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Sphingosine-1-Phosphate Rapidly Increases Cortisol Biosynthesis and the Expression of Genes Involved in Cholesterol Uptake and Transport in H295R Adrenocortical Cells

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Abstract

In the acute phase of adrenocortical steroidogenesis, adrenocorticotrophin (ACTH) activates a cAMP/PKA-signaling pathway that promotes the transport of free cholesterol to the inner mitochondrial membrane. We have previously shown that ACTH rapidly stimulates the metabolism of sphingolipids and the secretion of sphingosine-1-phosphate (S1P) in H295R cells. In this study, we examined the effect of S1P on genes involved in the acute phase of steroidogenesis. We show that S1P increases the expression of steroidogenic acute regulatory protein (StAR), 18-kDa translocator protein (TSPO), low-density lipoprotein receptor (LDLR), and scavenger receptor class B type I (SR-BI). S1P-induced StAR mRNA expression requires Gα_i signaling, phospholipase C (PLC), Ca²⁺/calmodulin-dependent kinase II (CamKII), and ERK1/2 activation. S1P also increases intracellular Ca²⁺, the phosphorylation of hormone sensitive lipase (HSL) at Ser⁵⁶³, and cortisol secretion. Collectively, these findings identify multiple roles for S1P in the regulation of glucocorticoid biosynthesis.

Keywords

sphingosine-1-phosphate; StAR; H295R; cortisol; acute steroidogenesis

1. Introduction

In the zona fasciculata of the human adrenal cortex, cortisol is synthesized from cholesterol via the concerted action of P450 heme-containing monooxygenases (CYPs) and 3β-hydroxysteroid dehydrogenase (3β-HSD) enzymes (Miller WL, 2008, Payne AH and Hales DB, 2004, Sewer MB et al., 2007). Additionally, StAR, 18-kDa translocator protein (TSPO), cholesterol hydrolase HSL, low-density lipoprotein receptor (LDLR), and scavenger receptor class B type I (SR-BI) are equally important in assuring adequate amounts of substrate for steroid hormone production. Cortisol biosynthesis is primarily regulated by the peptide hormone ACTH, which upon binding to the melanocortin 2 receptor, activates a

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cAMP/PKA-dependent pathway. Acutely, ACTH promotes cholesterol uptake and mobilization from intracellular stores to the inner mitochondrial membrane, where the first enzymatic step in steroid hormone production occurs. In the chronic phase of steroidogenesis, ACTH coordinately activates the transcription of all genes in the steroid hormone biosynthetic pathway, thus maintaining optimal steroidogenic capacity (Arlt W and Stewart PM, 2005, Dammer EB et al., 2007, Sewer MB and Waterman MR, 2003).

Import of free cholesterol into the inner mitochondrial membrane is facilitated by the formation of a macromolecular protein complex at the outer mitochondrial membrane containing TSPO, StAR, the TSPO-associated protein PAP7, and the regulatory subunit RI α of PKA (Hauet T et al., 2002, Liu J et al., 2006). StAR is essential for cholesterol trafficking (Caron KM et al., 1997b) and is rapidly synthesized in response to activation of the ACTH signaling pathway (Stocco DM and Clark BJ, 1996). The regulation of StAR transcription has been extensively studied [reviewed in (Manna PR et al., 2009a)], and the nuclear receptor steroidogenic factor 1 (SF-1) is required for StAR gene expression (Caron KM et al., 1997a) via its binding to a cAMP-responsive region located within the first 350 base pairs upstream of the transcription initiation site (Clark BJ and Combs R, 1999, Sandhoff TW et al., 1998, Sugawara T et al., 1996). Alternatively, Ca²⁺ influx through L-type Ca²⁺ channels abrogates StAR transcription by inducing the expression of the transcription factor DAX1 (dosage-sensitive sex reversal, adrenal hypoplasia critical region, on chromosome X, gene 1), a repressor of the StAR gene (Pandey AK et al., 2010). ACTH/cAMP rapidly increases StAR mRNA and protein levels (Lin D et al., 1995, Sewer MB and Waterman MR, 2001, Stocco D et al., 2005) as well as its enzymatic activity through PKA-mediated phosphorylation events at conserved serine residues (Arakane F et al., 1997). Further, the protein kinase C (PKC) signaling pathway also plays an integral role in StAR regulation by potentiating cAMP-stimulated StAR expression and phosphorylation (Manna PR et al., 2009c). Of note, Manna *et al.* have recently shown that different PKC isoforms, including PKC α , PKC δ , PKC ϵ and PKD, exhibit diverse effects on PMA-mediated StAR transcription and steroidogenesis (Manna PR et al., 2011). Finally, A-kinase anchoring protein 121 (AKAP121) enhances the post-transcriptional regulation of StAR by recruiting type II PKA regulatory subunit α (PKAR2 α) and StAR mRNA transcripts to the outer mitochondria membrane, thus promoting spatially localized protein synthesis and increasing steroidogenic efficiency (Dyson MT et al., 2008). Similarly, HSL, which is responsible for the neutral cholesteryl ester hydrolase activity in steroidogenic tissues (Kraemer FB and Shen WJ, 2002), plays a vital role in steroid hormone production by increasing the availability of free cholesterol. HSL interacts with StAR and this partnering between the two proteins facilitates the transport of cholesterol from lipid droplets to mitochondria (Luo Y et al., 2011).

Sphingolipids are structural components of cell membranes and key signaling mediators of many cellular processes (Cuvillier O, 2002, Goni FM and Alonso A, 2006, Hannun YA, 1996, Huwiler A et al., 2000, Kihara A et al., 2007, Lucki N and Sewer MB, 2008, Merrill AH Jr et al., 1999, Merrill Jr. AH, 2002, Ogretmen B, 2006, Zeidan YH and Hannun YA, 2007). Several studies have reported various roles for sphingolipids in adrenal and gonadal steroidogenesis (Brizuela L et al., 2006, Budnik LT et al., 1999, Degnan BM et al., 1996, Kwun C et al., 1999, Lucki N and Sewer MB, 2008, Lucki N and Sewer MB, 2009, McClellan DR et al., 1997, Meroni SB et al., 2000, Ozbay T et al., 2004, Porn MI et al., 1991, Santana P et al., 1996, Urs AN et al., 2006). As a bioactive lipid mediator, S1P regulates a broad array of physiological functions, including cell proliferation and survival (Olivera A et al., 1999, Olivera A and Spiegel S, 1993, Spiegel S and Milstien S, 2002), chemotaxis (Hla T et al., 1999, Wang F et al., 1999, Yamamura S et al., 1996), and protection against ceramide-mediated apoptosis (Cuvillier O et al., 1996). S1P has also been shown to stimulate cortisol production in zona fasciculata bovine adrenal cells in a PKC-

