THE ROLE OF SPHINGOLIPIDS IN CORTISOL SYNTHESIS IN THE ADRENAL CORTEX

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THE ROLE OF SPHINGOLIPIDS IN CORTISOL SYNTHESIS IN THE ADRENAL CORTEX

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To my father, Mahmut Selçuk, my mother, Meliha Selçuk

and

my lovely husband Serkan Özbay

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF SYMBOLS AND ABBREVIATIONS

DHEA	Dehydroepiandrosterone
ACTH	Adrenocorticotropic hormone
cAMP	Cyclic AMP
PKA	cAMP-dependent protein kinase
MC2R	Melanocortin 2 receptor
HSD	Hydroxysteroid dehydrogenase
P450aldo	Aldolase
P450aro	Aromatase
P450scc	Cholesterol side chain cleavage enzyme
CYP17	Cytochrome P450 C17α-hydroxylase/ C17-20 lyase
SF1	Steroidogenic factor-1
PSF	Polypyrimidine-tract binding protein associated splicing factor
So	Sphingosine
SM	Sphingomyelin
SMase	Sphingomyelinase
S1P	Sphingosine-1-phosphate
DAG	Diacylglycerol
EDG	Endothelial differentiation gene
hCG	Human chorionic gonadotropin
PKC	Protein kinase C
TNFα	Tumor necrosis factor α
H295R	Human adrenocortical carcinoma cells
Jeg3	Human choriocarcinoma cells
MA-10	Murine Leydig cells
StAR	Steroidogenic acute regulatory protein
FSH	Follicle stimulating hormone
ER	Endoplasmic reticulum
SREBP	Sterol regulatory element binding protein
bHLH	basic helix-loop-helix leucine zipper
SCAP	SREBP cleavage activating protein
Bt_2cAMP	Dibutyryl cAMP
ALLN	N-acetyl-Leu-Leu-norleucinal
siRNA	Small interfering RNA
DME/F12	Dulbecco's modified Eagle's/ F12
HPLC-ESI-MS/MS	High performance liquid chromatography-electrospray ionization-tandem mass spectrometry
MRM	Multiple reaction monitoring
TLC	Thin layer chromatography
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
ChIP	Chromatin immunoprecipitation
TSA	Trichostatin A
PVDF	Polyvinylidene difluoride
dhS1P	Dihydro-sphingosine-1-phosphate
SRE	Sterol regulatory element
MKP-1	Mitogen activated protein kinase phosphatase-1

SUMMARY

In the human adrenal cortex, adrenocorticotropin (ACTH) activates steroid hormone biosynthesis by acutely increasing cholesterol delivery to the mitochondria and chronically up-regulating the transcription of steroidogenic genes (including CYP17). Sphingolipids are a diverse family of phospholipids and glycolipids that mediate a wide variety of cellular processes, including apoptosis, proliferation, and survival. Sterol regulatory element binding proteins (SREBPs) are a family of transcription factors that regulate genes that are involved in cholesterol biosynthesis and fatty acid metabolism. In this study, we investigated the role of sphingolipids in ACTH-dependent steroidogenesis. H295R human adrenocortical cells were treated with ACTH or dibutyryl cAMP (Bt_2cAMP) for various time periods and the content of several sphingolipid species was quantified by mass spectrometry. Both ACTH and Bt_2cAMP decreased cellular amounts of sphingomyelin, ceramides, sphingosine (So) and sphingosine-1-phosphate (S1P). However, both ACTH and Bt_2cAMP increased the activity of sphingosine kinase and the amounts of S1P released into the media. Both So and S1P increased CYP17 mRNA expression and increased cortisol biosynthesis. This increase in CYP17 transcription occurs by promoting SREBP binding to an SRE at -450/-436 basepairs upstream of the transcription initiation site. Furthermore, chromatin immunoprecipitation (ChIP) assays revealed that Bt_2cAMP and S1P treatment results in an increase in acetylation of histone H3 and SREBP1 binding to CYP17 promoter. Additionally, transient transfection studies using wild type or mutated hCYP17 promoters and RNA interference (RNAi) assays confirmed the role of SREBP1 in mediating the stimulatory effect of S1P on CYP17 transcription. In summary, our studies demonstrate a link between sphingolipid

metabolism and ACTH-dependent steroidogenesis which requires the activation of SREBP1 in human adrenal cortex.

CHAPTER I
INTRODUCTION

Steroid hormones, such as cortisol and aldosterone, are essential regulators of a wide variety of cellular processes and are synthesized from cholesterol in steroidogenic tissues such as the adrenal cortex, ovaries, testes, and placenta. Figure 1 summarizes the major steroid biosynthesis pathways in steroidogenic tissues in human. The fate of pregnenolone is determined by the tissue in which steroidogenesis takes place. Aldosterone, a C21, 17-deoxy steroid, is synthesized in the glomerulosa layer of the adrenal cortex (Figure 1). In fasiculata and reticularis layers of the adrenal cortex, 17α-hydroxylation of pregnenolone leads to glucocorticoid production (Figure 1). C19, 17-ketosteroids, such as dehydroepiandrosterone (DHEA) and androstenedione, are synthesized in reticularis of the adrenal cortex after 17α-hydroxylation and subsequent C17-20 scission. Other sex steroids such as testosterone and estradiol are synthesized in testes and ovaries, respectively.

Steroid hormone biosynthesis in the adrenal cortex involves the coordinate action of several steroid hydroxylase cytochrome P450 enzymes whose genes (CYP) are transcriptionally activated by the peptide hormone adrenocorticotropin (ACTH) through a cAMP/cAMP-dependent protein kinase (PKA) signaling pathway [1]. As shown in Figure 2, when released from the anterior pituitary, ACTH binds to the melanocortin 2 receptor (MC2R), on the surface of fasiculata and reticularis layers of the adrenal cortex cells. This binding activates adenylyl cyclase which leads to a subsequent increase in intracellular cAMP. cAMP then activates PKA, which in turn induces gene transcription.

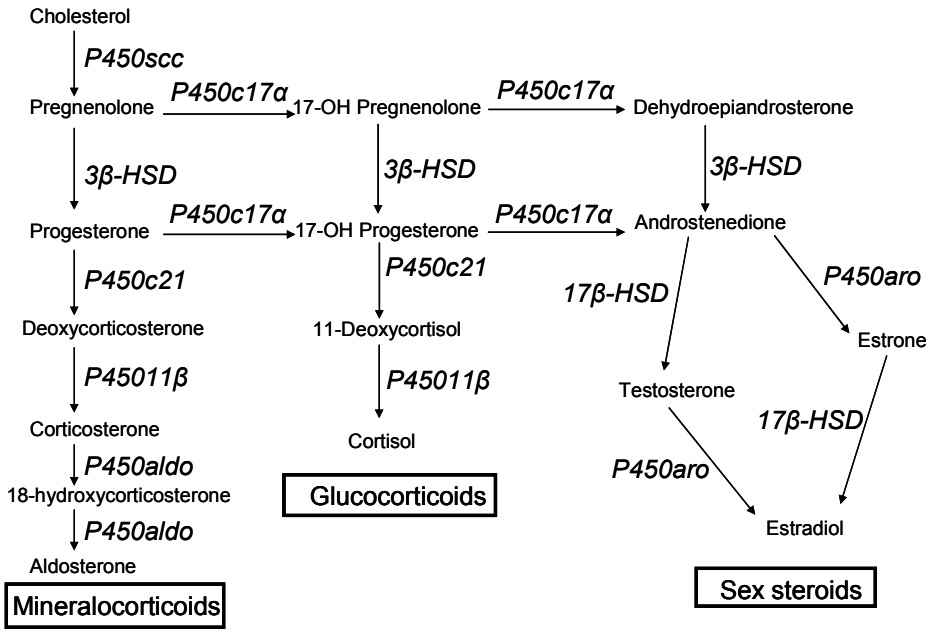


Figure 1. Major steroidogenic pathways in the human
HSD: hydroxysteroid dehydrogenase, P450aldo: aldolase, P450aro: aromatase

Once steroid hormones are produced, they are secreted to the circulation. When these molecules enter the target cell, they act as ligands for zinc-finger nuclear receptor of transcription factors. This steroid hormone/nuclear receptor complex regulates transcription by activating or repressing the target gene, resulting in a wide range of physiological effects in a broad variety of cell types [2].

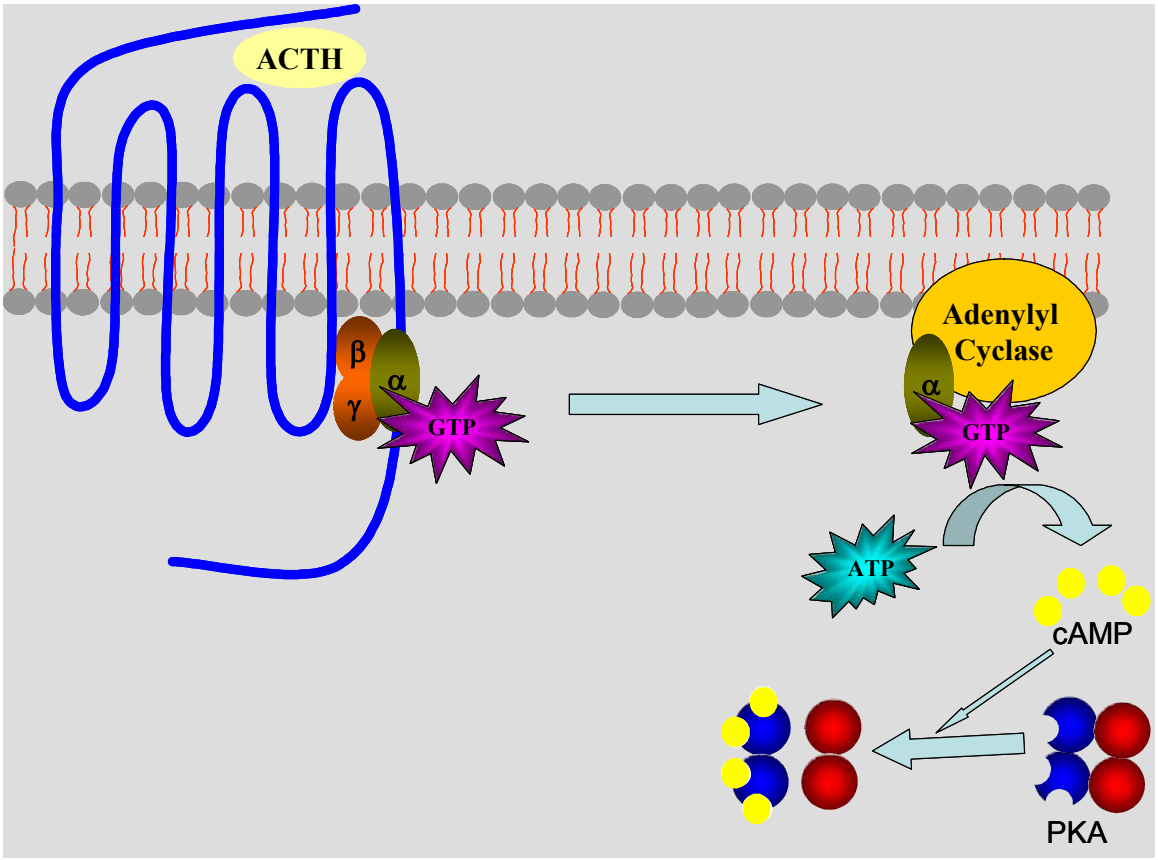


Figure 2. Mechanism of ACTH receptor activation
The binding of ACTH to the MC2R, a G-protein coupled receptor, leads to the activation of G_α and in turn, adenylyl cyclase. ATP is converted to cAMP by the action of adenylyl cyclase. cAMP binds to the regulatory subunits of PKA (blue), which releases the catalytic subunits (red).

