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Regulation of Adrenocortical Steroid Hormone Production by RhoA-Diaphanous 1 Signaling and the Cytoskeleton

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Abstract

The production of glucocorticoids and aldosterone in the adrenal cortex is regulated at multiple levels. Biosynthesis of these hormones is initiated when cholesterol, the substrate, enters the inner mitochondrial membrane for conversion to pregnenolone. Unlike most metabolic pathways, the biosynthesis of adrenocortical steroid hormones is unique because some of the enzymes are localized in mitochondria and others in the endoplasmic reticulum (ER). Although much is known about the factors that control the transcription and activities of the proteins that are required for steroid hormone production, the parameters that govern the exchange of substrates between the ER and mitochondria are less well understood. This short review summarizes studies that have begun to provide insight into the role of the cytoskeleton, mitochondrial transport, and the physical interaction of the ER and mitochondria in the production of adrenocortical steroid hormones.

Keywords

adrenal cortex; cAMP; cytoskeleton; diaphanous 1; cortisol

1. Introduction

Steroid hormone biosynthesis in the adrenal cortex is controlled by multiple regulatory mechanisms, ranging from transcriptional, post-translational, to substrate transport. Over the past several decades numerous labs have provided insight into the molecular mechanisms by which signaling pathways control steroid hormone output. For example, the transcription of genes that are required for the uptake and transport of cholesterol and for its metabolism into cortisol and aldosterone requires several transcription factors (Miller and Auchus, 2011, Rainey and Nakamura, 2008, Sewer et al., 2007, Sewer et al., 2008), including steroidogenic factor 1 (El-Khairi et al., 2011, Hoivik et al., 2010, Schimmer and White, 2010), nerve growth factor 1B (Bassett et al., 2004, Bassett et al., 2004), members of the specificity protein family (e.g. Sp1, Sp3) (Huang et al., 2005, Lin et al., 2001, Rui et al., 2008), sterol regulatory element binding protein 1 (Ozbay et al., 2006), GATA proteins (Fluck and Miller, 2004, Jimenez et al., 2003, Nakamura et al., 2007), and cAMP response element modulator (Meier and Clark, 2012, Sugawara et al., 2006, Zwermann et al., 2007). Moreover, emerging

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evidence implicates the need for the assembly of macromolecular protein complexes that facilitate the movement of cholesterol into the inner mitochondrial membrane (Bose et al., 2002, Bose et al., 2008, Liu et al., 2006, Rone et al., 2012) and for regulatory mechanisms that govern the selective transfer of electrons (Miller, 2005). Additionally, the function of varied proteins involved in adrenocortical steroidogenesis is regulated in response to signaling cascades that promote the activation of kinases such as cAMP-dependent protein kinase (PKA) and extracellular-signal regulated kinase (ERK) (Le and Schimmer, 2001, Manna et al., 2011, Okamoto et al., 2004, Ramanjaneya et al., 2011, Romero et al., 2006). Finally, roles for bioactive sphingolipids in the control of cortisol and aldosterone secretion are also well established (Brizuela et al., 2007, Brizuela et al., 2006, Lucki and Sewer, 2008, Lucki et al., 2012, Rabano et al., 2003). While the regulatory mechanisms mentioned above are highlighted in many articles, this brief review will focus on the mechanisms that may enable substrate transport between the ER and mitochondria during steroid hormone production in the adrenal cortex.