and Ca^{2+} -dependent manner (Rabano M et al., 2003), and promote aldosterone secretion in bovine glomerulosa cells via the phosphoinositide 3-kinase (PI3K)/Akt and mitogen-activated protein kinase (MAPK/ERK) pathways (Brizuela L et al., 2007, Brizuela L et al., 2006). S1P also mediates cAMP-dependent cortisol secretion in H295R human adrenocortical cells by promoting the SREBP-1-dependent transcription of CYP17 (Ozbay T et al., 2006).

S1P not only functions as an intracellular messenger (Hla T et al., 2001, Olivera A and Spiegel S, 2001) but also exerts many of its effects through cell surface G-protein coupled receptors (Alvarez SE et al., 2007). The mechanism of S1P export from cells is not completely understood, however, studies have provided evidence for the involvement of the ATP-binding cassette (ABC) family of transporters in this process (Anada Y et al., 2007, Kobayashi N et al., 2006, Mitra P et al., 2006, Takabe K et al., 2010). Five S1P receptors (S1PR₁₋₅) have been identified (Sanchez T and Hla T, 2004, Spiegel S and Milstien S, 2000), four of which (S1PR₁, S1PR₂, S1PR₃, S1PR₅) are expressed in H295R steroidogenic cells (Ozbay T et al., 2006). Differences in signaling through these receptors are primarily due to differential coupling to G-proteins. S1PR₁ signals through $\text{G}\alpha_i$ (Ancellin N and Hla T, 1999, Windh RT et al., 1999) whereas S1PR₂ and S1PR₃ couple to $\text{G}\alpha_i$, $\text{G}\alpha_q$, and $\text{G}\alpha_{13}$ (Ancellin N and Hla T, 1999). S1PR₄ associates with $\text{G}\alpha_i$ and $\text{G}\alpha_{12/13}$ (Graler MH et al., 2003, Van Brocklyn JR et al., 2000, Yamazaki Y et al., 2000) and S1PR₅ couples to $\text{G}\alpha_{i/o}$ and $\text{G}\alpha_{12}$ (Malek RL et al., 2001).

We have shown that ACTH rapidly stimulates sphingolipid metabolism in H295R cells (Ozbay T et al., 2004). ACTH and the cAMP analog Bt₂cAMP decrease cellular amounts of sphingomyelin, ceramide, and sphingosine, while simultaneously increasing the secretion of S1P (Ozbay T et al., 2004). The S1P produced stimulates cortisol secretion from H295R cells by promoting the maturation and binding of sterol regulatory element binding protein 1 (SREBP1) to the CYP17 promoter, thereby inducing gene transcription (Ozbay T et al., 2006). These findings implicate S1P as a paracrine mediator of ACTH-dependent CYP17 transcription. Therefore, the aim of the present study was to characterize the role of S1P in mediating the acute phase of steroidogenesis in H295R cells. We show that S1P rapidly increases cortisol biosynthesis and the mRNA expression of multiple genes involved in the acute phase of steroid hormone production including StAR, TSPO, SR-BI, and LDLR. In addition, we demonstrate that S1P acutely increases the phosphorylation of HSL at Ser⁵⁶³ and show that S1P-stimulated StAR gene expression is pertussis toxin sensitive and dependent on the activation of PLC, CamKII, and ERK1/2.

2. Materials and Methods

2.1. Reagents

Bt₂cAMP was obtained from Sigma (St. Louis, MO). Adrenocorticotropin (ACTH 1–39) was purchased from American Peptide Co. (Sunnyvale, CA). S1P and VPC23019 were obtained from Avanti Polar Lipids Inc (Alabaster, AL) and prepared by dissolving in ethanol and dimethylamine, followed by evaporation and solubilization in 4 mg/ml fatty-acid-free BSA. Pertussis toxin (PTX), U0126, KN-93, U73122, U73343, and Fluo 3/AM were obtained from EMD Biosciences (La Jolla, CA).

2.2. Cell culture and Treatment

H295R adrenocortical cells (Rainey WE et al., 1994, Rainey WE et al., 2004, Staels B et al., 1993) were generously donated by Dr. William E. Rainey (Medical College of Georgia, Augusta, GA) and cultured in Dulbecco's modified Eagle's/F12 medium (DMEM/F12) (Mediatech, Manassas, VA) supplemented with 10% Nu-Serum I (BD Biosciences, Palo

Alto, CA), 1% ITS Plus (BD Biosciences, Palo Alto, CA), antibiotics, and antimycotics. H295R cells were sub-cultured in 12-well plates and pretreated for 18 h with 10 μ M VPC23019 (S1PR₁ and S1PR₃ inhibitor), 5 pg/ml PTX, 10 μ M U73122 (PLC inhibitor), 10 μ M U73343 (inactive PLC analog), 10 μ M KN-93 (CamKII inhibitor), or 10 μ M U0126 (MEK1 inhibitor) followed by treatment with 1 μ M S1P, 50 nM ACTH, or 0.4 mM Bt₂cAMP for 2 h.

2.3. Cortisol and DHEA Assay

Cells were cultured in 12-well plates and treated with 0.4 mM Bt₂cAMP or 1 μ M S1P for 3- to 48 h. Cortisol and DHEA released into the media were determined in triplicate against standards made up in DMEM/F12 medium using a 96-well plate enzyme-linked immunosorbent assays (ELISA; Enzo Life Sciences Inc., Plymouth Meeting, PA). Results are expressed as nanomoles per milligram of cellular protein in each sample.