1.1 Cytochrome P450 C17α-Hydroxylase/ C17-20 Lyase (CYP17)

CYP17 encodes a bifunctional enzyme, P450c17, that catalyzes both the 17α-hydroxylation of pregnenolone and progesterone required for cortisol biosynthesis and the cleavage of the C-C bond between 17th and 20th carbons of 17α-hydroxylated steroids to produce androgens (Figure 3) [1], [3], [4].

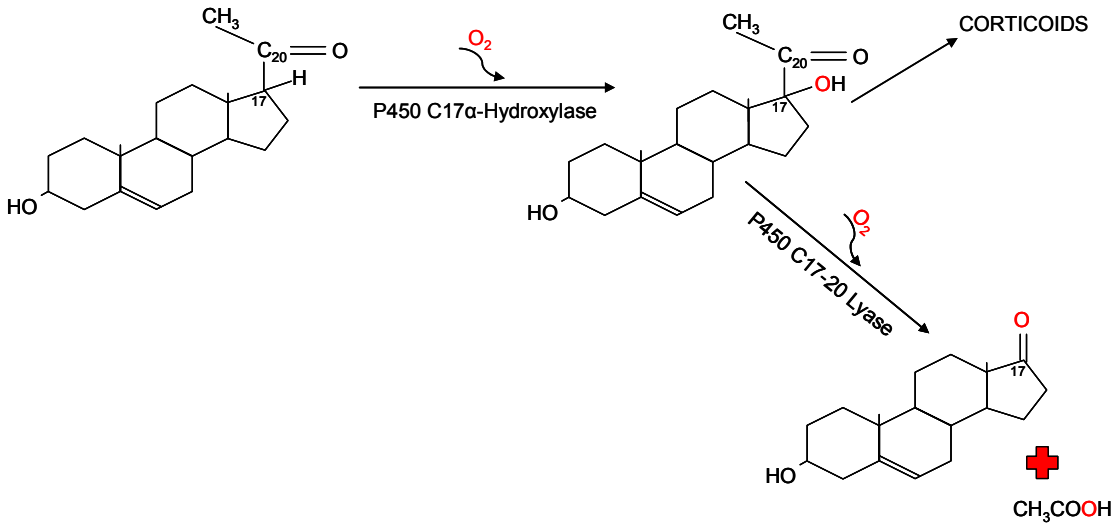


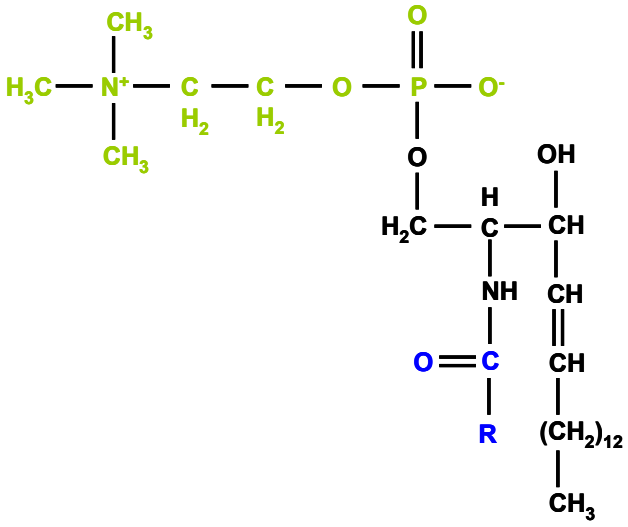
Figure 3. Enzymatic action of P450c17.
Reaction 1: hydroxylation at 17th carbon. Reaction 2: 17-20 bond scission to form 19 carbon steroids

In cholesterol synthesizing species, CYP17 is expressed in the fasiculata and reticularis of the adrenal cortex for both glucocorticoids and androgen biosynthesis and in the gonads for androgen biosynthesis [1]. Recently, it was shown that targeted deletion of CYP17 in mice caused a decrease in circulating testosterone, infertility, and changes in sexual behavior due to androgen imbalance [5]. Like all steroidogenic genes, CYP17 contains a binding site for steroidogenic factor-1 (SF-1). SF-1 was identified as a steroidogenic, tissue specific transcription factor that regulates the expression of steroid hydroxylase enzymes in the gonads and the adrenal cortex [6], [7]. SF-1 knockout mice revealed a complex endocrine phenotype, including the adrenal and gonadal agenesis [8], [9] and absence of ventromedial hypothalamic nucleus [10], [11]. It was previously

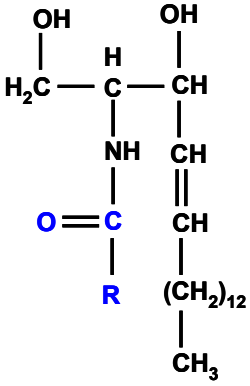
shown that the ACTH/cAMP-dependent increase in human CYP17 gene expression requires binding of a protein complex containing SF-1, $p54^{nrb}$, and polypyrimidine-tract binding protein associated splicing factor (PSF) [12]. The affinity of this SF-1/$p54^{nrb}$/PSF complex for region -57/-37 of the CYP17 promoter is induced by cAMP and is dependent on phosphatase activity [13], [14]. Although ACTH/cAMP plays a central role in regulating CYP17 gene expression, other signaling cascades and second messenger systems have been shown to modulate CYP17 gene expression [15], [16], [17].

1.2 Sphingolipids

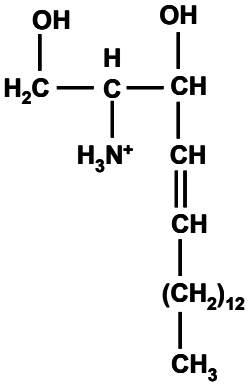
Sphingolipids are a diverse family of phospholipids and glycolipids that share a common sphingoid base backbone [such as sphingosine (So)], an amide-linked long chain fatty acid, and one of various polar head groups (Figure 4). Figure 5 outlines the sphingolipid pathway. Ceramide can be synthesized from palmitoyl CoA and serine in several steps or it can be produced from sphingomyelin (SM) by the action of sphingomyelinases (SMases). Ceramide can be cleaved by ceramidases to yield So and So can be phosphorylated by sphingosine kinases (SK) to form sphingosine-1-phosphate (S1P).



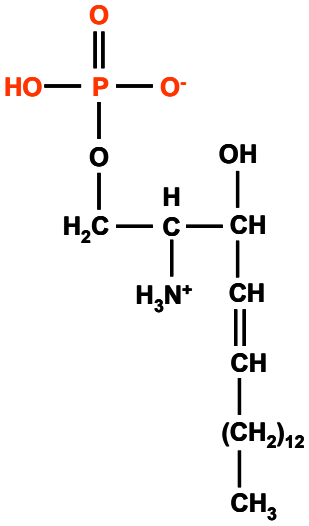
Sphingomyelin



Ceramide



Sphingosine



Sphingosine-1-Phosphate

Figure 4. Structures of SM, Ceramide, So, S1P

Black: sphingoid base backbone, **green:** phosphocholine, **blue:** amide linked fatty acid chain, **red:** phosphate group

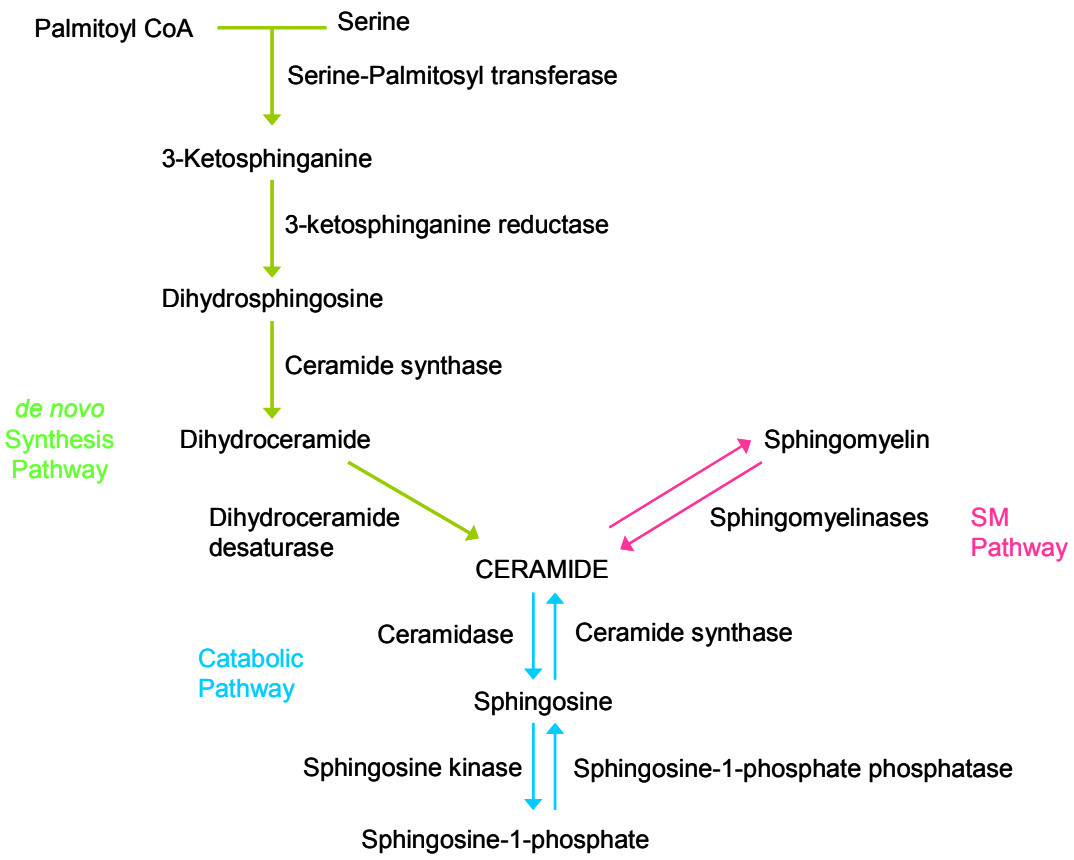


Figure 5. Sphingolipid Pathway

Sphingolipids serve as structural components of the cell membrane and key modulators of cellular processes [18], [19], [20], [21]. Sphingolipid metabolites like ceramide and S1P mediate cell-cell interactions, participate in signal transduction pathways and modulate the activity of various cellular proteins and receptors. Ceramide has been shown to act as a second messenger for events as diverse as differentiation, senescence, proliferation, cell cycle arrest, and apoptosis [20], [21], [19]. Ceramide has also been shown to serve an important role in coordinating the response of cells to

stress stimuli [22], [23], [24] and may regulate the production of inflammatory eicosanoids [25], [26]. Similarly So has been found to mediate varied cellular processes. Studies have been shown that So inhibits protein kinase C [27] and phosphatidic acid phosphohydrolase [28], while activating phospholipase D [29] and diacylglycerol (DAG) kinase [30].

