cAMP-induced steroidogenesis in MA10 Leydig cells (de Brito and Scorrano 2008; Duarte et al. 2012), and that VDAC1 and ATAD3a are parts of ER-mitochondria contact sites (Issop et al. 2012; Rone et al. 2012; Li and Rousseau 2012; Fang et al. 2010). Further indications for a direct transfer of ER cholesterol to mitochondria come from studies in caveolin-1-deficient cells and mice (Bosch et al. 2011a; Bosch et al. 2011b). Caveolin-1 is a cholesterol-binding protein best known for its role in the formation of plasma membrane caveolae, and may also contribute to the transport of cholesterol from the ER to the plasma membrane (Smart et al. 1996). Deficiency of caveolin-1 not only affects the plasma membrane, but also leads to mitochondrial cholesterol accumulation, elevated plasma steroid levels in caveolin-1-deficient mice and to increased pregnenolone synthesis in caveolin-1-deficient CHO cells expressing CYP11A1 (Bosch et al. 2011a; Bosch et al. 2011b). It has been proposed that in the absence of caveolin-1, cholesterol is retained in the ER and therefore moves to a greater extent from MAM to mitochondria (Bosch et al. 2011a; Bosch et al. 2011b). Cholesterol levels in the ER and Golgi of caveolin-1-deficient murine liver are indeed increased (Bosch et al. 2011b). Caveolin has also been reported to translocate into mitochondria from the plasma membrane (Fridolfsson et al. 2012), but was not found in mitochondrial fractions in other studies (Bosch et al. 2011a; Bosch et al. 2011b).

Based on the presence of VDAC1 and ATAD3a in ER-mitochondrial contact sites, the transport of cholesterol from MAM into mitochondria, if it occurs, may utilize a similar protein complex as the import of cholesterol into steroidogenic mitochondria. To date, none of the cholesterol-binding START proteins have been clearly localized to ER-mitochondria contact sites, and they may not be required for this pathway. In hepatoma cells, cholesterol biosynthesis inhibition or depletion of Stard1 both decrease mitochondrial cholesterol levels (Montero et al. 2008). ER stress increases contact site formation between ER and mitochondria (van Vliet et al. 2014; Bravo et al. 2011), and is associated with increased expression of Stard1, Stard4 and Stard5 (Soccio et al. 2005; Yamada et al. 2006; Barbero-Camps et al. 2014; Fernandez et al. 2013; Rodriguez-Agudo et al. 2012), and an increase in mitochondrial cholesterol levels (Barbero-Camps et al. 2014; Fernandez et al. 2013; Lluis et al. 2003). In neuroblastoma cells exposed to amyloid-β, mitochondrial cholesterol accumulation was prevented by treatment with an ER stress inhibitor, which also reduced the expression of Stard1 and Stard3 (Barbero-Camps et al. 2014). Taken together, these observations suggest that cholesterol can be transported from ER to mitochondria across contact sites, and that START proteins may stimulate this pathway, for example under conditions of ER stress. However, further investigation is required to determine how endogenously-synthesized cholesterol is imported into mitochondria, and to delineate the roles of MAM, START proteins and mitochondrial membrane proteins.