2.4. RNA isolation and real time RT-PCR

Total RNA was extracted using Isol-RNA Lysis Reagent (5 Prime, Inc., Gaithersburg, MD) and amplified using a One-Step SYBR Green RT-PCR Kit (Thermo Scientific Inc., Waltham, MA) and the following primer sets: β -actin (forward 5'-ACG GCT CCG GCA TGT GCA AG-3' and reverse 5'-TGA CGA TGC CGT GCT GCA TG-3'), StAR (forward 5'-GCT CTC TAC TCG GTT CTC-3' and reverse 5'-GCT GAC TCT CCT TCT TCC-3'), TSPO (forward 5'-GCA GAT TCC GTG ATT ACA GTG-3' and reverse 5'-TCC TCC TCG TCG TCA TCG-3'), HSL (forward 5'-CAC TAC AAA CGC AAC GAG AC-3' and reverse 5'-CCA GAG ACG ATA GCA CTT CC-3'), SR-BI (forward 5'-CCA TCC TCA CTT CCT CAA-3' and reverse 5'-CCA CAG GCT CAA TCT TCC-3'), LDLR (forward 5'-ACG GTG GAG ATA GTG ACA ATG-3' and reverse 5'-AGA CGA GGA GCA CGA TGG-3'). mRNA expression of StAR, TSPO, HSL, SR-BI, and LDLR was normalized to the transcript levels of β -actin and calculated using the delta-delta cycle threshold ($\Delta\Delta$ CT) method.

2.5. Measurement of Inositol 1, 4, 5-Trisphosphate (IP₃)

Cells were treated with 1 μ M S1P or 1 mM Bt₂cAMP for 5 to 60 min. Cells were harvested into PBS and centrifuged 5 min at 4,000 rpm. Supernatant was removed and the cells resuspended in 20% (w/v) perchloric acid on ice for 30 min. Cells were centrifuged and the supernatants neutralized with HEPES-KOH solution. Amounts of inositol 1, 4, 5-trisphosphate in each sample were measured by a radioreceptor assay with the D-myo-inositol 1, 4, 5-trisphosphate [³H] assay kit (TRK 1000, Amersham Biosciences, Piscataway, NJ).

2.6. Intracellular Ca²⁺Quantification

H295R cells were subcultured onto coverslips for 24 h and intracellular calcium was labeled with Fluo 3/AM (Burnier M et al., 1994, Orlicky J et al., 2004, Tsai J-H et al., 2010). Fluo 3/AM (5 μ M) was loaded into the cells in serum-free medium for 1 hour at 37 °C. 0.02% pluronic acid (Sigma, St. Louis, MO) was added to the medium to disperse Fluo 3/AM (EMD Biosciences). After the incubation, cells were washed three times with medium. Then cells were placed in a chamber with 0.5 ml medium for fluorescence imaging. Fluorescence images were acquired using UltraVIEW Vox Spinning Disk Confocal Microscope (Perkin Elmer Inc., Waltham, MA). Intensity ratio of Fluo 3/AM was quantified using the Volocity software (Perkin Elmer Inc.).

2.7. Western Blotting (WB)

For analysis of StAR protein expression, cells were treated with 1 μ M S1P (0 – 18 h). Phospho-ERK1/2 (pERK1/2) and total ERK2 expression was assessed in lysates purified from cells that were serum-starved for 30 h, pretreated with 10 μ M KN-93, or 5 pg/ml PTX for 1 h followed by treatment with 1 μ M S1P. Phospho-CamKII (pCamKII) and total CamKII expression was assessed in lysates purified from cells that were pretreated with 10 μ M VPC23019, 10 μ M U0126, or 5 pg/ml PTX for 1 h followed by treatment with 1 μ M S1P for 30 min. To assess the phosphorylation status of HSL, cells were treated for 15, 30, or 60 min with 1 μ M S1P. Cells were harvested into RIPA buffer (1X PBS, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS) containing 1X Protease Inhibitor Cocktail Set I (EMD Biosciences) and lysed by sonication (one 2 sec burst) followed by incubation on ice for 30 min. Lysates were centrifuged at 12,000 rpm for 15 min at 4°C and the supernatant collected for analysis by SDS-PAGE. Aliquots of each sample (30 μ g of protein) were run on 8% SDS-PAGE gels and transferred to Immobilon FL polyvinylidene difluoride (PVDF) membranes (Millipore). Blots were probed with anti-phospho-ERK1/2 (sc-7383, Santa Cruz), ERK2 (sc-154, Santa Cruz), StAR (sc-25806, Santa Cruz), phospho-Ser⁵⁶³-HSL (4139, Cell Signaling), HSL (sc-25843, Santa Cruz), phospho-Thr²⁸⁶-CamKII (06-881, Millipore), CamKII (04-1079, Millipore), or GAPDH (sc-25778, Santa Cruz). Expression was detected using an ECF western blotting kit (GE Healthcare, Piscataway, NJ) and visualized by scanning the blots on a VersaDoc 4000 imager (Bio-Rad). Protein concentrations were determined by the bicinchoninic acid (BCA) protein assay (Pierce, Rockford, IL).

2.8. Statistical Analysis

One-way ANOVA and Tukey-Kramer multiple comparison test were performed using GraphPad InStat software (GraphPad Software Inc., San Diego, CA). Significant differences from compared value were defined as $p < 0.05$ and denoted by asterisks (*) and carats (^).

3. Results

3.1. S1P stimulates cortisol biosynthesis

As previously discussed, we have demonstrated that ACTH and Bt₂cAMP rapidly stimulate sphingolipid metabolism (Ozbay T et al., 2004) and S1P secretion from H295R cells (Ozbay T et al., 2006). Additionally, S1P induces CYP17 transcription (Ozbay T et al., 2006). Therefore, in order to investigate the role of S1P in the acute phase of steroid hormone biosynthesis, we determined the effect of S1P on cortisol biosynthesis in H295R cells. Cells were treated for time points ranging from 3 to 48 h and the concentrations of steroid hormone secreted into the media were quantified as described in *Materials and Methods*. As shown in Fig. 1A, S1P maximally increased cortisol production by 2.5-fold at the 12-h time point. While S1P resulted in a significant increase in cortisol production at the 48 h time point (Fig. 1A), the bioactive sphingolipid was unable to evoke a sustained increase in DHEA secretion over the same time period (Fig. 1B). Bt₂cAMP treatment robustly stimulated the secretion of both cortisol and DHEA at all time points assayed, with a 7-fold increase in cortisol production and a 6-fold increase in DHEA biosynthesis at the 24 h time point.