2. Adrenocortical Steroidogenesis

Steroid hormones are essential regulators of varied physiological processes, including salt-water homeostasis, gluconeogenesis, and the inflammatory response. These molecules are synthesized in the adrenal cortex from cholesterol via a series of reactions that are catalyzed by members of the cytochrome P450 superfamily and 3β hydroxysteroid dehydrogenase, type 2 (HSD3B2). As depicted in Figure 1, in the first enzymatic reaction occurs in mitochondria, where P450 cholesterol side-chain-cleavage monooxygenase (CYP11A1) converts cholesterol to pregnenolone. Once pregnenolone is formed, it enters ER where it can be hydroxylated on carbon-17 by P450c17 α (CYP17A1) to form 17-hydroxypregnenolone or converted to progesterone by HSD3B2. CYP17A1 can also catalyze the formation of 17-hydroxyprogesterone. CYP17A1 also catalyzes a 17,20 lyase reaction on 17-hydroxypregnenolone and 17-hydroxyprogesterone, to form the adrenal androgens dehydroepiandrosterone (DHEA) and androstenedione, respectively. Notably, the final reactions in steroid hormone biosynthesis occur in mitochondria, where 11-deoxycortisol and deoxycorticosterone are converted to cortisol and corticosterone, respectively, by CYP11B1. Expression of aldosterone synthase (CYP11B2) in the zona glomerulosa of the adrenal cortex allows for the conversion of corticosterone to aldosterone. Given the organelle-specific expression of enzymes in the steroidogenic metabolic pathway, optimal hormone output dictates the need for regulatory mechanisms that facilitate the delivery of substrates between mitochondria and the ER.

3. Role of the Cytoskeleton in Organelle Positioning

The architectural integrity of cells is regulated by the cytoskeleton. However, the cytoskeleton also plays an integral role in mediating the ability of cells to respond to external and internal signaling cues. These responses are coordinated by the three major components of the cytoskeleton: microtubules, microfilaments, and intermediate filaments. Microtubules are comprised of polymerized tubulin via a process that is highly dynamic and regulated by cell signaling pathways that activate the hydrolysis of GTP. Consistent with the role of GTP in microtubule function, these polymers are typically associated with motor proteins such as kinesin and dynein. Microtubules regulate the intracellular transport of vesicles and organelles and the movement of flagella and cilia. Microfilaments are comprised of polymerized actin, which enable the assembly of cleavage furrows during cytokinesis. Intermediate filaments are composed of lamins, keratins, and vimentins. These fibers play a critical role in maintaining the spatial distribution of organelles and cellular structural integrity. In addition to these three structures, interactions between cytoskeletal components and effector proteins act to coordinate cellular processes such as vesicle and

organelle transport, mitosis and meiosis, gap junction-mediated cell communication, and cell migration.

Roles for all three components of the cytoskeleton have been found in the regulation of steroid hormone biosynthesis. Vimentin, an intermediate filament protein has been shown to play a role in lipid droplet migration in adrenal cells (Hall, 1997, Hall and Almahbobi, 1997). Light microscopic analysis of Y1 murine adrenal cells and bovine fasciculata cells have revealed that lipid droplets are attached to intermediate filaments (Almahbobi and Hall, 1990, Almahbobi et al., 1992). Moreover, Ca^{2+} , a modulator of adrenocortical steroidogenesis (Guagliardo et al., 2012, Sackmann et al., 2011, Wiederkehr et al., 2011), stimulates the phosphorylation of vimentin, a process thought to facilitate the reorganization of cellular components so as to promote hormone production in bovine fasciculata cells (Almahbobi et al., 1994). Recently, Shen and colleagues demonstrated that targeted disruption of vimentin in mice reduced cosyntropin-stimulated corticosterone production (Shen et al., 2012). The reduction in corticosterone secretion was concomitant with a decrease in human chorionic gonadotropin-stimulated progesterone production in female mice. Given that the interaction of vimentin with hormone sensitive lipase has been shown to regulate lipolysis and lipid droplet homeostasis in murine adipocytes (Shen et al., 2010) and importance of lipid droplet formation and trafficking for cholesterol delivery in adrenal and ovarian steroidogenesis, these findings implicate intermediate filaments as a key conduit in the delivery of cholesterol substrate.