The effects of So on proliferation are cell type specific, where So is antimitogenic in many cells, but promitogenic in others. S1P however, has been implicated as a second messenger in cellular proliferation and survival [31], [32], [33], [34], chemotaxis [35] and in protection against ceramide-mediated apoptosis [36]. Agents such as growth factors and cytokines have been shown to promote the biosynthesis of S1P [19], [31], [32], [37], [38], [39]. S1P has multiple second messenger roles. Many of the actions of S1P are mediated by serving as a specific ligand for several members of the G-protein coupled endothelial differentiation gene (EDG) receptor family [40], [41]. Previously, five different S1P receptors have been identified [42], [43], [44] which were renamed as $S1P_1$ (EDG-1), $S1P_2$ (EDG-5), $S1P_3$ (EDG-3), $S1P_4$ (EDG-6) and $S1P_5$ (EDG-8) [42]. The differences in signaling through these receptors are mainly a result of differential coupling to G-proteins. $S1P_1$ couples to G_i [45], [46], whereas $S1P_2$ and $S1P_3$ couple to G_i, G_q and G_{13} [46]. $S1P_4$ has been shown to associate with G_i [47], [48] and $G_{12/13}$ [49] and $S1P_5$ to couple to $G_{i/0}$ and G_{12} [50]. Interestingly, $S1P_2$ activates adenylyl cyclase [51], however since the receptor does not couple to G_s, the activation may occur through an indirect mechanism.

1.2.1 Relationship Between Sphingolipid Metabolism and Steroidogenesis

Over the past few years, several studies have examined the role of sphingolipids, primarily ceramide, on steroid hormone biosynthesis in both gonadal and adrenal cell lines [52], [53], [54], [55], [56], [57], [58], [59]. Ceramide, bacterial SMase (converts SM to ceramide), and dihydroceramide have all been shown to increase basal and human chorionic gonadotropin (hCG)-stimulated progesterone synthesis in MA-10 murine Leydig cells [52]. The mechanism by which ceramide induces progesterone production in these cells is unknown, however, since the concentration of ceramide used to increase progesterone biosynthesis was below that used to induce apoptosis, it was concluded that the mechanism by which ceramide stimulates progesterone production is not linked to the induction of apoptosis. Similar stimulatory effects of ceramide on steroid hormone production have also been found in JEG-3 human choriocarcinoma cells [53]. Exposure of MA-10 cells to SMase results in increased SM degradation, increased cholesterol movement from the cell surface into the mitochondria, and increased progesterone secretion [54]. SMase also enhanced cAMP-stimulated steroidogenesis, suggesting that sphingolipid metabolism may promote steroid hormone biosynthesis via increasing cholesterol movement to the inner mitochondrial membrane [54]. S1P was found to stimulate cortisol secretion in zona fasciculata bovine adrenal cells in a PKC and Ca^{2+}-dependent manner [55].

Studies have also been published demonstrating an inhibitory role of sphingolipids on steroid hormone production [59], [56], [58], [57]. Degnan *et al.* have shown that ceramide analogs have no effect on rat Leydig cell steroidogenesis, while SMase inhibits hCG-stimulated testosterone production [58]. Budnick *et al*. have reported that TNFα inhibits testosterone production by inducing ceramide accumulation, thereby resulting in

decreased steroidogenic acute regulatory (StAR) protein expression levels [59]. The role of ceramide on progesterone production was also examined in rat granulosa cells. Both SMase and ceramide inhibited follicle stimulating hormone (FSH)-stimulated progesterone biosynthesis and CYP11A1 and 3β-hydroxysteroid dehydrogenase mRNA expression levels [56]. However, in contrast to studies in rat Leydig cells, no effect of ceramide or SMase was found on cAMP production.

1.3 Sterol Regulatory Element Binding Proteins (SREBPs)

Sterol regulatory element binding proteins (SREBP1a, SREBP1c, and SREBP2) are a family of transcription factors that regulate the genes that encode for more than 30 enzymes that are involved in cholesterol, triacylglyceride, phospholipid biosynthesis, fatty acid desaturation, and cholesterol uptake [60], [61]. The activity of these transcription factors is controlled by transport from endoplasmic reticulum (ER) to Golgi (Figure 6). SREBPs have a basic helix-loop-helix leucine zipper (bHLH) domain at the N terminal that acts as DNA binding domain and two transmembrane domains. After translation in the ER, SREBPs bind to a protein called SREBP-cleavage activating protein (SCAP) which has a WD40-rich, long C terminal domain that binds to the C terminal of SREBPs and an N terminal domain that has eight transmembrane helices. SCAP has two functions; first to sense the absence of cholesterol and second to escort SREBP to Golgi in the absence of cholesterol. When SREBP enters Golgi, its bHLH domain is cleaved by site-1-protease and site-2-protease and the mature form translocates to nucleus to initiate transcription for target genes [60], [61].

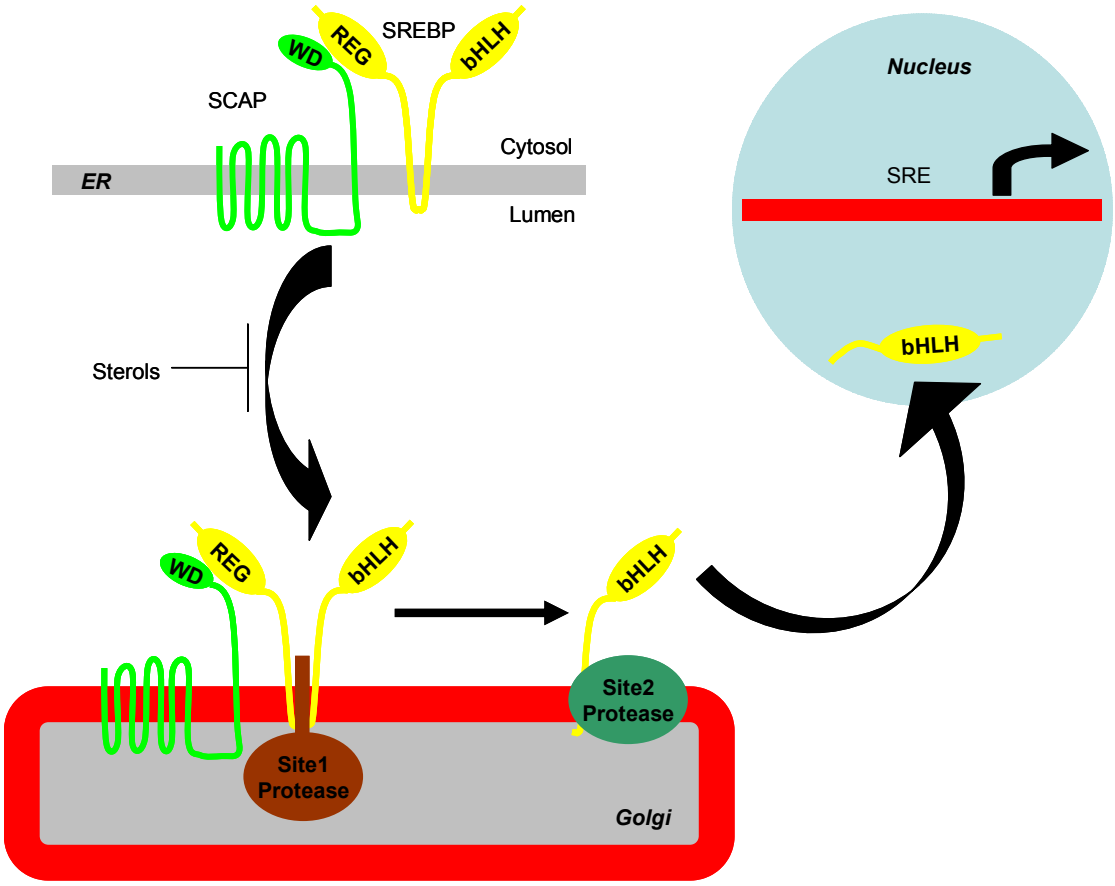


Figure 6. SREBP Maturation
In the presence of sterols SCAP-SREBP complex cannot leave ER. SCAP can sense the decreases in sterol level in the cell and escorts SREBP to Golgi. In Golgi SREBP is cleaved by two membrane associated proteases, and bHLH domain of SREBP translocates to nucleus where it binds to SRE to activate target gene transcription.

Interestingly, sphingolipids have been shown to induce SREBP-1 cleavage, leading to increase in intracellular cholesterol [62]. Furthermore, SREBP1a has been shown to activate transcription of the StAR protein which stimulates rapid steroid hormone synthesis by assisting the transportation of cholesterol from outer to inner mitochondrial membrane [63].

Although a number of studies have demonstrated the role of sphingolipids on steroidogenesis [52], [53], [54], [55], [56], [57], [58], [59], the role of sphingolipids on cortisol production in human adrenal cortex and the mechanism of action was not studied. This thesis will present the studies that establish a role for sphingolipid metabolites and SREBP1 in the biosynthesis of cortisol in the human adrenal cortex.

CHAPTER II
MATERIALS AND METHODS

2.1 Reagents

Dibutyryl cAMP (Bt_2cAMP) and N-acetyl-Leu-Leu-norleucinal (ALLN) were obtained from Sigma (St. Louis, MO). Sphingolipids were obtained from Avanti Polar Lipids Inc. N-Acetyl-D-erythro-sphingosine (C2-ceramide) was freshly prepared prior to each experiment by dissolving in ethanol. D-erythro-sphingosine (So) was prepared by dissolving in ethanol, followed by dilution in fatty acid free bovine serum albumin (Calbiochem, La Jolla, CA). D-erythro-S1P and dihydro-S1P (dhS1P) were prepared by solubilization in ethanol and dimethylamine, followed by evaporation and solubilization in 2 mM fatty acid free BSA. ACTH was obtained from Calbiochem (La Jolla, CA). Anti-SREBP1 (rabbit) and anti-SREBP2 (rabbit) were obtained from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). SREBP1, SREBP2, SK1, SK2 and non-specific siRNAs were obtained from Dharmacon (Lafayette, CO). Anti-SK1 and anti-SK2 were obtained from Exalpha Biologicals Inc. (Watertown, MA).

2.2 Cell culture

H295R adrenocortical carcinoma cells [64], [65] were generously donated by Dr. William E. Rainey (Medical College of Georgia, Augusta, GA) and cultured in Dulbecco's modified Eagle's/F12 (DME/F12) medium (Invitrogen, Carlsbad, CA) supplemented with

10% Nu-Serum I (BD Biosciences, Palo Alto, CA), 0.5% ITS Plus (BD Biosciences, Palo Alto, CA), antibiotics, and antimycotics. H295R cells are a subpopulation H295 cells that forms a monolayer in culture [64]. Unlike other adrenal cortex cell lines, H295R cells act as pluripotent adrenocortical cells that are able to produce each of the zone specific steroids [64]. Treatment of H295 cells with angiotensin II leads to the production of aldosterone and decreased levels of cortisol, whereas treatment with ACTH, cAMP or forskolin leads to increased levels of cortisol [64]. It was also reported that in H295 cells, cholesterol supplementation of the media increased the adrenal androgen levels compared to cortisol [66].