3.2. S1P induces the mRNA expression of various acute phase steroidogenic genes

To characterize the mechanism by which S1P acutely increases cortisol secretion, we determined the effect of S1P on the mRNA expression of multiple genes involved in the acute steroidogenic response. StAR is essential for the delivery of free cholesterol into the inner mitochondrial membrane (Caron KM et al., 1997b, Hauet T et al., 2002), the rate-

limiting step in steroid hormone biosynthesis. To determine the effect of S1P on the mRNA expression of StAR, H295R cells were treated for 0.5-, 1- or 2 h with 0.4 mM Bt₂cAMP or 1 μ M S1P and total RNA was isolated for quantification by qRT-PCR. As shown in Fig. 2A, S1P and Bt₂cAMP induced StAR mRNA expression by 2.0- and 1.8-fold after 1 h treatment, respectively. S1P further increased StAR expression at the 2-h time point by 5.0-fold, as compared to the 14-fold induction in StAR mRNA expression elicited by Bt₂cAMP (Fig. 2A).

In addition to examining StAR transcription, we quantified the mRNA expression of four additional genes that are involved in the biosynthesis of cortisol: TSPO, SR-BI, LDLR and HSL. TSPO forms a macromolecular complex with StAR to facilitate the import of cholesterol into the inner mitochondrial membrane (Papadopoulos V, 1993). SR-BI mediates the selective uptake of cholesteryl esters (CE) from high-density lipoprotein particles (HDL) in rodents and humans (Briand O et al., 2003, MA, 2009, Pilon A et al., 2003) and HSL has neutral cholesteryl ester hydrolase activity and is critical for hydrolyzing internalized CEs (Kraemer FB et al., 2004). Importantly, cAMP has been shown to regulate both HSL enzymatic activity and SR-BI mRNA expression (Krintel C et al., 2008). LDLR enables receptor-mediated endocytic delivery of CEs from LDL particles (Kraemer FB, 2007). In humans, LDLR provides most of the cholesterol necessary for steroid hormone production (Carr BR and Simpson ER, 1981). We examined the mRNA expression of these genes in H295R cells that were treated with 1 μ M S1P or 0.4 mM Bt₂cAMP for 2 h. As shown in Fig. 2B, S1P induced the mRNA expression of SR-BI, LDLR, and TSPO by 2.5-, 4.7-, and 4.5-fold, respectively. The mRNA expression of HSL was not affected by S1P; though Bt₂cAMP significantly increased the expression of all four genes.

3.3. S1P-mediated induction of StAR gene expression is pertussis toxin sensitive

As discussed earlier, many of the effects elicited by S1P are mediated through G-protein coupled S1P receptors (S1PR₁₋₅) that activate multiple downstream signaling cascades (Alvarez SE et al., 2007, Sanchez T and Hla T, 2004, Taha TA et al., 2004). In order to define the signaling pathway that mediates S1P-induced StAR gene expression, we determined the effect of the G α_i inhibitor PTX on S1P-dependent StAR gene expression. H295R cells were pretreated with 5 pg/ml PTX, followed by stimulation with 1 μ M S1P for 2 h. As shown in Fig. 3A, PTX prevented S1P-induced expression of StAR. Because multiple S1PR isoforms have been reported to couple to G α_i pathways (Hla T et al., 2001, Taha TA et al., 2004, Windh RT et al., 1999, Yamazaki Y et al., 2000), we next sought to identify the specific S1PR that mediates S1P-induced StAR transcription by pre-treating H295R cells with the S1PR₁/S1PR₃ antagonist VPC23019 followed by S1P stimulation. VPC23019 abolished S1P-mediated StAR gene expression (Fig. 3A). Taken together, these data suggest that S1P acutely activates StAR transcription through activation of G α_i -protein coupled receptors S1PR₁ and/or S1PR₃. Because we have previously found that ACTH/cAMP signaling promotes the secretion of S1P from H295R cells (Ozbay T et al., 2004, Ozbay T et al., 2006), we assessed the effect of PTX and VPC23019 on ACTH/cAMP-stimulated StAR mRNA expression and found that neither inhibitor affected the ability of Bt₂cAMP to induce StAR expression (Fig. 4B). However, VPC23019 significantly reduced ACTH-dependent StAR mRNA levels by 34% (Fig 4B). The ability of the S1PR_{1/3} antagonist to partially repress ACTH-stimulated StAR expression is consistent with a second messenger role for S1P in ACTH-dependent steroidogenesis. To further define the relationship between S1P-dependent StAR transcription and ACTH signaling, we examined the effect of S1P on ACTH- and Bt₂cAMP-induced on StAR mRNA expression. Neither Bt₂cAMP nor ACTH had an additive effect on the S1P response (Fig. 3C). However, unexpectedly ACTH-stimulated StAR transcription was suppressed in the presence of S1P (Fig. 3C).

3.4. S1P-dependent StAR transcription requires PLC activation

Signaling through $G\alpha_i$ affects multiple downstream effectors including adenylyl cyclase, the small G protein Rac, Src, PI3K/Akt, MAPK, and PLC. The inhibitory effect of PTX and VPC23019 on S1P-mediated StAR mRNA expression suggested that the stimulatory effect of S1P on gene expression occur through the interaction of S1P with $S1PR_{1/3}$. Therefore, in order to identify effectors in the signaling pathway downstream of $G\alpha_i$, we determined the effect of PLC inhibition on S1P-stimulated StAR transcription. H295R cells were pre-treated with 10 μ M U73122 followed by treatment with 1 μ M S1P for 2 h. PLC inhibition completely abolished S1P-induced StAR mRNA expression, whereas the inactive PLC analog U73343 had no significant effect on the ability of S1P to increase StAR mRNA expression (Fig. 4A).

3.5. S1P stimulates IP_3 release and increases cytoplasmic Ca^{2+}

Based on the findings described above demonstrating that S1P-stimulated StAR gene transcription is dependent on PLC activity, we quantified the amount of IP_3 release in response to S1P. H295R cells were treated with 1 μ M S1P for 5 to 60 min and IP_3 intracellular levels were determined as described in *Materials and Methods*. As shown in Fig. 4B, S1P significantly increased intracellular IP_3 by 2.5- and 4.8-fold after 5 and 15 min, respectively, compared to untreated controls. IP_3 levels in S1P-treated cells gradually decreased after 15 min of stimulation, although they remained significantly higher than untreated controls. Notably, Bt_2cAMP had no significant effect on IP_3 release (Fig. 4B), suggesting that the mechanism by which S1P induces StAR expression exhibits distinct elements when compared to Bt_2cAMP . The increase in IP_3 was concomitant with a 2.9- and 5.8-fold increase in cytosolic Ca^{2+} after 5 and 15 min of S1P stimulation, respectively (Fig. 4D). Bt_2cAMP also stimulated an increase in cytosolic Ca^{2+} , however, the magnitude of the response was lower than the increase elicited by S1P.