In addition to a role for the intermediate filament protein vimentin, a significant body of literature implicates microtubules in steroidogenesis. For example, using coherent anti-Stokes Raman Scattering Microscopy to visualize live cells, lipid droplets were shown to move along microtubule tracts in Y1 murine adrenocortical cells (Nan et al., 2006). The effect of chemical agents that modulate the stability of microtubules and microfilaments on both adrenal and gonadal steroid hormone biosynthesis has also been an area of active investigation for several decades (Cortese and Wolff, 1978, Hall and Almahbobi, 1997, Lee et al., 2001, Rainey et al., 1985, Rainey et al., 1984, Rajan and Menon, 1985, Sackett and Wolff J, 1986, Whitehouse et al., 2002). In fact, dating back to the late 1970s Cortese and Wolf demonstrated that cytochalasins B, D and E, inhibitors of actin polymerization that disrupt the formation of microfilaments, stimulate steroid hormone production in Y1 adrenocortical cells (Cortese and Wolff, 1978). These studies were supported by subsequent experiments demonstrating that cytochalasin D increased basal, but not adrenocorticotropin (ACTH)-stimulated corticosteroid secretion in Y1 adrenocortical cells (Rainey et al., 1984).

Perturbing microtubule polymerization with colchicine has been found to stimulate corticosterone production in Y1 cells (Lee et al., 2001, Sackett and Wolff, 1986), but to inhibit the secretion of cortisol in H295R human adrenocortical cells (Li and Sewer, 2010). The stimulatory effect of colchicine on steroid hormone biosynthesis observed in Y1 cells could also be observed in the Kin-8 murine adrenocortical cell line, suggesting that the effects of colchicine are independent of the cAMP signaling pathway (Sackett and Wolff, 1986). Intriguingly, colchicine increased the basal and cAMP-stimulated secretion of DHEA in H295R cells (Li and Sewer, 2010). Finally, a role for microtubules in the uptake and processing of lipoproteins was demonstrated in rodent luteal cells where colchicine reduced the lipoprotein-stimulated production of progesterone (Rajan and Menon, 1985).

Using time-lapsed video imaging of MitoTracker-stained mitochondria in H295R human adrenocortical cells, we demonstrated that both ACTH and the cAMP analog dibutyryl cAMP (Bt_2cAMP) rapidly increase the rate of mitochondria movement (Li and Sewer, 2010). To link the cytoskeleton to ACTH/cAMP-stimulated increases in the rate of mitochondrial trafficking, we found that treating cells with colchicine and nocodazole,

which both promote microtubule depolymerization, attenuated ACTH-stimulated mitochondrial movement, while latrunculin B, which inhibits actin polymerization, and paclitaxel, which promotes microtubule stabilization increased mitochondrial trafficking independent of ACTH signaling. The functional significance of changes in the rate of mitochondrial movement were established by quantifying the secretion of cortisol and DHEA, where both colchicine and nocodazole attenuated basal and Bt₂cAMP-stimulated cortisol secretion, while promoting the production of DHEA. In contrast, latrunculin-B and paclitaxel increased basal secretion of cortisol but decreased DHEA biosynthesis (Li and Sewer, 2010). Further, latrunculin-B and paclitaxel inhibited Bt₂cAMP-stimulated DHEA secretion. Our data indicated that microtubule polymerization is required for cortisol secretion, but not DHEA secretion and that actin polymerization negatively affects steroidogenesis. Moreover, our studies were consistent with the stimulatory effect of cytochalasins that was observed in Y1 cells, but in conflict with the inhibitory effect of paclitaxel found on basal and cAMP-stimulated steroid hormone production in Y1 adrenocortical cells, but not in bovine adrenocortical cells (Rainey et al., 1985). The inconsistencies observed with paclitaxel in these three adrenocortical cells suggest that there may be additional species-specific regulatory mechanisms that control steroidogenic capacity.