2.3 Analysis of sphingolipid molecular species

For sphingolipid measurements, cells were treated for 2 hours with 50 nM ACTH or 1 mM Bt_2cAMP and sphingolipids were analyzed by liquid chromatography-electrospray ionization-tandem mass spectrometry (LC-ESI-MS/MS) as described previously [67], [68]. Briefly, cells were pelleted into glass tubes, reconstituted in 0.5 ml methanol and 0.25 ml chloroform and then mixed with internal standards with sonication. The internal standards for quantization of the sphingolipids were obtained from Avanti Polar Lipids (Alabaster, AL). After incubating this mixture overnight at 48°C, 75 µl of KOH was added and half of the mixture was placed into a new tube, pelleted, dried and reconstituted in free base reconstitution mixture [methanol: water: acetic acid (50: 50:1) (v: v: v)]. The other half of the mixture was neutralized by adding glacial acetic acid, water and chloroform and centrifuged. The lower layer was dried and reconstituted in complex sphingolipid reconstitution mixture [methanol: chloroform (25: 75) (v: v)]. To

quantify the sphingolipids by HPLC-MS/MS using multiple reaction monitoring (MRM), extracts were loaded onto HPLC columns coupled to tandem mass spectrometer and precursor and product ion pairs were monitored in specific time frames. For the analysis of sphingoid base and sphingoid base-1-phosphate, reconstituted free base mixture was loaded into autosampler. After pre-equilibration of the reverse phase C_{18} column with 50: 50 reverse phase solutions A [water: methanol: acetic acid (69: 30:1) (v: v: v) containing 5mM ammonium acetate] and B [methanol: acetic acid (99:1) (v: v) containing 5mM ammonium acetate], the extracts were injected. After the start of monitoring ion transitions the column was first washed with 50: 50 mixture of reverse phase solution A and B, then treated with 100% reverse phase solution B linear gradient, washed with 100% reverse phase solution B, and lastly re-equilibrated with 50: 50 mixture of reverse phase solution A and B. Lipids were quantified using the areas under the peaks. For the analysis of complex sphingolipids, an aliquot of reconstituted complex sphingolipid mixture was diluted 5: 100 with normal phase solution A [acetonitrile: methanol: acetic acid (97: 2: 1) (v: v: v) containing 5 mM ammonium acetate] and loaded into autosampler. Silica column was pre-equilibrated with normal phase solution A and the sample was injected. After the start of monitoring ion transitions, the column was first washed with 100% normal phase solution A, then treated with 100% normal phase solution B [methanol: water: n-butanol/acetic acid (64: 15: 20: 1) (v: v: v: v) containing 5 mM ammonium acetate] linear gradient, washed with 100% normal phase solution B, and lastly re-equilibrated with 100% of normal phase solution A. Lipids were quantified using the areas under the peaks.

2.4 Sphingosine-1-phosphate in the media

To measure S1P released into the cell culture media, H295R cells were plated onto 100 mm dishes and then incubated in serum-free media containing 80 µCi [^{32}P]-orthophosphate for 12 hours. Cells then treated for 30 minutes or 2 hours with 50 nM ACTH or 1 mM Bt_2cAMP. Media and cells were collected and extracted with 2.5 volumes of chloroform/methanol/HCl (100:200:1, v/v), 1 volume of 2 M KCl and 1 volume of chloroform. The organic layer was dried, under nitrogen, resuspended in chloroform/methanol (95:5, v/v), and separated by thin layer chromatography (TLC). TLC plates were dried, sprayed with ninhydrin to visualize lipids, subjected to phosphorimaging (Fuji Film, Japan), and the amount of radioactivity measured normalized to the total protein content of each sample. S1P was identified by comparison to a nonradiolabeled standard.

2.5 Sphingosine kinase assay

Sphingosine kinase activity was assayed as described by Olivera *et al* [69]. Briefly, cells were plated onto 100 mm dishes and treated with 50 nM ACTH or 1 mM Bt_2cAMP for 5- to 60 minutes. After the desired treatment period, the cells were washed with ice-cold PBS, and then harvested into 200 µl of lysis buffer [20 mM Tris-HCl (pH 7.4), 20% glycerol, 1 mM dithiothreitol, 1 mM EDTA, 1 mM sodium orthovanadate, 10 mM $MgCl_2$, 15 mM sodium fluoride, 0.5 mM 4-deoxypyridoxine, and 1X protease inhibitor cocktail including AEBSF hydrochloride, aprotinin, E-64, EDTA disodium, leupeptin hemisulfate (Calbiochem)]. The cytosolic fraction was incubated with 5 µM D-*erythro*-sphingosine and [γ-^{32}P] ATP for 30 minutes at 37ºC. Sphingosine used in the reactions

was solubilized as a sphingosine-BSA complex as described above. Reactions were terminated by placing tubes on ice and adding 1 M HCl (20 µl). Eight hundred µl of chloroform/methanol/HCl (100:200:1, v/v) was added and the samples vortexed for 10 minutes, followed by the addition of 200 µl chloroform and 200 µl of 2 M KCl, vortexing again for 10 minutes and phase separation by centrifugation. An aliquot (50 µl) of the organic phase was spotted onto a Silica Gel 60 TLC plate and developed in 1-butanol/methanol/acetic acid/water (80:20:10:20, v/v). Standard unlabeled S1P was also spotted onto each plate and visualized by spraying with ninhydrin. The dried plates were exposed to a phosphorimager screen and the amount of radioactivity measured normalized to the total protein content of each sample.

2.6 Cortisol assay

Cells were cultured in twelve-well plates and treated with 1 mM Bt_2cAMP, 1 µM So, or 1 µM S1P for time periods ranging from 5 minutes to 24 hours. Cortisol released into the media was determined in triplicate against cortisol standards made up in DME/F12 medium using a 96-well plate enzyme-linked immune cortisol assay (Diagnostic Systems Corporation, Houston, TX). Results are expressed as nanomoles per milligram cellular protein.

2.7 RNA Isolation and Northern Blotting

Cells were cultured onto twelve-well plates and treated with 1 mM Bt_2cAMP, 0.1- to 10 μM So or 0.1- to 10 μM S1P for 12 hours. Total RNA was prepared from treated cells by acid-phenol extraction [70]. RNA was fractionated by agarose (1%) gel electrophoresis in the presence of 5% formaldehyde and transferred onto nylon transfer membrane filters (Millipore, Bedford, MA). A 1.2 kb cDNA fragment of CYP17 was used to detect CYP17 mRNA expression. cDNA fragments were radiolabeled with [γ-^{32}P]dCTP using a Random Primer Labeling Kit (Takara). Blots were hybridized overnight at 42ºC in 50% formamide, 5X SSPE, 1% SDS, 5X Denhardt's, 50 μg/ml tRNA, and ^{32}P-labeled cDNA fragments. The hybridized membranes were sequentially washed for 2 X 10 minutes in 2X SSC, 0.2% SDS and 2 X 5 minutes in 0.2X SSC, 0.2% SDS at 42°C. The amount of probe bound to the filter was quantified using a Fluorescence/Phospho-Imager (Fuji Film, Japan). Results were normalized to the content of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA.

2.8 RNAi and Real Time RT-PCR

In RNAi experiments, cells were subcultured onto twelve-well plates and 24 hours later media replaced with Optimem (Invitrogen, Carlsbad, CA). Cells were transfected with 150 nM of SREBP1, SREBP2, SK1, SK2 or 150nM of negative control siRNA using siIMPORTER. All siRNA oligonucleotides and siIMPORTER were obtained from Upstate (Lake Placid, NY). Seventy-two hours (SREBP1 and SREBP2) or forty-eight hours (SK1 and SK2) after the transfection, cells were treated with 1 mM Bt_2cAMP

or 1 μM S1P for 6 hours. Total RNA was extracted using TRIzol® (Invitrogen, Carlsbad, CA) and amplified using the iScript One-Step RT-PCR Kit with SYBR® Green (Bio-Rad, Hercules, CA) and an iCycler real-time thermocycler (Bio-Rad, Hercules, CA). The PCR primers shown in Table 1were used.

Table 1 RT-PCR Primers

CYP17	
Forward	5'-CTCTTGCTGCTTCACCTA
Reverse	5'-TCAAGGAGATGACATTGGTT
actin	
Forward	5'-ACGGCTCCGGCATGTGCAAG-3'
Reverse	5'-TGACGATGCCGTGCTGCATG-3'
SK1	
Forward	5'-CTGGCAGCTTCCTTGAACCAT-3'
Reverse	5'-TGTGCAGAGACAGCAGGTTCA-3'
SK2	
Forward	5'-CCAGTGTTGGAGAGCTGAAGGT-3'
Reverse	5'-GTCCATTCATCTGCTGGTCCTC-3'

PCR reactions were as follows:

i. 1 X 48ºC, 30 minutes

ii. 1 X 95ºC, 10 minutes

iii. 40 X 95ºC, 15 seconds, 60ºC, 1 minute

iv. Cool to 4ºC.

hCYP17 expression is normalized to actin and calculated using the delta delta cycle threshold (ΔΔCT) method. S1P receptor expression was determined using Taqman® gene expression probes and iScript One-Step RT-PCR Kit for Probes (Bio-Rad, Hercules, CA)

2.9 Transient transfection and reporter gene analysis

Cells were subcultured onto twelve-well plates and 24 hours later transfected with 500 ng of reporter plasmids containing 1100-, 700-, 300-, or 57-base pairs of the CYP17 promoter upstream of the transcription initiation site [12] using GeneJuice (Novagen, Madison, WI). The CYP17 1100-, 700-, and 300-pGL3 constructs were generated by PCR using a plasmid containing a 1.8 kb fragment of the CYP17 promoter fused to the luciferase gene in the pGL3 vector [generously donated by Dr. Janette M. McAllister (Pennsylvania State University, Hershey, PA)]. The CYP17 57-pGL3 plasmid was constructed by ligating double stranded oligonucleotides corresponding to the region -57/-2 of the CYP17 5' flank upstream of the luciferase gene in the pGL3 vector (Promega, Madison, WI). Cells were co-transfected with 10 ng of the Renilla luciferase plasmid (pRL CMV, Promega, Madison, WI) for normalization. Cells were then treated with 1 mM Bt_2cAMP, 1 μM So, or 1 μM S1P for 6 hours and harvested for dual luciferase assays (Promega, Madison, WI).

2.10 Chromatin Immunoprecipitation (ChIP)

For ChIP assays [71], [72] H295R cells (150 mm dishes) were stimulated with 50 nM ACTH, 1 mM Bt_2cAMP, 0.5 μM C2-ceramide, 1 μM So, 1 μM S1P, or 500 nM trichostatin A (TSA) for 1 hour or 4 hours. Cross-linking was performed by incubation in 1% formaldehyde (in PBS) for 10 minutes. The reaction was stopped by the addition of glycine (0.125 M final concentration), the cells washed, the nuclei harvested, and the lysates sonicated to obtain optimal DNA fragment lengths of 100 to 1000 base pairs.

The purified chromatin solutions were immunoprecipitated using anti-acetyl histone H3 (Upstate, Charlottesville, VA) or anti-SREBP1 and protein A/G plus (Santa Cruz Biotechnology, Santa Cruz, CA). The reaction was centrifuged at 4,000 rpm for 5 minutes and 50 µl of the supernatant (input) retained. The antibody/protein/DNA bound beads were subjected to a series of 5-minute washes: three times in RIPA buffer (1% Nonident P40, 0.5% sodium deoxycholate, 0.1% SDS in 1X PBS), three times in RIPA buffer plus 500 mM NaCl, three times in washing buffer (10 mM Tris-Cl, pH 8, 0.25 M LiCl, 1 mM EDTA, 1 mM EGTA, 1% NP-40, 1% sodium deoxycholate, 10 mM sodium butyrate, 20 mM β-glycerophosphate, and protease inhibitors), and three times in TE. The cross-links were reversed and protein digested using proteinase K (100 µg/ml). DNA was purified by phenol:chloroform extraction and ethanol precipitation. Precipitated DNA was amplified by PCR using the primer pairs shown in Table 2.

Table 2 ChIP Primers

CYP17	
Forward	5'-GGGGACCATTAACCCGACAGCCCTTATCGC-3' (-520/-491 CYP17)
Reverse	5'-GACAGATGACAGATTCAGGAGGGTCACAAG-3' (-331/-360 CYP17)
Forward	5'-CCGCCTCTCTCCCTTCTGGATATG-3' (-142/-119 CYP17)
Reverse	5'-GAGCTCCCACATGGTGGCTGGGTG-3' (+68/+45 CYP17)

PCR reactions were as follows.

i. 1 X 94ºC, 5 minutes

ii. 35 X 95ºC, 1 minutes, 55ºC, 1 minute, 72ºC, 2 minutes

iii. 1 X 72ºC, 10 minutes

iv. Cool to 4ºC.

PCR products were then subjected to agarose (2%) gel electrophoresis.