Functional consequences of altered mitochondrial cholesterol homeostasis

Conditions of mitochondrial cholesterol accumulation

Cholesterol influences fluidity, permeability, curvature, lateral domain formation and other biophysical characteristics of cellular membranes. It is therefore not surprising that changes in the cholesterol content of mitochondrial membranes are associated with alterations in mitochondrial function. While some studies have used various techniques to enrich isolated mitochondria with cholesterol in vitro, most evidence is derived from observations of mitochondrial function in (patho) physiological conditions with increased mitochondrial cholesterol levels, for example certain cancers (Montero et al. 2008; Baggetto et al. 1992; Parlo and Coleman 1984), myocardial ischemia (Rouslin et al. 1982; Sangeetha and Darlin 2009), aging (Paradies et al. 1994; Paradies and Ruggiero 1991; Paradies and Ruggiero 1990), hepatosteatosis following ethanol or high cholesterol feeding (Lluis et al. 2003; Colell et al. 1997; Coll et al. 2003; Mari et al. 2006), Alzheimer disease models (Fernandez et al. 2009; Barbero-Camps et al. 2014; Barbero-Camps et al. 2013), and NPC1-deficiency (Charman et al. 2010; Yu et al. 2005; Fernandez et al. 2009). Mice deficient in caveolin-1 and mice transgenic for SREBP2 also accumulate cholesterol in mitochondria (Fernandez et al. 2009; Bosch et al. 2011a; Barbero-Camps et al. 2013). Decreased mitochondrial cholesterol levels have been measured in kidney mitochondria from rats with hypothyroidism (Martinez-Abundis et al. 2007) and in rat heart tissue following exercise (Ziolkowski et al. 2013), and may also be decreased in brain mitochondria in Huntington disease (Eckmann et al. 2014). Here, we will focus on studies in which manipulations of cholesterol homeostasis or experiments in isolated mitochondria support a link between mitochondrial cholesterol content and functional effects.

Biophysical changes in mitochondrial membranes with high cholesterol levels

Incubation of isolated mitochondria with cholesterol in solution (Yu et al. 2005; Dietzen and Davis 1994), in liposomes (Madden et al. 1980; Hackenbrock and Chazotte 1986),

complexed to albumin (Martinez et al. 1988) or to Sephadex beads (Parlo and Coleman 1984; Dietzen and Davis 1994) leads to a rapid increase in mitochondrial membrane cholesterol levels without significant changes in other lipids. Most cholesterol delivered in vitro intercalates into the OMM; however, IMM cholesterol also increases (Montero et al. 2008; Coll et al. 2003; Colell et al. 2003). Key consequences of elevated mitochondrial cholesterol levels are decreased membrane fluidity and increased microviscosity; both in mitochondria enriched with cholesterol in vitro (Bosch et al. 2011a; Baggetto et al. 1992; Colell et al. 2003; Paradis et al. 2013), and in mitochondria isolated from cells or tissue where mitochondrial cholesterol levels are increased due to (patho) physiological conditions (Montero et al. 2008). The decrease in membrane fluidity correlates with the degree of cholesterol enrichment, and can be reversed by cholesterol extraction using methyl-β-cyclodextrin (MβCD) (Montero et al. 2008; Bosch et al. 2011a; Ziolkowski et al. 2010). Another biophysical consequence observed in cholesterol-enriched mitochondria and in mitochondria isolated from rat liver tumor tissue is a decrease in the passive proton permeability of the IMM (Baggetto et al. 1992).

Impaired mitochondrial glutathione import and oxidative stress

Elevated mitochondrial cholesterol levels and reduced membrane fluidity decrease 2-oxoglutarate carrier activity (Coll et al. 2003), which leads to reduced α-ketoglutarate transport and impaired glutathione (GSH) import into mitochondria (Fernandez et al. 2009; Bosch et al. 2011a; Barbero-Camps et al. 2014; Fernandez et al. 2013; Lluis et al. 2003; Colell et al. 1997; Mari et al. 2006; Barbero-Camps et al. 2013; Caballero et al. 2009; Llacuna et al. 2011). In vitro extraction of cholesterol from mitochondria or fluidization of the membrane without changing the cholesterol content normalize mGSH transport (Coll et al. 2003). Whether other transport processes are affected by cholesterol is less clear. Mitochondrial cholesterol has been reported to increase the export of citrate (Parlo and Coleman 1984) and to decrease phosphate and pyruvate transport (Paradies et al. 1999; Paradies et al. 1992), but these effects have not been consistently observed (Dietzen and Davis 1994).