3.6. Induction of StAR mRNA expression requires CamKII and ERK1/2 activation

The increases in IP_3 and Ca^{2+} led us to postulate that S1P-stimulated StAR expression requires CamKII. To test this hypothesis, H295R cells were treated for 2 h with 1 μ M S1P in the presence or absence of the CamKII inhibitor KN-93 (10 μ M) and StAR mRNA transcript levels were quantified by qRT-PCR. As shown in Fig. 5A, inhibiting CamKII activity attenuated S1P-induced StAR mRNA expression. Consistent with this finding, S1P stimulation activated CamKII after 30 min, and pre-treatment with VPC23019 and PTX abrogated S1P-stimulated CamKII phosphorylation (Fig. 5B). Because CamKs can crosstalk with many intracellular pathways, including MAPK signaling (Blanc A et al., 2004, Li N et al., 2009, Nguyen A et al., 2004, Schmitt JM et al., 2004), we next investigated the effect of S1P on the activation of ERK1/2. As shown in Fig. 5C, S1P activates ERK1/2 after 30 min of stimulation. To determine if ERK1/2 activation is upstream of CamKII, the phosphorylation status of ERK1/2 was quantified by western blotting from H295R cells pre-treated with 10 μ M KN-93 and stimulated with 1 μ M S1P for 15 or 30 min. As shown in Fig. 5D, pre-treatment with KN-93 prevented ERK1/2 phosphorylation in S1P-treated cells. Additionally, pre-treatment of H295R cells with the MEK1 inhibitor U0126 completely ablated S1P-induced StAR gene expression (Fig. 5E). Taken together, our results indicate that S1P-dependent StAR transcription requires the sequential activities of CamKII and ERK1/2.

3.7. S1P-mediated ERK1/2 activation is dependent on $G\alpha_i$ signaling

Our data show that ERK1/2 activation is required for S1P-dependent StAR gene transcription and that CamKII is upstream of ERK1/2. Moreover, $G\alpha_i$ and PLC are also required for the S1P response. Therefore, in order to determine if ERK1/2 phosphorylation

occurs through activation of G_{α_i} signaling, we carried out western blotting analysis of H295R cells pre-treated with 5 pg/ml PTX and stimulated with 1 μ M S1P for 15 or 30 min. As shown in Fig. 5F, suppression of G_i signaling by PTX prevents S1P-induced ERK1/2 phosphorylation.

3.8. S1P acutely increases StAR protein expression and HSL phosphorylation

To determine if the increase in StAR transcription resulted in an increase in protein expression, we quantified StAR protein levels in S1P-treated H295R cells by western blotting. As shown in Fig. 6A and B, S1P significantly increased StAR protein expression by 1.9- and 2.5-fold after 3 and 6 h of stimulation, respectively. Significantly, although S1P had no effect on HSL mRNA (Fig. 2B) or protein expression (data not shown), densitometric analysis of western blots revealed that S1P stimulated the phosphorylation of HSL at Ser⁵⁶³, a PKA target site that stimulates HSL activity (Carmen GY and Victor SM, 2006, Garton AJ et al., 1988, Stralfors P and Belfrage P, 1983, Stralfors P et al., 1984), by 2.4- and 2.8- fold after 15 and 30 min, respectively (Fig. 6C and 6D).

4. Discussion

ACTH promotes glucocorticoid production by coordinating a series of spatially and temporally distinct cellular processes. In what is classically known as the acute phase of steroidogenesis, ACTH-stimulated PKA activation leads to an increase in the hydrolysis of cholesterol esters and the subsequent trafficking of this substrate to the inner mitochondrial membrane, the site of the first enzymatic reaction in the steroid hormone biosynthetic pathway. During the acute phase, ACTH signaling rapidly induces the expression of StAR (Clark BJ et al., 1994, Stocco D et al., 2005) and the activation of HSL (Cook KG et al., 1982). Based on our previous studies showing ACTH-stimulated sphingolipid metabolism and S1P secretion (Ozbay T et al., 2006), we sought to define the role of S1P in the acute phase of steroidogenesis. We report herein that S1P induces the transcription of multiple acute steroidogenic genes, stimulates the phosphorylation of HSL at Ser⁵⁶³, and increases cortisol production. Further, we show that S1P-stimulated StAR gene expression occurs through an S1PR₁/S1PR₃-mediated G_{α_i} signaling pathway that involves PLC, CamKII, and ERK1/2 activation.

Although ACTH is the main regulator of cortisol biosynthesis, multiple additional signaling molecules can modulate steroidogenic gene transcription, including growth factors, prostaglandins, and phospholipids (Doi J et al., 2001, Schinner S et al., 2007, Shah BH et al., 2005, Watanabe M et al., 2006). S1P regulates aldosterone secretion in zona glomerulosa adrenocortical cells (Brizuela L et al., 2007, Brizuela L et al., 2006) and stimulates cortisol secretion in zona fasciculata bovine adrenal cells (Rabano M et al., 2003). We show that S1P is a novel modulator of acute steroid hormone biosynthesis in human adrenocortical cells. Although the effect of S1P and Bt₂cAMP were similar at earlier time points, the magnitude of the response elicited by Bt₂cAMP was significantly greater at later time points (Fig. 1A). Notably, S1P only increased DHEA secretion transiently at the 6 and 12 h time points (Fig. 1B). Because the biosynthesis of cortisol requires the movement of intermediate metabolites between the ER and mitochondria and we have previously shown that ACTH/cAMP-mediated mitochondrial movement plays a pivotal role in glucocorticoid biosynthesis (Li D and Sewer MB, 2010), it is tempting to speculate that S1P is stimulating cortisol production by facilitating substrate delivery to mitochondria. Indeed, we have found the S1P increases the rate of mitochondrial trafficking (Sewer MB and Li D, 2008).