2.11 SDS-PAGE and Western Blot Analysis

To confirm that RNAi of SREBP1, SREBP2, SK1 and SK2 was effective, cells were transfected with siRNA oligonucleotides targeted at SREBP1 or SREBP2 for 72 hours and cell lysates harvested for SDS-PAGE and Western blotting. Twenty-five µg of each sample was run on 10% SDS-PAGE gels, transferred to polyvinylidene difluoride membranes (PVDF, Pall Corporation, Pensacola, FL) and probed with anti-SREBP1, anti-SREBP2, anti-SK1 or anti-SK2. Protein expression was detected using an ECF Western Blotting Kit (Amersham Biosciences, Piscataway, NJ) and a Fluor/Phospho-Imager (Fuji Film, Japan).

To determine the effect of S1P on SREBP processing, cells were subcultured onto six-well plates and treated with 1 µM S1P for periods ranging from 1 to 4 hours. Two hours before harvesting, 25 µg/ml ALLN was added to inhibit proteome degradation of mature SREBP. For the 1-hour time point, cells were pretreated with ALLN prior to stimulation with S1P. Cells were washed twice in PBS, harvested into RIPA buffer (1% Nonident P40, 0.5% sodium deoxycholate, 0.1% SDS in 1X PBS) and lysed by passing 10 times through a 22-gauge needle. Lysates were centrifuged for 15 minutes at 4°C and the supernatant collected for analysis by SDS-PAGE. Aliquots of each sample (25 µg of protein) were run on 10% SDS-PAGE gels, transferred to PVDF membranes, and probed with anti-SREBP1. SREBP1 protein expression was detected and imaged as described above. For analysis of membrane and nuclear SREBP1, cells were plated onto 60 mm dishes and treated with 1 µM S1P or 1 µM dhS1P. Cells were isolated and membrane and nuclear extracts prepared using NE-PER (Pierce, Rockford, IL) containing protease inhibitors. Twenty-five µg of nuclear or membrane extracts were run

on 10% SDS-PAGE gels, transferred to PVDF membranes, probed with anti-SREBP1 and expression detected as described above.

CHAPTER III
RESULTS

3.1 ACTH stimulates sphingolipid metabolism in H295R cells.

Several studies have characterized the role of sphingolipids in steroid hormone biosynthesis and found both stimulatory and inhibitory roles for ceramide, So and S1P on hormone secretion [52], [53], [54], [55], [56], [57], [58], [59]. However, the effect of ACTH on cellular sphingolipid content is unknown. Thus, we treated H295R human adrenocortical cells for 2 hours with 50 nM ACTH or 1 mM Bt_2cAMP and isolated. Sphingolipids were extracted, identified by tandem mass spectrometry, and the amounts of these sphingolipids were quantified by liquid chromatography, tandem mass spectrometry (LC-ESI-MS/MS) using multiple reaction monitoring (MRM) as described in Materials and Methods.

As shown in Figure 7, both ACTH and Bt_2cAMP decreased cellular SM. Of the different SM molecules in H295R cells, both ACTH and Bt_2cAMP decreased C16 and C18 SM (Figure 8). Catabolism of complex sphingolipid molecules like SM is linked to increased production of bioactive species such as ceramide, So and S1P. Unexpectedly, we also observed ACTH/cAMP-stimulated reduction of ceramides, So and S1P (Figure 9). Bt_2cAMP treatment also decreased the amounts of multiple chain-length subspecies (Figure 10). We also analyzed the temporal effects of ACTH and Bt_2cAMP on cellular sphingolipid content. As seen in Figure 11 and Figure 12, treatment with either ACTH or Bt_2cAMP for 5 minutes caused a rapid decrease in cellular levels of ceramide, SM, So and S1P.

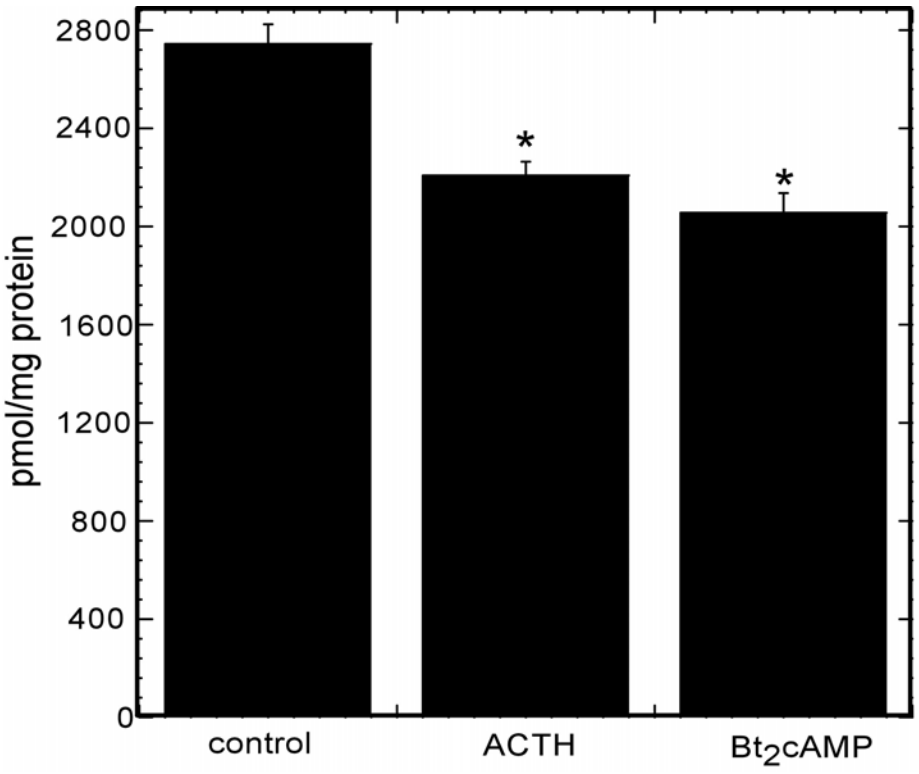


Figure 7. Amount of total endogenous SM

H295R cells treated for 2 hours with 50 nM ACTH or 1 mM Bt_2cAMP were harvested and assayed for SM by LC-ESI-MS/MS as described in Materials and Methods. Results are expressed as mean $\pm$SEM for 4 different experiments (n=3 per experiments) and normalized to total cellular protein. Statistical significance was calculated with respect to untreated control (*, $p<0.05$).

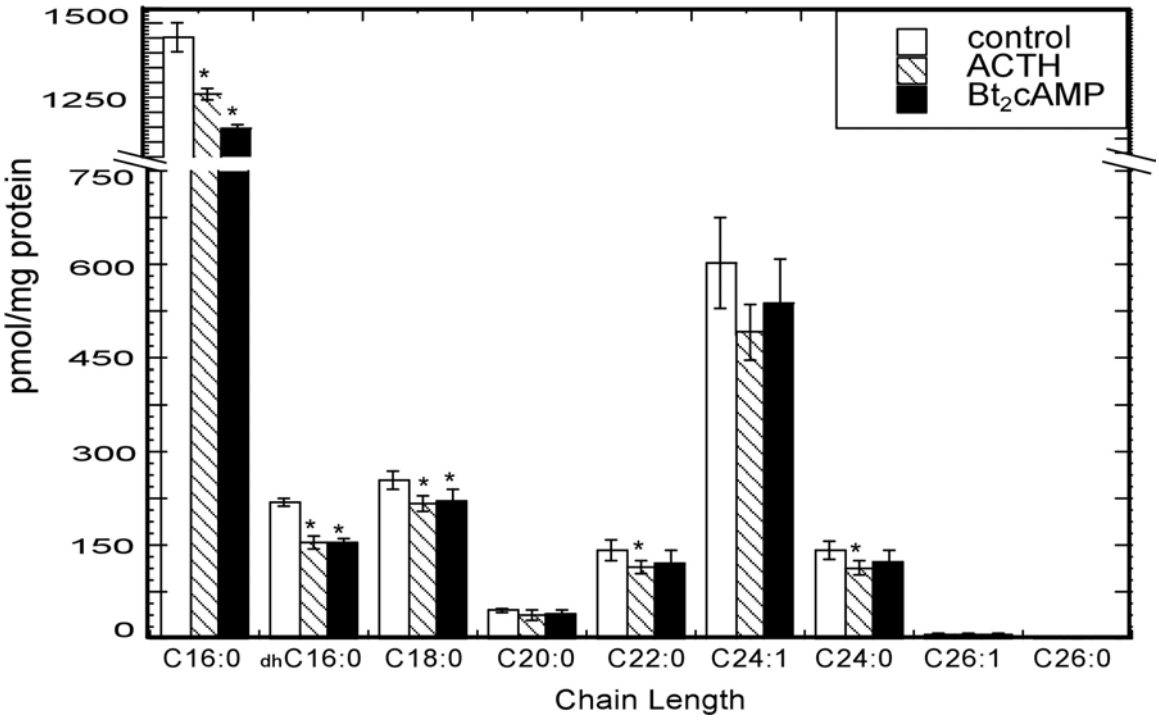


Figure 8. Amounts of SMs

H295R cells treated for 2 hours with 50 nM ACTH or 1 mM Bt_2cAMP were harvested and assayed for cellular content of individual SM molecules by LC-ESI-MS/MS as described in Materials and Methods. Results are expressed as mean $\pm$SEM for 4 different experiments (n=3 per experiments) and normalized to total cellular protein. Statistical significance was calculated with respect to untreated control (*, $p<0.05$).

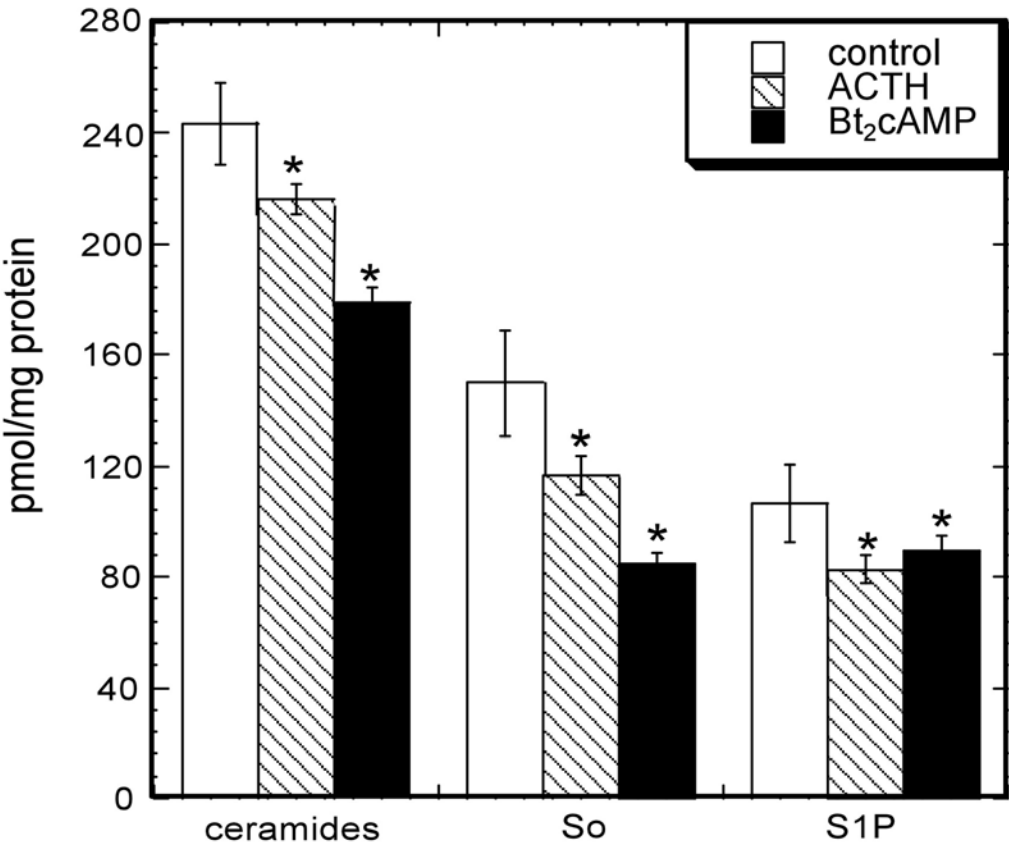


Figure 9. Amounts of total endogenous Ceramides, So and S1P
H295R cells treated for 2 hours with 50 nM ACTH or 1 mM Bt_2cAMP were harvested and assayed for ceramides, So and S1P by LC-ESI-MS/MS as described in Materials and Methods. Results are expressed as mean ±SEM for 4 different experiments (n=3 per experiments) and normalized to total cellular protein. Statistical significance was calculated with respect to untreated control (*, p<0.05).