GSH is a major antioxidant in mitochondria and also regulates key TCA cycle enzymes through glutathionylation (Mailloux et al. 2013; Murphy 2012). Since GSH is only synthesized in the cytosol, the impaired GSH import into mitochondria leads to a depletion of mitochondrial GSH (mGSH) and greater sensitivity to oxidative stress-inducing agents, which is reflected in higher ROS generation by mitochondria with increased cholesterol levels compared to control mitochondria following exposure to amyloid-β or TNFα (Fernandez et al. 2009; Mari et al. 2006). Mitochondrial cholesterol accumulation, lower mGSH levels, and increased ROS production are also observed in cells exposed to anthrax lethal toxin (Ha et al. 2012) or to acetaldehyde (Lluis et al. 2003), in a hepatoma cell line exposed to 10 nM insulin for 16 h (Mei et al. 2012), and in liver mitochondria from mice fed a high cholesterol diet (Llacuna et al. 2011). Depletion of Stard3 or inhibition of cholesterol biosynthesis normalizes mGSH levels in these experimental models (Ha et al. 2012; Lluis et al. 2003; Llacuna et al. 2011; Mei et al. 2012). Treatment with a membrane-permeable glutathione ethyl ester prevents cellular dysfunction and cell death in several cell models with increased mitochondrial cholesterol levels (Ha et al. 2012; Mailloux et al. 2013; Murphy 2012; Mari et al. 2009; Lash 2006; Mari et al. 2013), indicating that the depletion of mGSH is a key factor in the pathological consequences of mitochondrial cholesterol accumulation (Mari et al. 2013; Ribas et al. 2014).

Mitochondrial permeability transition and cell death

Mitochondrial membrane permeabilization in response to excessive cell stress is a key event in cell death (Sarosiek et al. 2013; Bender and Martinou 2013; Tait and Green 2010). At least two possible mechanisms appear to play a role: *i*) the permeabilization of both IMM and OMM by opening of an as yet undefined permeability transition pore complex, leading to depolarization, calcium release, mitochondrial swelling, and rupture of the OMM, and *ii*) the permeabilization of the OMM, mediated and regulated by members of the Bcl2 family, leading to the release of intermembrane space proteins, such as cytochrome c during apoptosis. Mitochondrial cholesterol influences both mechanisms of membrane permeabilization (Bosch et al. 2011b; Montero et al. 2010). Increased cholesterol levels in mitochondria inhibit permeability transition pore opening caused by the ANT ligand atractyloside, reactive oxygen species (ROS) or by high calcium levels (Montero et al. 2008; Colell et al. 2003). This effect is directly related to the decreased membrane fluidity caused by higher cholesterol levels, as treatment with agents that fluidize the membrane without changing its cholesterol content sensitize mitochondria to permeability transition (Montero et al. 2008; Colell et al. 2003). The inhibition of ANT-dependent membrane permeabilization was caused by increased levels of cholesterol in the IMM, as cholesterol enrichment of IMM mitoplasts also prevented atractyloside-induced permeability transition (Colell et al. 2003). Mitochondrial cholesterol accumulation also inhibits the permeabilization of the OMM by Bcl2 family members, as Bax oligomerization and subsequent pore formation are impaired when cholesterol levels in the membrane increase (Montero et al. 2008; Lucken-Ardjomande et al. 2008). Bax insertion into the membrane is not affected by cholesterol (Montero et al. 2008; Lucken-Ardjomande et al. 2008). Extraction of

cholesterol from hepatoma cells with MβCD decreases mitochondrial cholesterol levels and sensitizes cells to ROS- or calcium-induced OMM permeabilization (Montero et al. 2008). Interestingly, membrane permeabilization may also be inhibited in mitochondria with lower than normal cholesterol levels, as calcium-induced swelling was reduced in MβCD-treated rat liver mitochondria, and in mitochondria isolated from heart tissue following exercise, which also contained decreased levels of mitochondrial cholesterol (Ziolkowski et al. 2013; Ziolkowski et al. 2010).

