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Review

A single cell level measurement of StAR expression and activity in adrenal cells

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ABSTRACT

The Steroidogenic acute regulatory protein (StAR) directs mitochondrial cholesterol uptake through a C-terminal cholesterol binding domain (CBD) and a 62 amino acid N-terminal regulatory domain (NTD) that contains an import sequence and conserved sites for inner membrane metalloproteases. Deletion of the NTD prevents mitochondrial import while maintaining steroidogenesis but with compromised cholesterol homeostasis. The rapid StAR-mediated cholesterol transfer in adrenal cells depends on concerted mRNA translation, p37 StAR phosphorylation and controlled NTD cleavage. The NTD controls this process with two cAMP-inducible modulators of, respectively, transcription and translation SIK1 and TIS11b/Znf36l1. High-resolution fluorescence in situ hybridization (HR-FISH) of StAR RNA resolves slow RNA splicing at the gene loci in cAMP-induced Y-1 cells and transfer of individual 3.5 kB mRNA molecules to mitochondria. StAR transcription depends on the CREB coactivator CRTC2 and PKA inhibition of the highly inducible suppressor kinase SIK1 and a basal counterpart SIK2. PKA-inducible TIS11b/Znf36l1 binds specifically to highly conserved elements in exon 7 thereby suppressing formation of mRNA and subsequent translation. Co-expression of SIK1, Znf36l1 with 3.5 kB StAR mRNA may limit responses to pulsatile signaling by ACTH while regulating the transition to more prolonged stress.

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1. StAR integrates inter-membrane cholesterol transfer with mitochondrial electron transfer processes

The steroidogenic acute regulatory protein (StAR) initiates steroidogenesis by transferring cholesterol from outside the mitochondria to cytochrome P450 11A1 (CYP11A1) in the inner mitochondrial membrane (IMM) (Artemenko et al., 2001; Caron et al., 1997; Clark et al., 1994; Kiriakidou et al., 1996). Even after adrenocorticotropic hormone (ACTH) stimulation, cholesterol metabolism by CYP11A1 in adrenal mitochondria can exceed StAR mediated transfer so that cholesterol normally does not accumulate. ACTH stimulated cholesterol accumulation is produced by the CYP11A1 inhibitor aminoglutethimide (AMG) resulting in up to 3–5 cholesterol molecules per CYP11A1. This stimulation is paralleled by cholesterol–CYP11A1 complex formation (Jefcoate et al., 1973), which has been reproduced in cultured bovine adrenal cells (DiBartolomeis and Jefcoate, 1984). Turnover of this pool of reactive cholesterol at CYP11A1 is driven by reduced nicotinamide adenine dinucleotide phosphate (NADPH) generated most effectively by succinate dehydrogenase and the ATP-dependent NADH/NADPH transhydrogenase (NNT) (Hanukoglu and Jefcoate, 1980; Yamazaki et al., 1995). This process competes with transfer to IMM Cyp11b1 as shown by the opposing effect of cholesterol accumulation at CYP11A1 (Yamazaki et al., 1993). Mitochondrial intermembrane 3

Abbreviations: 3′EU, extended 3′UTR; ACTH, adrenocorticotropic hormone; AMG, aminoglutethimide; CBP, CREB binding protein; CREB, cAMP responsive element binding protein 1; CRTC, CREB regulated transcription coactivator; CBD, C-terminal cholesterol binding domain; CHX, cycloheximide; FISH, fluorescence in situ hybridization; HR-FISH, high resolution fluorescence in situ hybridization; IMM, inner mitochondrial membrane; MMP, metalloproteases; NNT, NADH/NADPH transhydrogenase; N-SIM, Nikon's structured illumination microscope; NNT, nicotinamide nucleotide transhydrogenase; NTD, N-terminal regulatory domain; OP, o-phenanthroline; PKA, protein kinase A; p-RNA, primary RNA; SIK1, salt inducible kinase 1; sp-RNA, spliced primary RNA; StAR, steroidogenic acute regulatory protein; TSPO, translocator protein; VDAC, voltage-dependent anion channel.

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beta-hydroxysteroid dehydrogenase (Hsd3b2) may have activity integrated with StAR activity (Rajapaksha et al., 2016) to relieve product inhibition of CYP11A1.

2. StAR functions through C-terminal cholesterol binding domain

StAR consists of two domains: the N-terminal domain (NTD), which includes about 62 amino acids, and the C-terminal domain (CBD), which forms cholesterol complexes and is the conserved core of the STARD family. The NTD has the typical positive charge characteristics of other mitochondrial import sequences in the initial N-terminal amino acids and additional sequences that provide an unusually appreciable helical content and unusual dual cleavage sites (Bose et al., 1999).

The crystal structures of the CBD of StAR/STARD1 and StARD3 are similar even though they have very different specialized NTD (Kang et al., 2010; Letourneau et al., 2015). Each complex has a single cholesterol molecule. The transgenic deletion of the StAR gene in mice reproduces the pathology of human adrenal lipidemic hyperplasia (ALH) (Bose et al., 2002; Parker et al., 1998). Mutations, which cause the human disease, concentrate in the cholesterol binding domain rather than the NTD (Sahakitrungruang et al., 2010). However, the R182 mutation retains full cholesterol exchange activity but does not stimulate activity at CYP11A1 (Baker et al., 2005; Barbar et al., 2009).