During the acute phase of steroidogenesis, ACTH-dependent cholesterol mobilization is mirrored by an increase in the transcription of genes essential for its uptake, transport, and de-esterification, including StAR and TSPO (Thomson M, 1997). Our data show that S1P

rapidly increases the transcription of TSPO, SR-BI, LDLR, and StAR (Fig. 2). Significantly, although S1P had no effect on HSL mRNA expression (Fig. 2B), phosphorylation of HSL at Ser⁵⁶³ was acutely stimulated S1P (Fig. 6C). As discussed earlier, the activity of HSL is regulated by phosphorylation at multiple residues (Anthonsen MW et al., 1998, Degerman E et al., 1990, Garton AJ and Yeaman SJ, 1990, Greenberg AS et al., 2001). However, the phosphorylation of HSL at Ser⁵⁶³ by PKA (Anthonsen MW et al., 1998) triggers HSL translocation to the surface of lipid droplets where it hydrolyzes cholesteryl esters (Clifford GM et al., 2000, Egan JJ et al., 1992). Moreover, HSL has been found to interact with StAR (Shen WJ et al., 2003). Therefore, our data identify multiple roles for S1P in facilitating increased substrate availability and delivery for cortisol biosynthesis (Fig. 7).

Most of the physiological effects elicited by S1P are the result of binding to tissue-specific S1P receptors (S1PR₁₋₅) (Alvarez SE et al., 2007, An S et al., 1998). In this manner, S1P has been shown to activate PLC, PI3K/Akt, and MAPK signaling as well as Ca²⁺ mobilization (Brizuela L et al., 2007, Brizuela L et al., 2006, Graler MH et al., 2003, Olivera A and Spiegel S, 1993, Spiegel S and Milstien S, 2003, Suhaiman L et al., 2010, Yamazaki Y et al., 2000). Our results demonstrate that S1P-dependent StAR gene expression occurs through a pertussis toxin-sensitive Gα_i-coupled receptor (Fig. 3A). Moreover, because the S1PR₁/S1PR₃ antagonist VPC23019 prevented S1P-induced gene expression, it is likely that S1P signals through one or both of these receptor isoforms. Notably, both isoforms are expressed in the H295R cells line (Ozbay T et al., 2006) and can couple to Gα_i (Ancellin N and Hla T, 1999, Brizuela L et al., 2007, van Brocklyn JR et al., 1998, Windh RT et al., 1999). Further, S1P and Bt₂cAMP did not have an additive effect on StAR mRNA expression (Fig. 3C), suggesting that these stimuli activate a common downstream effector. Conversely, S1P abrogated ACTH-stimulated StAR transcription (Fig. 3C). Because our data suggests that S1P signals through Gα_i to induce StAR mRNA expression (Fig. 3A) and ACTH activates a Gα_s-cAMP-PKA signaling pathway (Mountjoy KG et al., 1992), it is tempting to speculate that S1P-mediated Gα_i activation downregulates ACTH-dependent Gα_s signaling by inhibiting adenylyl cyclase (Taussig R et al., 1993). Gα_i-mediated downregulation of cAMP production by S1P has been previously reported (Jongsma M et al., 2009, Jun DJ et al., 2006, Means CK et al., 2008, Sensken SC et al., 2008).

Many reports support the activation of Ca²⁺ mobilization by S1P through GPCR-mediated PLC activation (Bornfeldt KE et al., 1995, Noh SJ et al., 1998, Okajima F et al., 1996, Suhaiman L et al., 2010). Consistent with these findings, we show that the transcriptional response evoked by S1P requires PLC activation and results in the accumulation of cytoplasmic IP₃ and Ca²⁺ (Fig. 4). It should be noted that S1P has been shown to increase cytosolic Ca²⁺ by stimulating release from intracellular stores and by activating Ca²⁺ influx (Bischoff A et al., 2001, Coussin F et al., 2002, Mathieson FA and Nixon GF, 2006). Although our studies do not distinguish between these two sources, the increase in IP₃ levels elicited by S1P (Fig. 4B) suggest that elevated cytosolic Ca²⁺ in response to S1P stimulation stems, at least in part, from intracellular stores. Notably, even though Bt₂cAMP also elevated cytosolic Ca²⁺ levels (Fig. 4D), it did so independently of cellular IP₃ levels (Fig. 4B), which supports the notion that the molecular mechanism of S1P-mediated glucocorticoid production displays unique components when compared to ACTH/cAMP signaling.

In order to further characterize the signaling molecules involved in the S1P transcriptional response, we show that downstream of PLC, CamKII and ERK1/2 activation are required events for S1P-dependent StAR gene transcription (Fig. 5). CamKII is a well-established Ca²⁺-activated kinase, thus its activation by increased cytoplasmic Ca²⁺ is plausible. In addition, S1P has been shown to activate this enzyme in vascular smooth muscle cells (Mathieson FA and Nixon GF, 2006). Consistent with previous reports on the crosstalk

between CamK and the MAPK/ERK pathway (Illario M et al., 2003, Li N et al., 2009, Nguyen A et al., 2004, Schmitt JM et al., 2004) and the activation of MAPK signaling by S1P (Brizuela L et al., 2007, Rakhit S et al., 1999, Wu J et al., 1995), we show that S1P rapidly activated ERK1/2 (Fig. 5B) in a CamKII-dependent manner (Fig. 5C). Further, ERK1/2 activation was required for S1P-dependent StAR mRNA expression (Fig. 5D). Importantly, MAPK/ERK activation in response to S1PR₁ and S1PR₃ couples to Gα_i (Brizuela L et al., 2007, Lee MJ et al., 1996, van Brocklyn JR et al., 1998, Windh RT et al., 1999, Zondag GC et al., 1998), further supporting our findings.