Mitochondrial bioenergetics

Mitochondrial cholesterol levels also influence bioenergetics; however, the underlying mechanisms and the overall consequences for energy homeostasis vary among model systems and are not yet fully understood. Several studies have found decreases in ATP synthesis, ATP hydrolysis or respiration in mitochondria enriched with cholesterol. Thus, the maximum ATP synthesis in the presence of an uncoupler and succinate was decreased in cholesterol-enriched rat liver mitochondria (Echegoyen et al. 1993), and cholesterol enrichment of isolated brain mitochondria decreased ATP synthesis in the absence of added energy substrates (Yu et al. 2005). Mitochondria isolated from two different rat tumor cell types or from a hepatoma cell line have higher cholesterol levels than rat liver mitochondria and show decreased ADP-dependent respiration and lower ATP production per oxygen, respectively (Campbell and Chan 2007; Baggetto et al. 1992). Oxidative phosphorylation requires energy substrate import and oxidation, activity of electron transport chain complexes I to IV, ATP/ADP transport, and ATP synthase activity. A decrease in oxidative phosphorylation could theoretically be due to alterations in any of these steps. However, measurements performed in the presence of succinate bypass most oxidative metabolism and complex I, and the activities of complexes II, III, and IV of the electron transport chain seem to be unchanged by cholesterol-enrichment (Yu et al. 2005; Rouslin et al. 1982). Therefore, the lower ATP synthesis/hydrolysis rates observed in cholesterol-rich mitochondria in the presence of succinate are likely due to lower ATP synthase activity and/or impaired ADP/ATP exchange. It is difficult to experimentally distinguish decreased ATP synthase activity from a defect in ADP/ATP exchange in isolated mitochondria. Decreased ATPase activity in submitochondrial particles (small, inside-out IMM vesicles) prepared from mitochondria with elevated cholesterol levels from ischemic heart tissue, suggests that under certain conditions, IMM cholesterol directly affects ATP synthase activity, likely through altered membrane microviscosity (Rouslin et al. 1982). Defects in ATP/ADP exchange were found by live-cell monitoring of mitochondrial ATP in CHO cells with elevated mitochondrial cholesterol levels due to NPC1-deficiency (Kennedy et al. 2014). Respiration defects in NPC1-deficient CHO cells are prevented by Stard3 depletion, indicating that the bioenergetic alterations in these cells are due to mitochondrial cholesterol accumulation (Kennedy et al. 2014). ATP and ADP are transported by ANT in the IMM and VDAC in the OMM; therefore, defects in either one of these can impair nucleotide exchange. A study by Echegoyen et al. suggests that the effects of mitochondrial cholesterol on ATP hydrolysis are OMM-dependent, because direct enrichment of IMM mitoplasts with cholesterol did not alter ATPase activity, even though it was decreased when cholesterol was added to whole mitochondria (Echegoyen et al. 1993). In artificial membranes, sterols influence VDAC folding and conformation (Bay et al. 2008; Hiller et al. 2008; Thinnes and Burckhardt 2012; Mlayeh et al. 2010). Cholesterol-induced conformational changes in VDAC could, therefore, potentially affect its conductivity or selectivity or could exert more subtle effects on VDAC through altered binding affinities. VDAC function is regulated by a range of binding partners, including hexokinase, tubulin, Bcl2 family proteins, and parkin (Pastorino and Hoek 2008; Abu-Hamad et al. 2008; Rostovtseva et al. 2012; Arbel et al. 2012; Geula et al. 2012; Sun et al. 2012; Manczak and Reddy 2012; Shoshan-Barmatz and Golan 2012; Maldonado and Lemasters 2012; Rostovtseva and Bezrukov 2008). Alterations in the association of cytosolic regulatory proteins would not become apparent following in vitro enrichment of isolated mitochondria with cholesterol, but could be observed when mitochondrial cholesterol levels are increased in vivo. However, clear evidence that cholesterol affects the binding of hexokinase or other cytosolic proteins is still lacking, and one study has reported that, vice versa, hexokinase binding to VDAC increases OMM cholesterol levels (Campbell and Chan 2007). Some studies have found no effects of increased mitochondrial cholesterol levels on respiration or import of radiolabeled ADP (Dietzen and Davis 1994; Colell et al. 2003). Also, cholesterol-loading of prostate cancer cells with cholesterol/cyclodextrin complexes increased cellular and mitochondrial cholesterol levels, but did not change mitochondrial respiration (Prabhu et al. 2013). These seeming discrepancies among different studies may in part be due to different experimental conditions, but it is also possible that increasing mitochondrial cholesterol beyond a certain threshold does not further affect bioenergetics (Baggetto et al. 1992), and thus limits the effect of additional mitochondrial cholesterol enrichment in cancer cells. Moreover, different mechanisms of mitochondrial import could increase cholesterol levels mainly in the OMM or in both mitochondrial membranes, which could in turn have different functional consequences. While cholesterol accumulation in the IMM can directly impair ATP synthase activity, increased OMM cholesterol levels likely act mainly on OMM proteins such as VDAC, or exert secondary effects on the IMM (Montero et al. 2010).

