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Review

The role of mitochondrial fusion and StAR phosphorylation in the regulation of StAR activity and steroidogenesis

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ABSTRACT

The steroidogenic acute regulatory (StAR) protein regulates the rate-limiting step in steroidogenesis, *i.e.* the delivery of cholesterol from the outer (OMM) to the inner (IMM) mitochondrial membrane. StAR is a 37-kDa protein with an N-terminal mitochondrial targeting sequence that is cleaved off during mitochondrial import to yield 30-kDa intramitochondrial StAR. StAR acts exclusively on the OMM and its activity is proportional to how long it remains on the OMM. However, the precise fashion and the molecular mechanism in which StAR remains on the OMM have not been elucidated yet. In this work we will discuss the role of mitochondrial fusion and StAR phosphorylation by the extracellular signal-regulated kinases 1/2 (ERK1/2) as part of the mechanism that regulates StAR retention on the OMM and activity.
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1. Introduction

The regulation of steroidogenesis is predominantly controlled by pituitary-synthesized trophic hormones, the luteinizing (LH) (Dufau, 1998), the follicule-stimulating (FSH) and adrenocorticotropin hormones (ACTH), angiotensin II (Ang II) (Gallo-Payet and Battista, 2014; Manna and Stocco, 2005) and many other factors, such as cytokines (Bornstein et al., 2004; Haidan et al., 2000) and growth factors (Manna and Stocco, 2005). Normal function of both ovary and testis is long recognized to be dependent on LH and FSH. Primarily, LH acts on internal theca in the ovary and in Leydig cells in the testis, while FSH regulates the function of granulosa cells in the ovary and Sertoli in the testis. Ang II is considered to be the main hormonal stimulus of the zona glomerulosa, whereas ACTH is the main stimulus of both zona fasciculata and reticularis of the adrenal cortex.

Abbreviations: StAR, steroidogenic acute regulatory; OMM, outer mitochondrial membrane; IMM, inner mitochondrial membrane; ERK1/2, extracellular signal-regulated kinases 1/2; LH, luteinizing hormone; FSH, follicle-stimulating hormone; ACTH, adrenocorticotropin hormone; Ang II, angiotensin II; hCG, human chorionic gonadotropin; CAH, congenital adrenal hyperplasia; PKA, cAMP-dependent protein kinase A; PKC, protein kinase C; IMS, mitochondrial intramembranous space; VDAC1, voltage dependent anion channel; ATAD3a, ATPase family AAA domain containing 3A; MAPKs, mitogen activated protein kinases; AKAPs, A-kinase anchor proteins; 8Br-cAMP, 8-bromoadenosine 3′,5′-cyclic monophosphate; MKP, mitogen-activated protein kinase phosphatase; pERK, phospho-ERK; GST, glutathione S-transferase; MEK1/2, mitogen-activated protein kinase kinase 1/2; SHP2, src homology 2-containing phosphotyrosine phosphatase 2; ACSL4, Acyl-CoA synthetase 4; AA, arachidonic acid; AA-CoA, arachidonoyl-CoA; PP2A, serine/threonine 2A; Mfn, mitofusin; ER, endoplasmic reticulum; OPA1, optic atrophy 1; MAM, mitochondria associated membrane; Drp1, dynamin related protein 1.

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The classical mechanism utilized in trophic hormone-responsive steroid synthesis consists of an increase in the activation of a second messenger, which in turn, triggers a regulatory cascade resulting in the mobilization and delivery of cholesterol from the outer (OMM) to the inner (IMM) mitochondrial membrane (Crivello and Jefcoate, 1980; Privalle et al., 1983). Indeed, intramitochondrial cholesterol transport is the rate-limiting and regulating step in steroidogenesis, and is dependent on *de novo* protein synthesis, as inhibitors of protein synthesis have been shown to decrease the steroidogenic response (Davis and Garren, 1968; Ferguson, 1963).

This review will focus on the current level of understanding concerning the regulation of cholesterol transport and the role of protein phosphorylation with a view to prospective research. It will discuss viewpoints that may help to unravel the mechanisms governing Steroidogenic Acute Regulatory protein (StAR) retention in the OMM and steroidogenesis.

2. Steroidogenic Acute Regulatory protein (StAR)

StAR protein was discovered in search of the factor that triggers the rate-limiting and regulating step in acute steroidogenesis (Alberta et al., 1989; Krueger and Orme-Johnson, 1983; Pon et al., 1986; Stocco and Sodeman, 1991). The purification of the protein and the cloning and sequencing of the murine cDNA were accomplished in 1994 (Clark et al., 1994). In the following years, both the mouse and human structural genes for StAR and this amino acidic sequence (284 residues in mouse, 285 residues in human) were isolated and characterized (Clark et al., 1994; Sugawara et al., 1995). These characterizations allowed for the study of transcription regulation and structural analysis of StAR.

An analysis of several publications on the matter reveals a close correlation between StAR gene tissue-specific expression and tissue capacity to produce steroids, which indicates StAR specific role in steroidogenesis. Transient transfection experiments demonstrated that StAR cDNA-derived protein expression in MA-10 cells increased steroid synthesis in the absence of LH or human chorionic gonadotropin (hCG). In addition, transient transfection of COS-1 cells with StAR cDNA increased the conversion of cholesterol to pregnenolone (Lin et al., 1995; Stocco and Clark, 1996; Sugawara et al., 1995).