Given the integral role that the cytoskeleton has been shown to play in steroidogenesis, it is likely that cell signaling promotes the selective interaction of effector proteins with microtubules, microfilaments, and intermediate filaments to enable the spatiotemporal trafficking of proteins and lipid substrates. Studies establishing the physical association between vimentin and hormone sensitive lipase (Shen et al., 2010) and hormone sensitive lipase and steroidogenic acute regulatory protein (Shen et al., 2003) lend support to this premise. Indeed, we have evidence that diaphanous 1 (DIAPH1) interacts with tubulin, actin, vimentin and other proteins in H295R human adrenocortical cells that may collectively act to facilitate the efficient transport of substrates during steroid hormone production (Li *et al.*, unpublished observations, 2012).

4. The Role of Rho-DIAPH1 Signaling in Cortisol Secretion

As discussed above, both our studies using agents that perturb the cytoskeleton and the work of other laboratories provided compelling evidence for the integral role that microtubules, microfilaments, and intermediate filaments play in regulating adrenocortical steroid hormone production. However, in order to further understand the mechanism by which ACTH signaling coordinates changes in the cytoskeletal architecture and how these changes impinge on mitochondrial and lipid droplet trafficking, we examined the role of effector proteins that are known to interact with components of the cytoskeleton. One family of proteins that is a well-documented regulator of the cytoskeleton and of cell and organelle migration is the Rho proteins.

Rho GTPases are members of the large family of low-molecular weight proteins that regulate varied cellular processes, including vesicle and organelle trafficking, phagocytosis, cell migration, as well as cytoskeletal dynamics (Bar-Sagi and Hall, 2000, Etienne-Manneville and Hall, 2002, Hall, 2005, Jaffe and Hall, 2005, Raftopoulou and Hall, 2004). Signal transduction cascades stimulate the activation of Rho proteins by facilitating the exchange of GDP for GTP. In the GTP-bound form, Rho proteins act to relay extracellular signals to intracellular effectors. Indeed, we found that ACTH and Bt₂cAMP rapidly increase the cellular content of active Rho in H295R cells, while overexpression of a GFP-tagged dominant negative RhoA inhibited mitochondrial movement and cortisol production, but increased the secretion of DHEA (Li and Sewer, 2010). The identification of RhoA as a mediator of ACTH/cAMP-stimulated mitochondrial movement and optimal cortisol output,

prompted us to investigate RhoA effector proteins. Although Rho-associated protein kinase is implicated in regulating the enzymatic activity of CYP17A1 in H295A human adrenocortical cells (Tee et al., 2008), we found no role for this kinase in mitochondrial movement. However, DIAPH1, a Rho effector was identified as a key regulator of mitochondrial movement and steroid hormone production.

DIAPH1 is a member of the diaphanous-related formin family of Rho effector proteins that regulate cell and organelle motility and migration by interacting with microtubules and microfilaments (Madaule Watanabe et al., 1997). DIAPH proteins contain an N-terminal Rho-binding domain, two formin-homology regions, and a C-terminal diaphanous-autoregulatory domain (DAD) that stabilizes the proteins in an inactive conformation in the absence of Rho binding. DIAPH1 is activated upon the binding of Rho, which promotes the dissociation of intramolecular interactions between the N-terminal region and the DAD (Alberts, 2001, Li and Higgs, 2003, Li and Higgs, 2005, Otomo et al., 2005, Rose et al., 2005). Activated cell signaling promotes the release of the intermolecular autoinhibitory interactions via a process that is accelerated by Rho-GTP (Lammers et al., 2005). Therefore, truncation mutants that lack the DAD or the Rho binding domain are constitutively active (Ishizaki et al., 2001, Li and Higgs, 2003, Li and Higgs, 2005, Watanabe et al., 1999). We have found that treatment of H295R cells with ACTH or Bt₂cAMP resulted in the rapid activation of RhoA and stimulated the interaction of RhoA with DIAPH1 (Li and Sewer, 2010). Moreover, overexpression of a dominant negative RhoA mutant impaired mitochondrial movement and reduced cortisol secretion (Li and Sewer, 2010). Overexpression of a DIAPH1 constitutively active mutant (Ishizaki et al., 2001, Yamana et al., 2006) rendered mitochondria in cells expressing the GFP-tagged mutant virtually immobile (Li and Sewer, 2010). A model for the role of RhoA and DIAPH1 is shown in Figure 2. There is precedence for a role for DIAPH1 in controlling mitochondrial movement, where Minin *et al.*, have found that lysophosphatidic acid inhibits mitochondrial movement in CV-1 cells by acting through a RhoA and DIAPH1-dependent mechanism (Minin et al., 2006). Given the role of DIAPH1 in regulating mitochondrial trafficking and adrenocortical steroid hormone biosynthesis, it will be important to further define the molecular mechanism by which this protein acts and to determine how ACTH/cAMP signaling regulates the function of this protein. We have preliminary data to demonstrate that DIAPH1 is a component of a larger protein complex and is phosphorylated in response to cAMP signaling (Li *et al.*, unpublished observations, 2012), however further studies are required to fully elucidate the role of DIAPH1 in adrenocortical steroidogenesis.