Mitochondrial cholesterol in disease

Increased mitochondrial cholesterol levels have been observed in many different disease models that are also associated with well-characterized alterations in mitochondrial function.

Steatohepatitis describes an advanced stage of liver disease with a combination of lipid accumulation in the liver and inflammation, which develops following excessive lipid accumulation (steatosis) in the liver. Depending on the lipid composition of the diet, steatosis in mice involves predominantly triacylglycerol accumulation, or predominantly cholesterol accumulation. Only the accumulation of cholesterol, but not triacylglycerols, in the liver was associated with increased mitochondrial cholesterol levels, decreased mGSH levels and an increased susceptibility to inflammatory agents such as TNF/Fas ligand (Mari et al. 2006), indicating that steatosis with cholesterol accumulation is more likely to progress to steatohepatitis. Similar associations of increased mitochondrial cholesterol levels, mGSH depletion and susceptibility to TNF/Fas ligand were observed in livers of NPC1-deficient mice (Mari et al. 2006), and in alcohol-induced steatosis (Colell et al. 1997). Leptin-deficient mice (ob/ob mice), which show excessive weight gain and accumulation of both triacylglycerols and cholesterol in the liver, also have increased liver mitochondrial cholesterol levels and are more susceptible to liver inflammation (Mari et al. 2006). In these animal models, mitochondrial cholesterol accumulation was associated with increased ROS production and an increased susceptibility to ischemia/reperfusion injury in the liver, which was ameliorated by treatment with cholesterol biosynthesis inhibitors (Llacuna et al. 2011). Moreover, SREBP2 and Stard1 expression were increased in livers with diet-induced cholesterol accumulation, suggesting that the build-up of mitochondrial cholesterol in steatosis is largely caused by increased import of endogenously-synthesized cholesterol (Llacuna et al. 2011; Caballero et al. 2009).