ERK1/2-mediated StAR gene expression is well established (Gyles SL et al., 2001, Manna PR et al., 2006, Manna PR et al., 2007) and both PKA and PKC signaling can activate ERK1/2 (Manna PR et al., 2007). Importantly, PKA (reviewed in (Arakane F et al., 1997, Stocco DM, 2000)) and PKC (Fiedler EP et al., 1999, Manna PR et al., 2007) signaling regulate StAR transcription. Our data identify S1P as a novel regulator of StAR gene expression via the activation of the MAPK/ERK pathway. Regulation of StAR gene transcription involves multiple transcription factors including the bZIP family of transcription factors (cAMP-regulatory element binding protein (CREB)/CRE modulator (CREM)/activating transcription factor (ATF)), which binds to conserved 5'-CRE half-sites (Manna PR et al., 2009b, Manna PR et al., 2003). In addition, increased Ca²⁺ and subsequent activation of CamK leads to CREB phosphorylation in a MAPK-dependent manner (Wu GY et al., 2001). Further, S1P was shown to partially activate CREB in this manner (Coussin F et al., 2003). Thus, it is tempting to speculate that S1P-induced StAR transcription is mediated, at least in part, through ERK1/2-dependent CREB phosphorylation. Moreover, because ERK1/2 regulate StAR function by phosphorylation at Ser²³² (Poderoso C et al., 2009), it is possible that S1P stimulation triggers StAR phosphorylation in addition to inducing its gene expression. Future studies are needed to test these hypotheses as well as uncover other regulatory proteins involved in S1P-dependent acute steroidogenic gene expression.

We (Ozbay T et al., 2006) and others (Brizuela L et al., 2007, Brizuela L et al., 2006, Rabano M et al., 2003) have reported that S1P mediates steroid hormone biosynthesis by activating various intracellular cascades. Rabano et al. (2003) demonstrated that S1P-mediated cortisol secretion in bovine adrenocortical cells is dependent on PKC and intracellular Ca²⁺ in a pertussis toxin-sensitive manner. Our results support a similar mechanism of action for S1P in human H295R cells, although we demonstrate that PLC activation is also required. Importantly, we show that S1P induces the phosphorylation of HSL at Ser⁵⁶³, providing evidence that S1P regulates steroidogenesis by acting via multiple. Given that ACTH/cAMP promotes the secretion of S1P from H295R cells (Ozbay T et al., 2006), it is plausible that adrenocortical cells might utilize an ACTH/S1P feed-forward mechanism to facilitate rapid steroidogenic output and fine-tune sustained hormone production. In summary, we demonstrate that S1P acts at multiple levels to promote cortisol production.

Highlights

- > S1P induces StAR, TSPO, LDLR, and SR-BI expression and cortisol secretion.
- > StAR expression requires a Ca²⁺-dependent pathway that involves PLC, CamKII, and ERK1/2.
- > S1P rapidly stimulates the phosphorylation of hormone sensitive lipase (HSL) at Ser⁵⁶³.

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Abbreviations

S1P	sphingosine-1-phosphate
ACTH	adrenocorticotropin
PLC	phospholipase C
StAR	steroidogenic acute regulatory protein
TSPO	translocator protein
CamKII	Ca ²⁺ /calmodulin-dependent kinase II
IP₃	inositol 1,4,5-triphosphate
PKA	protein kinase A
PKC	protein kinase C
HSL	hormone sensitive lipase
LDLR	low-density lipoprotein receptor
SR-BI	scavenger receptor class B type I
SF-1	steroidogenic factor 1
MAPK	mitogen-activated protein kinase
ERK	extracellular signal-regulated kinase
PI3K	phosphoinositide 3-kinase
S1PR	S1P receptor
Bt₂cAMP	dibutyryl-cAMP
PTX	pertussis toxin

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Fig. 1. S1P increases cortisol secretion

H295R cells were treated for time periods ranging from 3 to 48 h with Bt₂cAMP (0.4 mM) or S1P (1 μ M) as described in *Materials and Methods*. Cortisol (A) or DHEA (B) released into the media was quantified by ELISA. Values represent the mean $\pm$ SEM of 3 experiments, each performed in triplicate and normalized to the total cellular protein content. Asterisks (*) denote $p < 0.05$ for agonist-stimulated hormone production versus vehicle-treated controls.

Fig. 2. S1P acutely increases steroidogenic gene expression

(A) H295R cells were treated with 0.4 mM Bt₂cAMP or 1 μ M S1P for 30 min to 2 h. StAR mRNA levels were quantified by qRT-PCR and normalized to the mRNA expression of β -actin. (B) H295R cells were treated with 0.4 mM Bt₂cAMP or 1 μ M S1P for 2 h and mRNA expression of SR-BI, HSL, LDLR, and TSPO was quantified by qRT-PCR and normalized to the mRNA levels of β -actin. Data graphed are expressed as percent of control group mean and represent the mean $\pm$ SEM of 3 experiments, each performed in triplicate. Asterisk (*) denotes $p < 0.05$ for Bt₂cAMP- or S1P-stimulated mRNA expression versus vehicle-treated control.

Fig. 3. S1P-stimulated StAR gene expression occurs through receptor-mediated G_{α_i} signaling (A) H295R cells were treated for 2 h with 1 μ M S1P in the presence or absence of 10 μ M VPC23019 or 5 pg/ml PTX. Total mRNA was isolated and StAR mRNA expression was quantified by qRT-PCR and normalized to the mRNA levels of β -actin. Data graphed represent the mean $\pm$ SEM of 3 experiments, each performed in triplicate. Asterisk (*) denotes $p < 0.05$ compared to vehicle-treated controls. (B) H295R cells were pre-treated with 10 μ M VPC23019 or 5 pg/ml PTX for 18 h and then stimulated for 2 h with 50 nM ACTH or 0.4 mM Bt₂cAMP. Total mRNA was isolated and StAR mRNA expression was quantified by qRT-PCR and normalized to the mRNA levels of β -actin. Data graphed represent the mean $\pm$ SEM of 3 experiments, each performed in triplicate. Asterisks (*)

denote $p < 0.05$ compared to vehicle-treated control and carat (^) denotes statistically different from ACTH-treated group ($p < 0.05$). (C) H295R cells were treated for 2 h 1 μ M S1P in the presence or absence of 0.4 mM Bt₂cAMP or 50 nM ACTH. Total mRNA was isolated and StAR mRNA expression was quantified by qRT-PCR and normalized to the mRNA levels of β -actin. Data graphed represent the mean $\pm$ SEM of 3 experiments, each performed in triplicate. Asterisk (*) and carat (^) denote statistically different ($p < 0.05$) compared to vehicle-treated controls or ACTH-treated, respectively.