We have previously shown that activation of the ACTH signaling pathway in H295R human adrenocortical cells stimulates the degradation of complex sphingolipids and increases the secretion of the bioactive sphingolipid, sphingosine-1-phosphate (S1P) (Ozbay et al., 2004, Ozbay et al., 2006). The S1P produced is a ligand for the G protein-coupled S1P receptors and acts in a paracrine or autocrine manner to increase intracellular signaling and CYP17 gene expression (Ozbay et al., 2006). Notably, we have also shown that S1P increases mitochondrial movement in H295R cells (Sewer and Li, 2008). Given that the ability of A-kinase activating protein 13 to bridge PKA and RhoA signaling in HEK293 human embryonic kidney cells is stimulated by lysophosphatidic acid, a member of the S1P receptor family (Diviani et al., 2001), these studies collectively suggest that S1P may act as a mediator in ACTH-stimulated mitochondrial movement and cortisol biosynthesis. We have preliminary evidence showing that S1P increases the phosphorylation of DIAPH1 (Li and Sewer, unpublished observations, 2012), the functional significance of which is still unclear.

It is also possible that other Rho effector proteins may be activated in response to ACTH signaling. Miro proteins (Fransson et al., 2003, Fransson et al., 2006, Guo et al., 2005),

named for mitochondrial Rho, are similar to the Rho GTPases in their N-terminal GTPase domains, but they also contain EF-hand calcium-binding motifs (Kawasaki et al., 1998, Nelson and Chazin, 1998), and an additional putative GTPase domain in the C terminus which is diverged from the Rho GTPases. Since mitochondrial movement is associated with Ca^{2+} (Yi et al., 2004) and Miro1/2 have EF hands (Fransson et al., 2006), future efforts may probe the effect of ACTH and Bt_2cAMP on intracellular Ca^{2+} concentrations. Miro1 regulates Ca^{2+} -dependent mitochondrial movement as well as mitochondrial fusion-fission dynamics, where overexpression of Miro1 in H9c2 myocardial cells increased mitochondrial movement and fusion (Saotome et al., 2008). As shown in Figure 3, both Bt_2cAMP and S1P rapidly increase cellular Ca^{2+} concentrations in H295R cells, thus it is tempting to speculate that ACTH/cAMP signaling may increase intracellular S1P and Ca^{2+} , which may act as second messengers in conferring increased mitochondrial trafficking, and ultimately steroid hormone production.