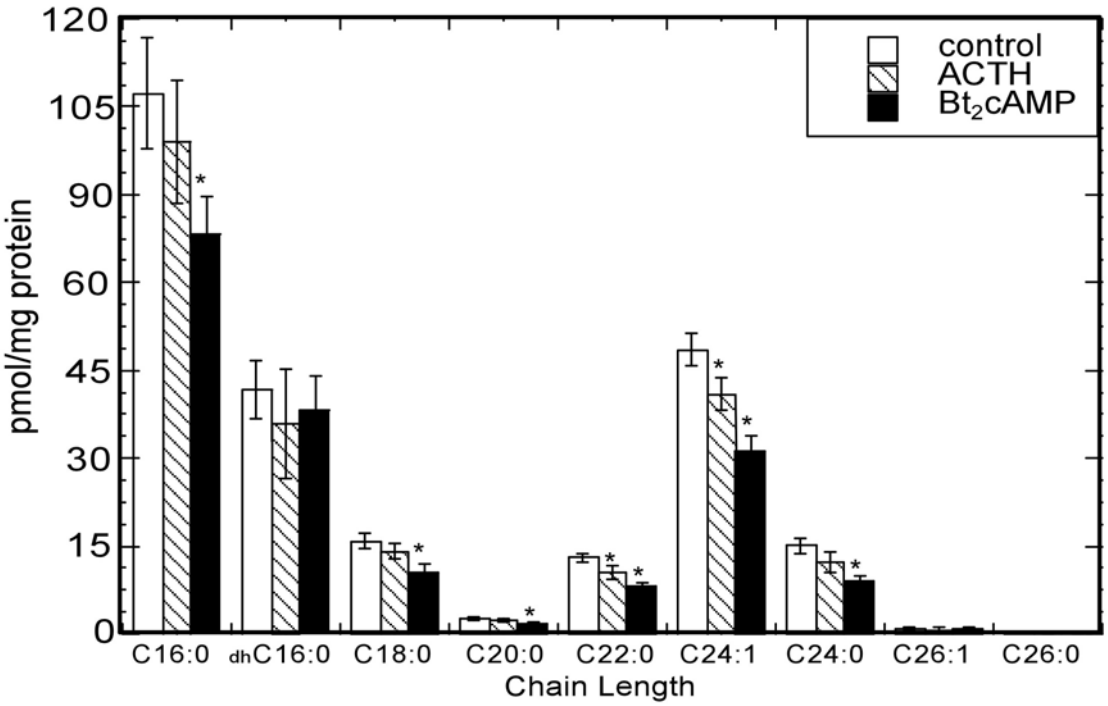


Figure 10. Amounts of ceramides

H295R cells treated for 2 hours with 50 nM ACTH or 1 mM Bt_2cAMP were harvested and assayed for cellular content of individual ceramide molecules by LC-ESI-MS/MS as described in Materials and Methods. Results are expressed as mean $\pm$SEM for 4 different experiments (n=3 per experiments) and normalized to total cellular protein. Statistical significance was calculated with respect to untreated control (*, $p<0.05$).

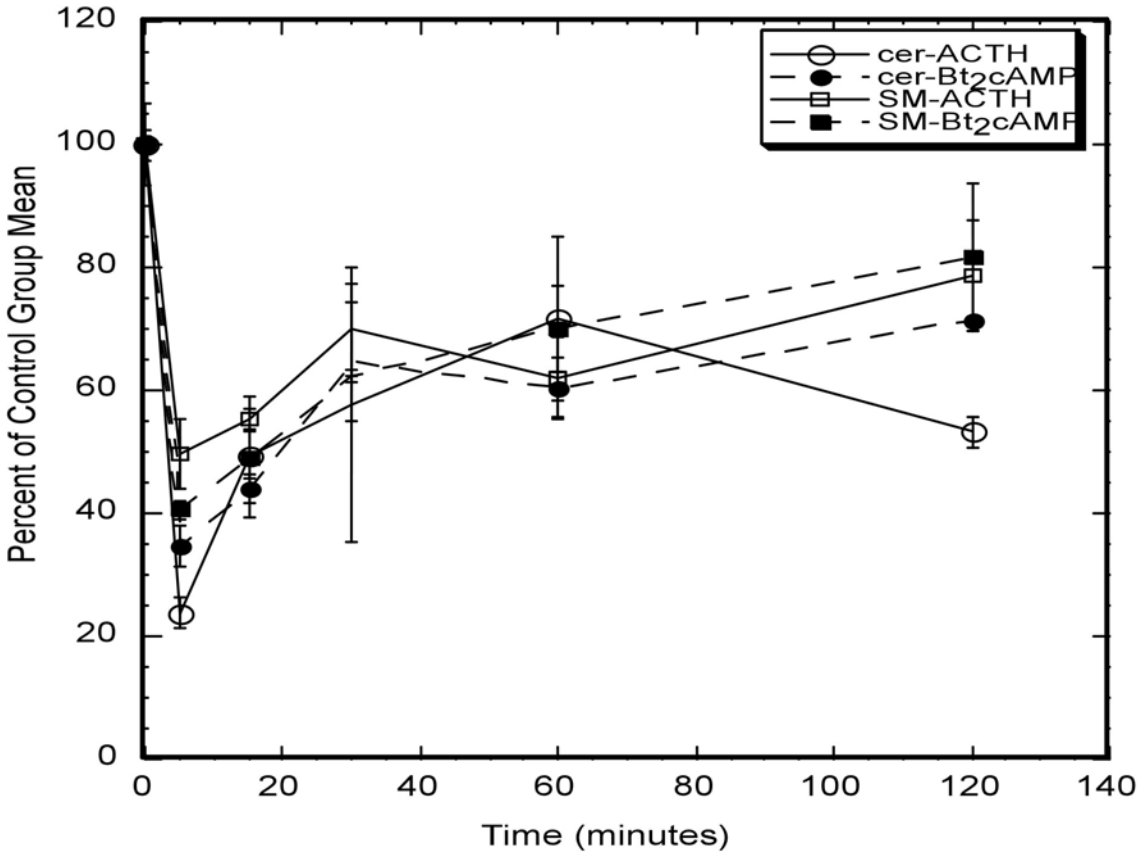


Figure 11. Amounts of SM and ceramide rapidly decrease with ACTH and Bt_2cAMP H295R cells treated with 50 nM ACTH 1 mM Bt_2cAMP for 5-, 15-, 30-, 60- or 120-minutes were harvested and assayed for total cellular SM and ceramides as described in Materials and Methods. Data is expressed as percent of control group mean.

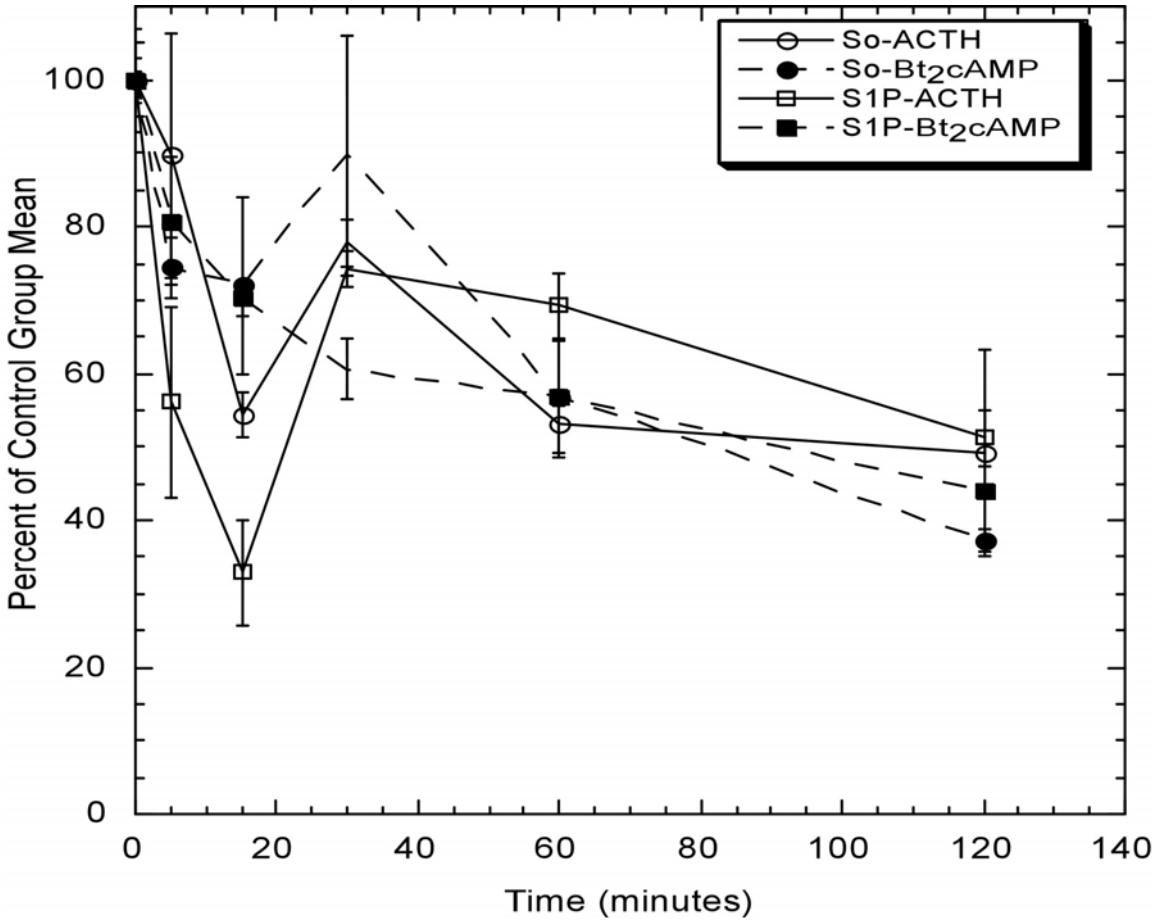


Figure 12. Amounts of So and S1P rapidly decrease with ACTH and Bt_2cAMP H295R cells treated with 50 nM ACTH 1 mM Bt_2cAMP for for 5-, 15-, 30-, 60- or 120-minutes were harvested and assayed for total cellular So and S1P as described in Materials and Methods. Data is expressed as percent of control group mean.