Mitochondrial cholesterol levels are not only increased in NPC1-deficient livers, but also in the brains of NPC1-deficient mice and in NPC1-deficient cell lines (Charman et al. 2010; Yu et al. 2005; Fernandez et al. 2009; Kennedy et al. 2014). Mitochondrial cholesterol accumulation was observed within days following NPC1-depletion in cultured cells (Kennedy et al. 2014), suggesting that it may be an early event in the development of NPC disease. Mitochondrial dysfunction in NPC1-deficiency is indicated by observations of increased oxidative stress, mGSH depletion, alterations in energy metabolism, decreased mitochondrial DNA content and mRNA expression, and impaired mitophagy in NPC1-deficient cell and animal models (Fernandez et al. 2009; Porter et al. 2010; Cluzeau et al. 2012; Vazquez et al. 2012; Fu et al. 2010; Kennedy et al. 2013; Ordonez et al. 2012). In addition, alterations in metabolic gene expression and increased lactate levels in both NPC1-deficient brain and CHO cells suggest a shift towards greater reliance on glycolysis for ATP production (Kennedy et al. 2014; Kennedy et al. 2013). NPC1-deficient CHO cells show decreased mitochondrial respiration and impaired mitochondrial ATP/ADP exchange (Kennedy et al. 2014). Downregulation of Stard3 expression in NPC1-deficient CHO cells by RNA interference reduces mitochondrial cholesterol levels and prevents metabolic alterations and oxidative stress (Kennedy et al. 2014). Mitochondrial cholesterol levels, lactate secretion and oxidative stress also correlated in CHO cells, in which mitochondrial cholesterol levels were manipulated through the expression of mutants of the NPC2 protein with different transport characteristics (Kennedy et al. 2014). These findings indicate that the accumulation of endosome-derived cholesterol in mitochondria causes mitochondrial abnormalities in NPC1-deficiency; however, the extent to which this mechanism contributes to overall mitochondrial dysfunction and neuropathology is unknown.

Mitochondrial cholesterol levels are also increased in the brains of a murine model of Alzheimer disease (APP/PS mice) (Fernandez et al. 2009; Barbero-Camps et al. 2014; Barbero-Camps et al. 2013) and in neuroblastoma cells exposed to amyloid-β (Barbero-Camps et al. 2014). Mitochondrial cholesterol accumulation in these models is associated with ER stress, increased expression of SREBP2 and Stard1, and with higher levels of VDAC and the Sigma-1-receptor in MAM, suggesting increased cholesterol transport from the ER to mitochondria. Given that neuroblastoma cells exposed to amyloid-β have higher levels of endosomal cholesterol and higher Stard3 mRNA levels than untreated cells (Barbero-Camps et al. 2014; Mohamed et al. 2012), increased transport of endosomal cholesterol to mitochondria may also contribute to the mitochondrial cholesterol accumulation. The hypothesis that mitochondrial cholesterol accumulation and mGSH depletion contribute to Alzheimer disease pathology is further supported by findings that *i*) overexpression of SREBP2 exacerbates mitochondrial cholesterol accumulation and certain aspects of neuropathology in APP/PS mice (Barbero-Camps et al. 2013), *ii*) treatment of neuroblastoma cells with membrane-permeable GSH-ethyl ester reduced amyloid-β-induced neurotoxicity without preventing ER stress (Barbero-Camps et al. 2014), and *iii*) in vivo treatment of APP/PS/SREBP-2 mice with GSH-ethyl ester decreased tau pathology and amyloid-β deposition in the brain (Barbero-Camps et al. 2013).

While in steatohepatitis and neurodegeneration the build-up of mitochondrial cholesterol is associated with susceptibility to injury and cell death, elevated mitochondrial cholesterol levels in cancer cells increase the resistance to cell death induced by chemotherapeutic agents (Montero et al. 2008). This chemoresistance is associated with impaired OMM permeabilization by Bax pore formation (Montero et al. 2008;