5. Organelle trafficking and the cytoskeleton

Our previous studies identified a novel role for mitochondrial trafficking in enabling adrenocortical steroid hormone biosynthesis (Li and Sewer, 2010). Although much is known about the physiological significance of mitochondrial transport in neurons (Chada and Hollenbeck, 2004, Davis and Clayton, 1996, Morris and Hollenbeck, 1993, Morris and Hollenbeck, 1995), the role of dynamic mitochondrial movements in steroidogenesis is less clear. Mitochondria have been visualized in association with microfilaments (Morris and Hollenbeck, 1995), microtubules (Ball and Singer, 1982, Yi et al., 2004), and intermediate filaments (Stromer and Bendayan, 1990, Summerhayes et al., 1983) in various cells types. Further, mitochondria-bound motor proteins, such as kinesins and dyneins, facilitate the movement of organelles along cytoskeletal fibers (Gross, 2004, Hollenbeck and Saxton, 2005, Kwok and Kapoor, 2007, Miki et al., 2005, Tanaka et al., 1998, Verhey and Hammond, 2009). Interestingly, kinesin is required to align vimentin and microtubules (Gyoeva and Gelfand, 1991). Moreover, the mobility of vimentin filaments has been shown to be dependent on microtubules (Pralad et al., 1998). Given published reports establishing that vimentin plays a central role in adrenocortical steroid hormone production (Shen et al., 2012) and that vimentin interacts with hormone sensitive lipase and lipid droplets (Shen et al., 2010), it is tempting to speculate that coupling to kinesin and microtubules may provide a mechanism by which cholesterol substrate is transported in adrenocortical cells. However, our preliminary data identifying vimentin and kinesin as DIAPH1 binding partners (Li *et al.*, unpublished observations, 2012), suggest that tethering to microtubules, and possibly kinesin motors, may also play a role in the movement of substrates between ER and mitochondria. Further studies are warranted to elucidate the precise molecular mechanism by which ACTH/cAMP-stimulated, microtubule-dependent mitochondrial movement regulates adrenocortical steroid hormone biosynthesis.

6. Communication between ER and mitochondria

As summarized earlier, cortisol and aldosterone biosynthesis occurs via a series of metabolic reactions that occur in ER and mitochondria (Figure 1). A large body of research has been carried out to define the mechanisms by which cholesterol is imported, deesterified, and transferred to the inner mitochondrial membrane; the site of the first enzymatic reaction (Connelly, 2009, Connelly and Williams, 2004, Miller, 2007, Rone et al., 2009, Stocco et al., 2005). However, the factors that control the exchange of substrates between the ER and mitochondria for subsequent reactions in the multi-step pathway are unknown. The discontinuous localization of enzymes in a single metabolic pathway in two organelles – ER and mitochondria – dictate the need for regulatory mechanisms that facilitate the inter-organelle movement of substrates. As discussed above, our findings demonstrating that

ACTH stimulates the rate of mitochondrial movement and that impairing microtubule polymerization attenuates cortisol production, while concomitantly increasing DHEA secretion suggest a role for regulated communication between the ER and mitochondria during adrenocortical steroidogenesis (Li and Sewer, 2010). Moreover, given that transfer of 11-deoxycortisol from ER to mitochondria is a requisite for the final reaction in cortisol production (Figure 1), we postulate that ACTH/cAMP enables steroidogenesis by regulating mitochondrial trafficking and efficient communication between ER and mitochondria.

There is precedence for the functional significance of morphological changes in adrenocortical cell structure upon activation of hormone synthesis (Rainey et al., 2004). Analysis of rat adrenocortical cells using electron microscopy has revealed the formation of plasma membrane microvillar channels that are thought to serve as conduits for lipoprotein entry (Reaven et al., 1989). Intriguingly, the formation of these structures requires the expression of SR-B1 (scavenger receptor class B, type 1), a plasma membrane receptor that mediates the uptake of high density lipoproteins into steroidogenic cells and hepatocytes (Williams et al., 2002). In a recent study, Duarte *et al.*, reported that cAMP stimulation of MA-10 murine Leydig cells promoted the fusion of mitochondria into elongated, tubular structures (Duarte et al., 2012). Using RNA interference the authors demonstrated that silencing mitofusin 2 (MFN2) reduces cAMP-stimulated mitochondrial fusion and progesterone synthesis and that the mRNA expression of MFN2 is rapidly induced by cAMP and human chorionic gonadotropin (Duarte et al., 2012). Significantly, there is evidence to support a role for MFN2 in regulating organelle positioning. MFN2 has been shown to be required for the movement of mitochondria in murine neurons, where targeted disruption of MFN2 in mice selectively impaired the trafficking of mitochondria, without affecting the movement of other cellular organelles (Misko et al., 2010). Interestingly, the researchers also found that silencing Miro2 (both Miro1 and Miro2 were shown to interact with MFN1 and MFN2) expression in cultured mouse neurons also impaired mitochondrial transport. Although MFN2 is required for both mitochondrial fusion and mitochondrial movement, neither microtubules nor microfilaments have been found to play a role in regulating mitochondrial fusion (Mattenberger et al., 2003, Misko et al., 2010). Collectively, these studies suggest that the MFN2-dependent fusion of mitochondria that contributes to progesterone synthesis in MA-10 murine Leydig cells (Duarte et al., 2012) is uncoupled from a putative role of MFN2 in regulating mitochondrial movement.