3.2 ACTH/cAMP activate sphingosine kinase activity.

The changes in cellular sphingolipid content in response to ACTH/cAMP described above warranted further characterization of the relationship between steroidogenesis and sphingolipid metabolism in the human adrenal cortex. We hypothesized that this increase in sphingolipid turnover may be due to a stimulatory effect of ACTH/cAMP on enzymes in the sphingolipid metabolic pathway. Thus we carried out enzyme assays as described by Olivera *et al* [69] to determine if ACTH/cAMP was activating SK activity. The cytosolic fraction from the H295R cells treated with 50 nM ACTH or 1 μM Bt_2cAMP for different time points ranging from 5 to 60 minutes was incubated with D-*erythro*-sphingosine and [γ-^{32}P] ATP and amount of S1P produced is measured by TLC.

As shown in Figure 13, exposure of H295R cells to ACTH and Bt_2cAMP resulted in rapid increases in SK activity. Maximal increases in enzyme activity were observed at 15 minutes for both agents and returned to basal levels after 30 minutes. Incubation of H295R cells for 15 minutes with ACTH increased SK activity 3.2-fold, whereas Bt_2cAMP resulted in a 4.8-fold increase in catalytic activity (Figure 13).

3.3 ACTH/cAMP promotes S1P secretion into the media.

Since ACTH and Bt_2cAMP stimulate SK activity (Figure 13), we speculated that this increase should be mirrored by an increase in the cellular concentrations of S1P. However, our mass spectrometric analysis showed that both ACTH and Bt_2cAMP treatments caused a decrease in cellular amounts of S1P (Figure 9, right panel). In the

view of the fact that S1P is an important second messenger and some cells secrete S1P [73], [74]; the amounts of S1P in the medium were also analyzed. H295R cells were incubated in [^{32}P]-orthophosphate for 12 hours followed by 50 nM ACTH or 1 mM Bt_2cAMP treatment. Both media and the cells were separated and analyzed for S1P content by TLC.

As shown in Figure 14, ACTH and Bt_2cAMP evoked increases in S1P in the medium that paralleled the decreases in cellular S1P. These findings strongly suggest that, ACTH and cAMP stimulate SM metabolism, which results in decreases in cellular SM, ceramide, So and S1P and increased secretion of S1P into the extracellular space.

3.4 Sphingolipids stimulate cortisol biosynthesis.

As previously discussed, studies carried out in primary bovine zona fasiculata adrenal cells have found that S1P stimulates cortisol secretion [55]. Based on the effect of ACTH/cAMP on cellular sphingolipid content and SK activity, we carried out experiments to determine the effect of So and S1P on cortisol biosynthesis in H295R human adrenocortical cells. Cells were treated for time points ranging from 5 minutes to 24 hours and cortisol released into the media was determined with enzyme-linked immune cortisol assay.

As shown in Figure 15, both So and S1P maximally increased cortisol production at the 3-hour time point, whereas Bt_2cAMP maximally activated cortisol secretion at 24 hours. The increase in cortisol levels after treatment with So and S1P was transient and decreased to near control levels after 12 hours, whereas Bt_2cAMP-treated cells continued to secrete cortisol after 24 hours (Figure 15).

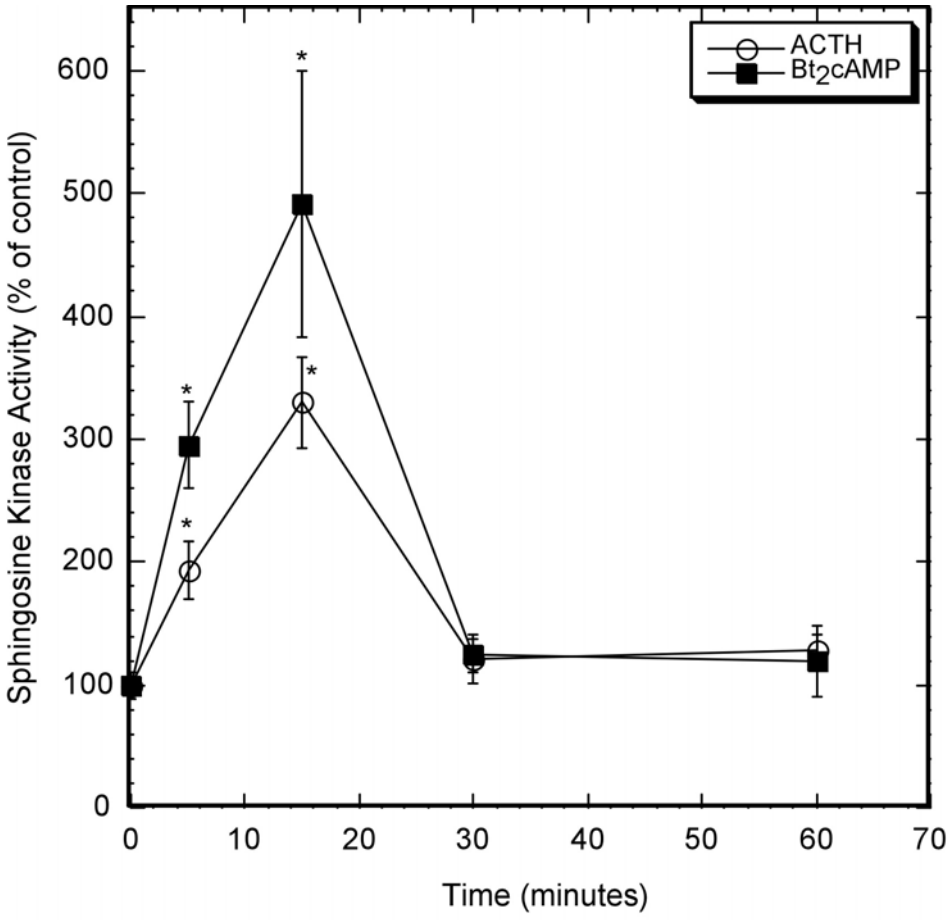


Figure 13. ACTH and Bt_2cAMP activate SK activity in H295R cells

H295R cells were treated for time periods ranging from 5- to 60- minutes with 50 nM ACTH or 1 mM Bt_2cAMP. SK activity was measured as described in Materials and Methods. Radiolabeled S1P was quantified by densitometric analysis. The amount of radioactivity measured normalized to the total protein content of each sample. Results are expressed as percent of control group mean $\pm$ SEM form three separate experiments, each performed in triplicate. *, Statistically different from control, $p<0.05$.

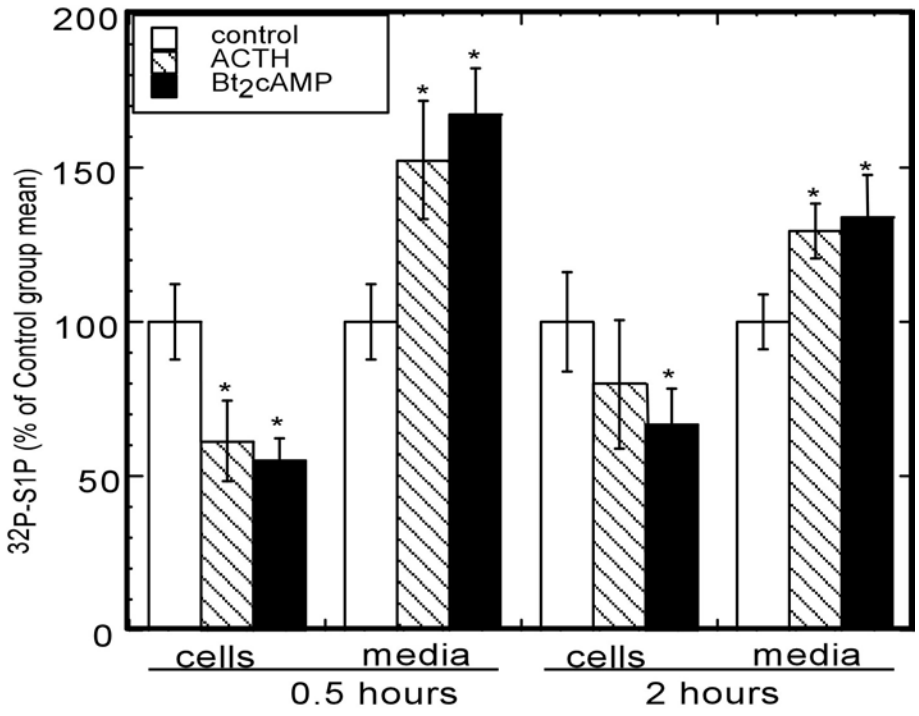


Figure 14. ACTH and Bt_2cAMP increase S1P secretion into the media
H295R cells were incubated with 80 μCi [^{32}P]-orthophosphate then treated with 50 nM ACTH or 1 mM Bt_2cAMP for 30 minutes or 2 hours. Both the media and cells were collected for assay of S1P levels by TLC. The amount of radioactivity measured normalized to the total protein content of each sample. Results were expressed as mean ± SEM form three separate experiments, each performed in triplicate. Statistical significance was calculated with respect to untreated control (*, p<0.05).

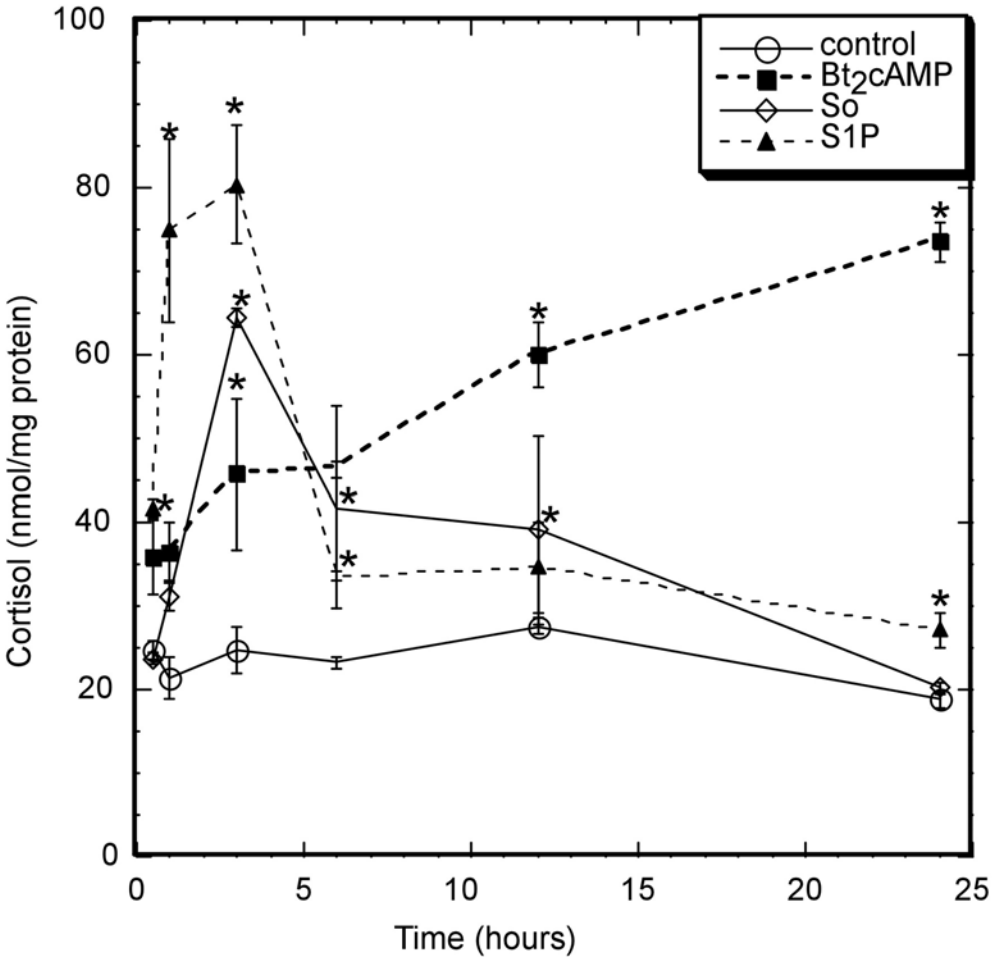


Figure 15. Sphingosine and S1P increase cortisol secretion
H295R cells were treated for time periods ranging from 5 minutes to 24 hours, as described in Materials and Methods, with 1 mM Bt_2cAMP, 1 μM So or 1 μM S1P. The amount of cortisol released into the media was determined by enzyme-linked immune assay. Values represent the mean ± SEM of 2 experiments, each performed in triplicate. *, p<0.5 for agonist –stimulated steroidogenesis versus untreated control.