Lucken-Ardjomande et al. 2008). Cholesterol biosynthesis inhibitors and depletion of Stard1 lower mitochondrial cholesterol levels and sensitize hepatocellular carcinoma cells to chemotoxicity (Montero et al. 2008), suggesting that the high cholesterol synthesis observed in many cancer cells may indirectly support survival by increasing mitochondrial cholesterol levels. In addition, Stard3 expression is associated with higher stage breast and prostate cancers (Tomasetto et al. 1995; Moog-Lutz et al. 1997; Stigliano et al. 2007; Cai et al. 2010); and Stard3 depletion decreases cell proliferation of human breast cancer cell lines (Cai et al. 2010), suggesting that Stard3-mediated mitochondrial cholesterol import can also promote cancer cell survival through alterations in mitochondrial cholesterol levels or by promoting steroidogenesis (Stigliano et al. 2007). Increased TSPO expression in several types of cancer is often associated with increased cancer aggressiveness (Fafalios et al. 2009; Carmel et al. 1999; Batra and Alenfall 1994; Katz et al. 1990); however, given that TSPO function is not limited to cholesterol transport, this correlation does not necessarily demonstrate a linkage to mitochondrial cholesterol. Cancer cells are also characterized by alterations in energy metabolism to support continuous growth and proliferation. A key characteristic of this metabolic change is the so-called Warburg effect of aerobic glycolysis, whereby cancer cells rely heavily on glycolysis for ATP synthesis even in the presence of oxygen (Maldonado and Lemasters 2012; Metallo and Vander Heiden 2013; Cairns et al. 2011; Dang 2012). One hypothesis states that mitochondrial cholesterol accumulation increases hexokinase translocation to mitochondria (Pastorino and Hoek 2008; Campbell and Chan 2008), which would increase aerobic glycolysis in cancer cells. Decreased oxidative phosphorylation, as observed in several models of mitochondrial cholesterol accumulation, would also increase glycolysis; however, there is little evidence that mitochondrial dysfunction is the main cause for the Warburg effect (Boland et al. 2013; Koppenol et al. 2011). Thus, it is still unclear how far mitochondrial cholesterol contributes to bioenergetic changes in cancer or surrounding stromal cells. Similarly, further investigation is required to determine whether an increase in mitochondrial cholesterol levels promotes other aspects of mitochondrial function that promote cell survival and proliferation of cancer cells (Boland et al. 2013).

Conclusion

Mitochondrial cholesterol homeostasis has received increasing attention over recent years as it has become clear that mitochondrial cholesterol levels are altered in a range of pathological conditions and affect several aspects of mitochondrial function. While mitochondrial cholesterol import was first described in the context of steroidogenesis and Stard1 function, recent studies have addressed mitochondrial cholesterol homeostasis in non-steroidogenic cells. START domain proteins are key players in mitochondrial cholesterol import. Stard1 interacts with a complex of VDAC, TSPO and ATAD3a to transfer cholesterol to the IMM. Whether other START proteins act through a similar mechanism is not yet clear. Transport of cholesterol to the OMM can be achieved through endosomal Stard3, members of the cytosolic Stard4 subfamily, or across mitochondrial contact sites with the ER or lipid droplets; however, direct evidence for some of these pathways is still lacking. Mitochondria can receive cholesterol from all other subcellular membranes, creating considerable redundancy in mitochondrial cholesterol import pathways. The regulation of these different pathways is largely unknown. In many cases, mitochondrial cholesterol import increases due to increased cholesterol availability, for example due to endosomal cholesterol accumulation, increased cholesterol biosynthesis, closer contact between ER and mitochondria during ER-stress, or following non-physiological methods of cholesterol loading. This implies that there may be little regulation of the transport mechanism itself. Even if mitochondrial cholesterol accumulation is largely a secondary effect, it still has consequences for mitochondrial function, most prominently a decrease in mGSH import and alterations in oxidative phosphorylation. These alterations increase the sensitivity to other insults, such as oxidative stress or conditions that increase the need for oxidative phosphorylation. In cancer cells, the decreased susceptibility to OMM permeabilization appears to outweigh other potentially detrimental effects of mitochondrial cholesterol accumulation, and protect the cells from stress-inducing agents. The degree to which differences in the amount or distribution of cholesterol in OMM and IMM or other cellular characteristics, such as the sensitivity to oxidative stress, determine the overall effect of mitochondrial cholesterol accumulation on cellular function is unknown. Future work to elucidate the mechanisms of mitochondrial cholesterol import and the functional consequences of alterations in these pathways could reveal additional strategies to manipulate cell survival.

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