The StAR activity under hormonal control is mediated by phosphorylation at S194 by cAMP and protein kinase A (PKA) in fasciculata cells, and by Ca–dependent kinases in glomerulosa cells (Dyson et al., 2009; Elliott et al., 1993). A second phosphorylation by extracellular signal-regulated kinase (ERK) at S232 affects mitochondrial import (Duarte et al., 2014). A large number of cholesterol molecules transferred for each newly synthesized StAR protein (Artemenko et al., 2001). This high turnover suggests that cholesterol activation of the CBD directs receptor-like activity for StAR. The cholesterol induced conformational change in StAR, which delivers a more flexible structure matches this concept. Such complexes are active on the mitochondrial outer membrane (OMM) where they may enrich cholesterol at sites proximal to the IMM mitochondrial permeability transition pore (mPTP). StAR transfer of cholesterol is inhibited by cholesterol sulfate with the consequence that cholesterol sulfatase can enhance activity (Sugawara and Fujimoto, 2004).

Protein cross-linking and many other studies like the yeast two-hybrid system suggest coordination with voltage-dependent anion channel (VDAC) proteins and translocator protein (TSPO)/peripheral benzodiazepine receptor (PBR) (Li et al., 2001; Prasad et al., 2015; Shanmughapriya et al., 2015). The systemic TSPO-ko mice retain full steroidogenic activity in the testis much as is seen in MA-10 cells (Tu et al., 2015). It is also seen for an SF1-cre directed conditional loss of TSPO in the testis (Tu et al., 2016). By contrast, this same mouse shows a loss of ACTH induced glucocorticoid synthesis in the adrenal cortex. There appears to be a difference in the StAR-mediated cholesterol transfer process in the testis compared to the adrenal cortex. We have emphasized here that adrenal cells can produce peak steroidogenesis at very low levels of StAR mRNA thus pointing to a much more efficient process. The integration of TSPO and the StAR NTD into a distinctive adrenal process provides one explanation for the difference. The established effect of TSPO on mitochondrial Ca-sensitive permeability channels also suggests that mitochondria may rapidly adapt with alternative controls over membrane contacts that enhance StAR activity.

Cholesterol transfer depends on the continuous translation of the 37kd StAR pre-protein with concomitant phosphorylation by PKA. Thus, Inhibition with cycloheximide (CHX) stops ACTH-stimulated steroidogenesis within 5 min, while causing an accumulation of cholesterol in the OMM that remains inaccessible to IMM CYP11A1 (Pon et al., 1986; Privalle et al., 1983). This cholesterol accumulation in intact mitochondria generated by CHX is comparable to that generated by inhibition of CYP11A1 by AMG. For CHX, the cholesterol is in the OMM and inaccessible to CYP11A1, whereas for AMG the pool forms cholesterol-CYP11A1 complexes and is completely converted in minutes to pregnenolone after removal of the inhibitor. This early data indicates that cholesterol can enter the OMM and any associated membranes without StAR. The CBD can function in COS1 cells as a TOM 20 chimera, thus demonstrating that activity on the OMM alone effects cholesterol transfer to the IMM (Bose et al., 2002). However, the rate and efficiency here are possibly 100 times lower than in adrenal cells suggesting that other factors including the NTD can enhance the turnover. This activity has been reconstituted with rat adrenal mitochondria and StAR CBD. Soluble *N*-ethylmaleimide-sensitive factor attachment protein receptors (SNAREs) were shown to be co-activators of StAR activity (Lin et al., 2016).

The inter-membrane cholesterol barrier is readily breached by mild treatments such as elevated Ca or hypotonic media. This is an issue for many early assays for StAR cholesterol transfer activity. The metabolism of this artificially transferred cholesterol is supported by NADPH generated by isocitrate but not by succinate in conjunction with the nicotinamide nucleotide transhydrogenase (NNT) (Yamazaki et al., 1995). This precaution is realized in the SNARE assay by using succinate to support CYP11A1 activity.

3. What is the role of the StAR NTD?

The sequence encompassing these NTD cleavage sites is highly conserved across species (Yamazaki et al., 2006). The cleavage pattern for bovine StAR in COS1 cells is similar to that for native StAR in MA-10 cells. Mutation of the conserved cleavage sites in bovine StAR singly and in combination show a complex additive cleavage process involving two separate processing pathways. The double mutation of these sites slowed but did not prevent NTD cleavage or decrease cholesterol metabolism in transfected COS1 cells. The IMM metalloproteases (MMP) that cleave the StAR NTD appear likely to interact with OMM VDAC1 each as participants in the mitochondrial permeability core complex (Shanmughapriya et al., 2015). This pore complex mediates the Ca inhibition of mitochondrial cholesterol metabolism, which is blocked by cyclosporine, a drug that binds to cyclophilin, a component of the pore complex (Kowluru et al., 1995). NTD-modulatory activity beyond the mitochondrial import function may involve the StAR 30–62 sequences, which are downstream of the first conserved MMP cleavage site. The MMP cleavage enzymes are located on the inner face of the IMM thus requiring transfer of p37 StAR to this point for cleavage. Recent evidence suggests that this cleavage facilitates interaction of StAR with the VDAC2, which then facilitates both cholesterol transfer and cleavage of this sequence (Prasad et al., 2015).

The role of IMM proteases in StAR activity is well demonstrated by the inhibition of cholesterol metabolism in Y-1 cells by o-phenanthroline (OP), a metalloprotease inhibitor (Artemenko et al., 2001). OP prevents NTD cleavage while inhibiting Br-cAMP-induced cholesterol metabolism but without any effect on the CYP11A1 cleavage of 20-hydroxy-cholesterol. When OP is washed out, the metabolism of cholesterol to pregnenolone continues as the cleavage of StAR ensues, but now without the need for the further synthesis of StAR. Four phosphorylated forms of p30-BAC/p32/rodent have been captured in 2D gels (Artemenko et al., 2001; Epstein and Orme-Johnson, 1991). A plausible model is that an IMM

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