The crucial role of StAR in the regulation of steroidogenesis has been inferred from patients suffering from lipoid congenital adrenal hyperplasia (lipoid CAH), an autosomal recessive disorder in which both adrenal and gonadal steroid biosynthesis is severely impaired due to mutations in the StAR gene (Bose et al., 1996; Lin et al., 1995). The targeted disruption of the StAR gene in mice resulted in a phenotype that is essentially identical to that found in lipoid CAH in humans (Caron et al., 1997; Hasegawa et al., 2000). StAR expression, activation and extinction are regulated by protein kinase A (PKA), protein kinase C (PKC), as well as a host of other signaling pathways (Manna and Stocco, 2005; Stocco and Clark, 1996).

3. The role of StAR in acute steroidogenic response: retention in the OMM and steroidogenesis

StAR mechanism of action has been extensively studied, although its complete understanding remains elusive. StAR is synthesized as a 37-kDa protein, imported into mitochondria via a typical mitochondrial leader sequence and cleaved to a 30-kDa intramitochondrial form (Allen et al., 2006; Granot et al., 2007a; Jefcoate, 2002; Miller, 2013). Upon reaching the matrix, the 30-kDa StAR is degraded, with a half-life of 4–5 hours (Granot et al., 2007a; Midzak et al., 2011). Inside the matrix, StAR protein levels are controlled by the ATP-dependent Lon protease (Granot et al., 2007b). It has been recently pointed out that acute accumulation of StAR protein in the matrix provokes a “mitochondria to nucleus” signaling which, in turn, activates selected transcription of genes encoding mitochondrial proteases relevant for enhanced clearance of StAR (Bahat et al., 2014). Moreover, we have observed that the presence of mitochondrial extracellular signal-regulated kinases (ERK) is strictly necessary for protecting StAR from unknown proteases to avoid further degradation, which constitutes one of the mechanisms involved in the regulation of mitochondrial StAR levels (Duarte et al., 2014).

It was initially suggested that 37-kDa StAR is a “precursor” and that 30-kDa StAR is the biologically active “mature form”. Crystallography has indicated that 30-kDa StAR acts in the intramembranous space (IMS) to shuttle cholesterol from the OMM to the IMM (Tsujishita and Hurley, 2000). Additional data have also proposed that IMM proteolysis in adrenal cells is essential in cholesterol fluxes, with a role of StAR in the IMM (Artemenko et al., 2001; Jefcoate, 2002). In addition, it has been shown that disrupting mitochondrial membrane potential ($\Delta\Psi m$) prevents the appearance of 30-kDa StAR in the mitochondria and inhibits steroidogenesis, which suggests that StAR import into the mitochondrial matrix and its subsequent processing are necessary for normal steroidogenesis (Allen et al., 2006; Artemenko et al., 2001; Granot et al., 2007a).

However, deletion of 62 N-terminal amino acids results in a cytoplasmic form of StAR (N-62 StAR) which retains complete activity and appears to interact with the OMM (Black et al., 1994; Miller, 2007). Moreover, when expressed in cytoplasm or added to mitochondria *in vitro*, both the 37- and 30-kDa StAR forms are equally active (Arakane et al., 1996).

It was suggested that StAR mitochondrial leader serves to target the C-terminus of StAR to unidentified receptors or effectors on the OMM, *i.e.* that the mitochondrial import machinery is important in increasing the local effective concentration of StAR on the OMM, thus transiently augmenting StAR action before it is terminated by StAR import into the mitochondria (Arakane et al., 1998; Miller and Strauss, 1999). This model suggests that the active form of StAR is a partially unfolded form, where an N-terminal domain enters the mitochondria, whereas the partially unfolded molten globule form of the C-terminus interacts with the OMM. Direct evidence has been presented that StAR exists as such a molten globule. Limited proteolysis has identified an N-terminal domain that retains a significant degree of structure, while the C-terminal domain is less tightly folded at the low pH that StAR may experience at the mitochondrial membrane. These data suggest that this tightly folded N-terminal domain makes StAR pause as it enters the mitochondria, increasing the opportunity for the C-terminus to exert its activity.

StAR is constitutively active when immobilized on the OMM, but it is inactive when localized to the IMS or to the matrix (Bose et al., 2002). Cells expressing StAR immobilized on the outside of the OMM by fusion to the C-terminus of Tom20 achieve maximal constitutive steroidogenesis, while StAR bound to Tim9 in the IMS is inactive. The Tim9/StAR protein was found to be active with steroidogenic mitochondria *in vitro*, which shows that this protein was inactive in transfected cells because of its IMM location, not because of its structure. Similarly, StAR was also inactive when localized in the mitochondrial matrix (Bose et al., 2002).

Mitochondrial protein-import assays of StAR with a modified leader peptide confirmed that StAR acts exclusively on the OMM: slowing down StAR mitochondrial entry produces an increase in activity, whereas speeding its entry up generates a decrease. Again, StAR activity was determined by its occupancy time on the OMM.

These data further demonstrate that StAR acts exclusively on the OMM (Arakane et al., 1996; Bose et al., 2002), and its activity in promoting steroidogenesis is proportional to its residency time in such location (Bose et al., 2002). Thus, it is StAR cellular localization, not its cleavage, that determines whether it is active or not. StAR has a sterol-binding pocket that accommodates a single molecule of

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