Our studies on the role of ACTH/cAMP-stimulated mitochondrial trafficking in regulating cortisol production hint at a role for this microtubule-dependent movement in the transport of substrates between the ER and mitochondria (Li and Sewer, 2010). Ongoing studies in the lab are testing the hypothesis that cAMP signaling activates cortisol biosynthesis by facilitating the physical interaction between the ER and mitochondria. High-resolution microscopy of multiple cell types has demonstrated that the ER forms contact sites not only with mitochondria, but also with other organelles, including Golgi, peroxisomes, lipid droplets, and lysosomes (Elbaz and Schuldiner, 2011, Friedman and Voeltz, 2011, Rowland and Voeltz, 2012). Contact sites between the ER and mitochondria have been shown to occur on acetylated microtubules in COS-7 cells, where the ER forms tube-like structures that associate with mitochondria (Friedman et al., 2010). The ER and mitochondria have also been found to form contact sites called ER-mitochondria encounter structures (ERMES) in *Saccharomyces cerevisiae*, which facilitate the exchange of Ca^{2+} and phospholipids between the two organelles (Kornmann et al., 2009). Interestingly, the yeast Miro1 homolog, Gem1, controls the number and size of the ERMES complexes (Kornmann et al., 2011). Given that Gem1 is a GTPase that serves as a Ca^{2+} -sensitive switch (Saotome et al., 2008), it is likely that signaling cues that activate guanine nucleotide exchange may regulate the function of ERMES complexes, the capacity of the organelles to exchange components, and ultimately metabolic processes. These studies establishing a key role for Gem1 in regulating contacts

between the ER and mitochondria make Miro1 (discussed above) an attractive candidate for future studies on the factors that contribute to ACTH/cAMP-stimulated mitochondrial movement and inter-organelle substrate exchange.

Notably, MFN2 has been found to not only enable the fusion of two mitochondria (Koshiba et al., 2004, Santel and Fuller, 2001), but to also tether mitochondria to ER (de Brito and Scorrano, 2008). However, a recent study using electron microscopy to compare ER-mitochondria contact sites in wild type and MFN2 knockout fibroblasts indicates that ER-mitochondria tethering can occur in the absence of MFN2 (Cosson et al., 2012). The effect of MFN2 knockdown on progesterone production in MA-10 Leydig cells make it tempting to speculate that activation of the ACTH/cAMP signaling pathway may promote the fusion of ER and mitochondria for transfer of substrates (e.g. 11-deoxycortisol, deoxycorticosterone) between organelles during cortisol production. However, the role of this protein in adrenocortical steroidogenesis is unclear. Ultrastructural analysis of H295R human adrenocortical cells for the existence of ER-mitochondria contact sites is an area of ongoing investigation in our laboratory.