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Fig. 4. S1P-mediated StAR transcription is dependent on PLC activation

(A) H295R cells were pretreated with U73122 or U73343 (10 μ M) and then stimulated for 2 h with 1 μ M S1P. Total RNA was isolated and StAR mRNA expression assessed by qRT-PCR and normalized to the mRNA levels of β -actin. Data are graphed as percent of control group mean and represent the mean $\pm$ SEM of 3 experiments, each performed in triplicate. Asterisks (*) denote statistically different from the control group ($p < 0.05$). (B) H295R cells were treated with 1 μ M S1P or 0.4 mM Bt_2cAMP for time periods ranging from 5 to 60 min. IP₃ content was determined by radioreceptor assay as described in *Materials and Methods*. Data graphed are expressed as pmol of IP₃ per milligram of protein and represent the mean $\pm$ SEM of 2 experiments, each performed in triplicate. Asterisks (*) denote $p < 0.05$ of S1P-treated cells versus vehicle-treated controls. (C) Representative immunofluorescence live cell images of cytosolic Ca^{2+} in H295R cells treated for 0 to 15 min with S1P (top panel) or Bt_2cAMP (bottom panel). Cytosolic Ca^{2+} was visualized using Fluo 3/AM (green). (D) Quantification of cytosolic Ca^{2+} in H295R cells treated for 5 to 15 min with 0.4 mM Bt_2cAMP or 1 μ M S1P using the Volocity software. Data are graphed as mean intensity over area and represent the mean $\pm$ SEM of 4 experiments, each performed in triplicate.

Fig. 5. S1P-stimulated StAR transcription requires CamKII and ERK1/2 activation

(A) H295R cells were pretreated with 10 μ M KN-93 and then stimulated with 1 μ M S1P for 2 h. Cells were harvested and total RNA isolated for analysis of StAR mRNA expression by qRT-PCR and normalized to the mRNA levels of β -actin. Data graphed are expressed as percent of control group mean and represent the mean $\pm$ SEM of 3 experiments, each performed in triplicate. Asterisk (*) denotes $p < 0.05$ for S1P-stimulated mRNA expression versus vehicle-treated control. (B) H295R cells were pre-treated with 10 μ M VPC23019 or 5 ng/mL PTX for 1 h, and then treated with 1 μ M S1P for 30 min. Cell lysates were harvested and separated by SDS-PAGE. CamKII activity was assessed in cell lysates by western blotting using an antibody against phospho-Thr²⁸⁶-CamKII (top panel) or CamKII (bottom panel). Data are expressed as fold change in phospho-CamKII over control group mean $\pm$ STD DEV, normalized to total CamKII, and represent the densitometric analysis of western blots from three independent experiments, each performed in triplicate.

(C) H295R cells were serum-starved for 30 h, treated for 15 or 30 min with 1 μ M S1P, and cell lysates were harvested and separated by SDS-PAGE followed by western blotting using anti-phospho-ERK1/2 (top panel) and ERK2 (bottom panel) antibodies. Data are expressed as fold change in phospho-ERK1/2 over control group mean $\pm$ STD DEV, normalized to total ERK2 and represent the densitometric analysis of western blots from three independent experiments, each performed in triplicate. (D) H295R cells were serum-starved for 30 h, pre-treated with 10 μ M KN-93 for 1 h, and then treated for 15 or 30 min with 1 μ M S1P. Some cells were treated with 25 ng/ml EGF for 10 min. ERK activity was assessed in cell lysates by western blotting using an antibody against phospho-ERK1/2 (top panel) or ERK2 (bottom panel). Data are expressed as fold change in phospho-ERK1/2 over control group mean $\pm$ STD DEV, normalized to total ERK2 and represent the densitometric analysis of western blots from three independent experiments, each performed in triplicate. (E) H295R cells were treated for 2 h with 1 μ M S1P in the presence or absence of 10 μ M U0126. Total RNA was isolated and StAR mRNA expression was quantified by qRT-PCR and normalized to the mRNA levels of β -actin. Data graphed represent the mean $\pm$ SEM of 3 separate experiments, each performed in triplicate. Asterisk (*) denotes statistically significant difference ($p < 0.05$) compared to vehicle-treated control. (F) H295R cells were serum-starved for 30 h, pre-treated with 5 pg/ml PTX for 1 h, and then treated for 15 or 30 min with 1 μ M S1P. Some cells were treated with 25 ng/ml EGF for 10 min (positive control). Cell lysates were harvested and separated by SDS-PAGE followed by western blotting using antibodies against phospho-ERK1/2 (top panel) or ERK2 (bottom panel). Data are expressed as fold change in phospho-ERK1/2 over control group mean $\pm$ STD DEV, normalized to total ERK2 and represent the densitometric analysis of western blots from three independent experiments, each performed in triplicate.

Fig. 6. S1P increases StAR protein expression and HSL phosphorylation

(A). H295R cells were treated with 1 μ M S1P for 0 to 18 h and cell lysates were harvested and separated by SDS-PAGE followed by western blotting analysis using anti-StAR and anti-GAPDH antibodies. (B). Densitometric analysis of western blots of StAR protein expression, normalized to GAPDH protein content in cells treated for 0 to 18 h with 1 μ M S1P. Data graphed represent the mean $\pm$ SEM of 2 separate experiments, each done in triplicate. Asterisks (*) denote statistically significant difference ($p < 0.05$) versus untreated controls. (C) H295R cells were treated with 1 μ M S1P for 15 to 60 min and cell lysates were harvested and separated by SDS-PAGE followed by western blot analysis using antibodies

against phospho-Ser⁵⁶³-HSL (top panel) and anti-HSL (bottom panel). **(D)** Data are graphed as fold change in phospho-HSL over control group mean $\pm$ STD DEV, normalized to total HSL and represent the densitometric analysis of western blots from three independent experiments, each performed in triplicate.

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Fig. 7. Proposed model for S1P-mediated cortisol biosynthesis in H295R cells

S1P binds to S1PR₁ and/or S1PR₃ and activates G_i. G_i couples to PLC, thereby increasing intracellular IP₃ and subsequently cytosolic Ca²⁺. Intracellular Ca²⁺ accumulation activates CamKII, which in turn mediates the phosphorylation of ERK1/2. Acute activation of the S1P signaling pathway culminates in an induction of StAR gene expression and an increase in cortisol secretion. S1P also stimulates PKA-mediated phosphorylation of HSL at Ser⁵⁶³, which increases cholesterol ester hydrolysis and substrate availability for cortisol production.