In addition to the likelihood that the ER and mitochondria tether to facilitate the inter-organelle exchange of substrates during glucocorticoid and aldosterone biosynthesis, it is equally plausible that substrate delivery occurs via a protein-mediated process. Although the presence of a pregnenolone-binding protein has not been demonstrated, our preliminary finding that DIAPH1 interacts with members of the oxysterol-binding-protein related protein (ORP) family (Li *et al.*, unpublished observations, 2012) suggests that substrate (e.g. 11-deoxycortisol, deoxycorticosterone) transport may be protein dependent. ORPs are a family of lipid binding proteins that are implicated in lipid metabolism by serving as transporters between organelles, signaling mediators, and sensors of cellular lipid concentrations (Fairn and McMaster, 2008, Lehto et al., 2001, Oikkonen and Levine, 2004, Ridgway, 2010). Members of the ORP family have been shown to bind to cholesterol and oxysterols and are thus implicated in cholesterol metabolism (Du et al., 2011, Wang et al., 2002). However, ORPs have also been shown to bind to phospholipids, notably phosphatidic acid and phosphatidylinositides (Stefan et al., 2011, Xu et al., 2001). We are currently testing the hypothesis that ORPs may play a role in adrenocortical steroidogenesis by enabling the exchange of substrates between the ER and mitochondria.

Additionally, the role of ER-mitochondria tethering and the formation of contact sites also has implications for other metabolic pathways where the enzymes are localized in different organelles. For example, phosphatidylcholine synthesis also requires the exchange of substrates between mitochondria and the ER. We also posit that ER-mitochondrial interaction, and possibly regulated mitochondrial movement, may also play a key role in the exchange of substrates between organelles in the metabolism of cholesterol to form bile acids in the alternative/acidic pathway, where CYP7A1 and CYP7B1 are microsomal and CYP27A1 is mitochondrial. Inter-organelle substrate delivery may also play a role in regulating the concentrations of active vitamin D in certain tissues, where the activating enzyme, CYP27B1 is localized in mitochondria and the inactivating enzyme, CYP24A1, is in the ER.

7. Summary and Future Directions

Though our understanding of the complex regulatory mechanisms that control adrenocortical steroid hormone biosynthesis is increasing, it is clear that many questions still remain with regard to the factors that control the movement of substrates between ER and mitochondria during steroidogenesis. It is still unclear how ACTH/cAMP signaling controls this process. Moreover, the precise molecular mechanism(s) by which proteins such as

DIAPH1 and cytoskeletal components act to regulate steroid hormone biosynthesis is an area of investigation. Approaches such as live cell video imaging, mass spectrometry, RNA interference, and electron microscopy are likely to provide insight into the factors that contribute to optimal hormone output.

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Highlights

- ACTH/cAMP-stimulated adrenocortical steroidogenesis is regulated by microtubules, microfilaments, and vimentin.
- ACTH/cAMP signaling increases the rate of mitochondrial movement in H295R cells via a RhoA-DIAPH-dependent pathway.
- Inter-organelle transport of substrates between mitochondria and the ER during adrenocortical steroidogenesis may require ER-mitochondria tethering and/or protein-mediated transport.

Fig. 1.
Diagram of the steroid hormone biosynthetic pathway in the human adrenal cortex.

Fig. 2.

Model for the role of RhoA and diaphanous 1 (DIAPH1) in facilitating inter-organelle substrate exchange. ACTH activates the synthesis of intracellular cAMP and PKA activity, which rapidly increases the production of sphingosine-1-phosphate (S1P) and activates RhoA. GTP-bound RhoA interacts with its effector DIAPH1, which interacts with cytoskeleton components, including microtubules. S1P may act as a second messenger to stimulate and increase in intracellular Ca^{2+} , which may be required for microtubule-dependent tethering of ER and mitochondria and the transfer of 11-deoxycortisol from ER to mitochondria for the final enzymatic step in cortisol biosynthesis.

Fig. 3. H295R cells were plated onto cover slips and incubated with Fura3AM. Cells were then stimulated for 5 min with 400 μM Bt₂cAMP or 1 μM S1P and intracellular Ca^{2+} imaged by fluorescence microscopy.