3.5 S1P induces steroidogenic gene expression.

Given that ACTH and Bt_2cAMP alter the sphingolipid profile in H295R cells (Figure 7 to Figure 12) and that sphingolipids stimulate cortisol secretion (Figure 15), we carried out studies to further explore the relationship between sphingolipid metabolism and steroidogenesis. Cells were treated with Bt_2cAMP or sphingolipids and CYP17 mRNA expression was assayed by Northern blotting.

Figure 16 shows a representative Northern blot from analysis of cells treated with Bt_2cAMP, So or S1P. Both So and S1P increased CYP17 mRNA expression compared to control whereas highest increase was evoked by Bt_2cAMP treatment. The effects of So and S1P on CYP17 mRNA expression was also dose dependent and the maximum increase in CYP17 mRNA expression was 2.0–fold with 10 μM So and 3.6–fold with 1 μM S1P (Figure 17) whereas Bt_2cAMP induced hCYP17 mRNA expression 5.2 fold.

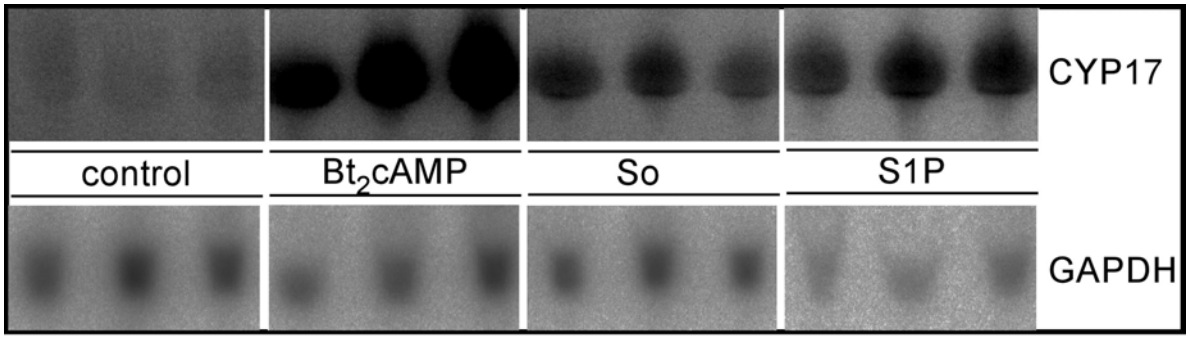


Figure 16. Sphingolipid- dependent CYP17 mRNA expression
Representative Northern blot showing increase in CYP17 mRNA expression after 12-hour treatment with 1 mM Bt_2cAMP, 1 μM So or 1 μM S1P. Northern blotting and hybridization to a radiolabeled CYP17 cDNA (upper panel) or GAPDH cDNA (lower panel) were performed as described in Materials and Methods.

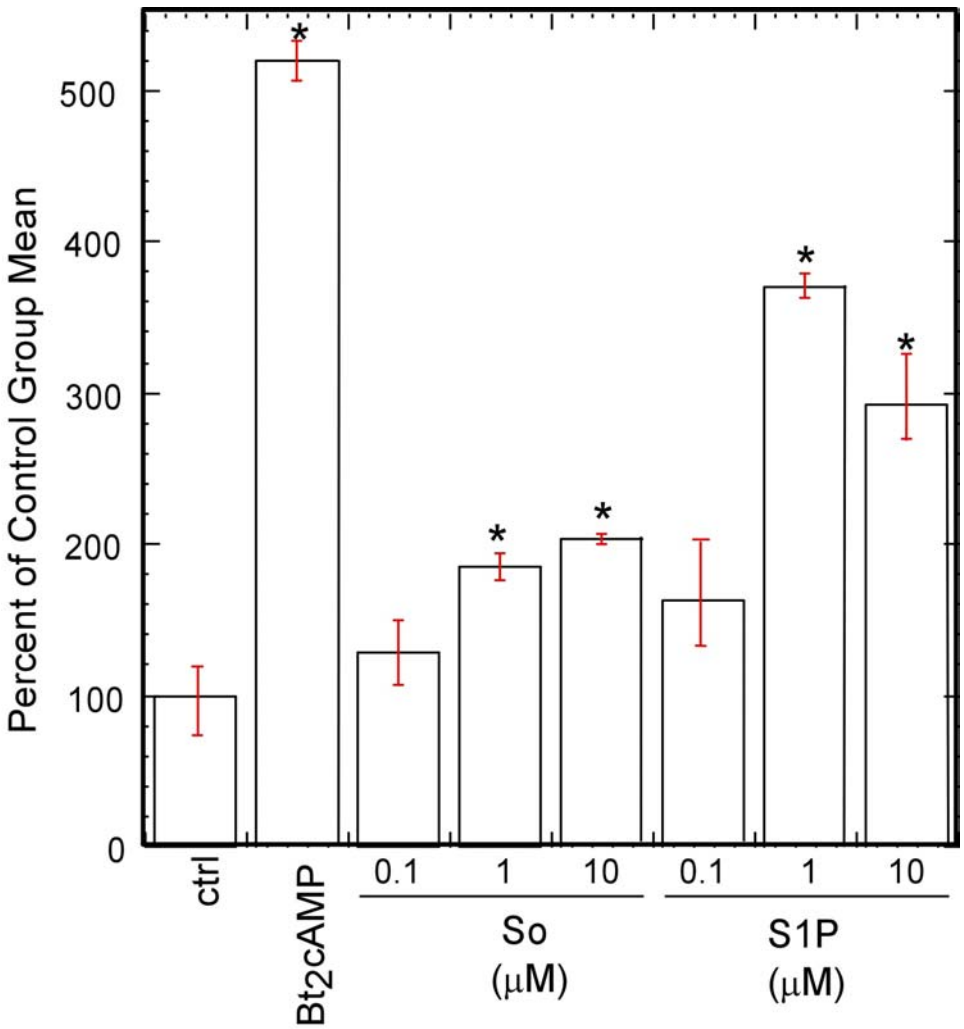


Figure 17. Dose dependency of sphingolipid-dependent CYP17 mRNA expression H295R cells were treated with 1 mM Bt_2cAMP, 0.1 μM-10 μM So or 0.1 μM-10 μM S1P for 12 hours and total RNA was extracted. Densitometric analysis of Northern blots probed for CYP17 mRNA expression. Data graphed represents the mean ± the standard error from 4 separate experiments, each performed in triplicate. CYP17 mRNA expression is normalized to the mRNA content of GAPDH and expressed as a percentage of the control group mean. *Asterisk* indicates statistically different from control; $p < .05$.

3.6 cAMP-dependent CYP17 mRNA expression requires SK1.

Since SK activity was increased by Bt_2cAMP treatment (Figure 13) and both So and S1P increased the CYP17 expression (Figure 16 and Figure 17) we postulated that phosphorylation of So to S1P by SK may be an important step in the cAMP-stimulated induction of CYP17 mRNA expression. We used RNAi to determine if cAMP-stimulated CYP17 transcription requires SK. To determine the effect of SK silencing on CYP17 mRNA expression, H295R cells were transfected with SK1 and SK2 small interfering oligonucleotides for 48 hours as described in Materials and Methods followed by exposure to Bt_2cAMP. Total RNA was extracted for analysis by real time RT-PCR.

Quantitative RT-PCR results revealed that silencing both SK1 and SK2 attenuated the stimulatory effect of Bt_2cAMP on CYP17 mRNA expression (Figure 18). However, suppression of neither SK1 nor SK2 was able to block cAMP-dependent CYP17 transcription completely. Since it was previously demonstrated that cAMP/PKA signaling activates transcription of CYP17 by promoting binding of SF-1 to the promoter [12], it is plausible that there are two distinct pathways that is activated by cAMP, one includes activation of sphingolipid pathway and the other includes activation of SF-1, which end up with an increase in CYP17 mRNA expression. We also confirmed the suppression of SK1 and SK2 mRNA expression by real time RT-PCR (Figure 19) and protein expression by Western blotting (Figure 20).

3.7 Sphingolipids stimulate CYP17 transcriptional activity.

As described above, sphingolipids rapidly stimulate adrenal steroidogenesis (Figure 15). To discern the mechanism by which sphingolipids increase CYP17 mRNA

(Figure 16 and Figure 17), we performed transient transfection assays using plasmids containing varying lengths of CYP17 promoter fused to the luciferase gene.

Treatment of both So and S1P increased the transcriptional activity of the 1100- and 700-pGL3 CYP17 reporter constructs, while having no significant effect on the CYP17 57- and 300-pGL3 plasmids (Figure 21). In contrast to the effect of So and S1P, Bt_2cAMP significantly stimulated the luciferase activity of all plasmids tested (Figure 21) as seen previously [12]. These findings suggest that the mechanism by which sphingolipids induce CYP17 mRNA expression is distinct from SF-1-dependent CYP17 transcription and that the region of the CYP17 promoter required for sphingolipid-stimulated gene transcription lies between -700 and -300 base pairs of the promoter. *In silico* analysis [75] of the -700/-300 region revealed a putative binding site for the SREBP family of transcription factors. Thus, we determined the effect of mutating this site on ability of S1P to stimulate CYP17 reporter gene activity. As shown in Figure 22, mutation of the putative SRE attenuated the stimulatory effects of S1P on CYP17 transcriptional activity.

3.8 Sphingolipids increase acetylation of histone H3 at the CYP17 promoter.

To determine the effect of sphingolipids on chromatin structure, we next examined the effect of S1P on the acetylation of histone H3 at the CYP17 promoter. Acetylated histones are found in chromatin regions that are transcriptionally active. H295R cells were treated with Bt_2cAMP, ceramide, So, S1P or the histone deacetylase inhibitor, trichostatin (TSA) for 4 hours, followed by chromatin immunoprecipitation (ChIP). Cells were incubated with formaldehyde to covalently link DNA to proteins

followed by cell lysis and sonication obtain obtimal chromatin lengths. Chromatin was immunoprecipitated using antibody against acetylated histone H3. DNA-protein interactions were dissociated and DNA was purified for PCR using primers that amplified regions -142/+45 and -520/-360. Although it was previously shown that ACTH/cAMP-dependent gene transcription occurs upon binding of a complex containing SF-1/p54^{nrb}/PSF to region -57/-37 of the CYP17 promoter [12], studies described herein show that S1P activates CYP17 gene transcription by stimulating the binding of a trans-acting factor(s) to a more distal region of promoter (-436/-448).

As shown in Figure 23, Bt_2cAMP, So and S1P increased the acetylation of histone H3 associated with the region -520/-360 of the CYP17 gene. TSA also increased the acetylation of histone H3 at the CYP17 promoter (Figure 23). These findings are in contrast to the results of PCR using primers designed to amplify the -142/+45 region of the CYP17 promoter, where only Bt_2cAMP and TSA significantly increased the acetylation of histone H3 (Figure 24).

Since sphingolipids stimulated SREBP1 cleavage [62] and SREBP1a mediates increased transcription of StAR [63], we also performed ChIP assays using an antibody to SREBP1. As shown in Figure 25, ACTH, Bt_2cAMP and S1P increased binding to the -520/-360 region of the CYP17 gene.

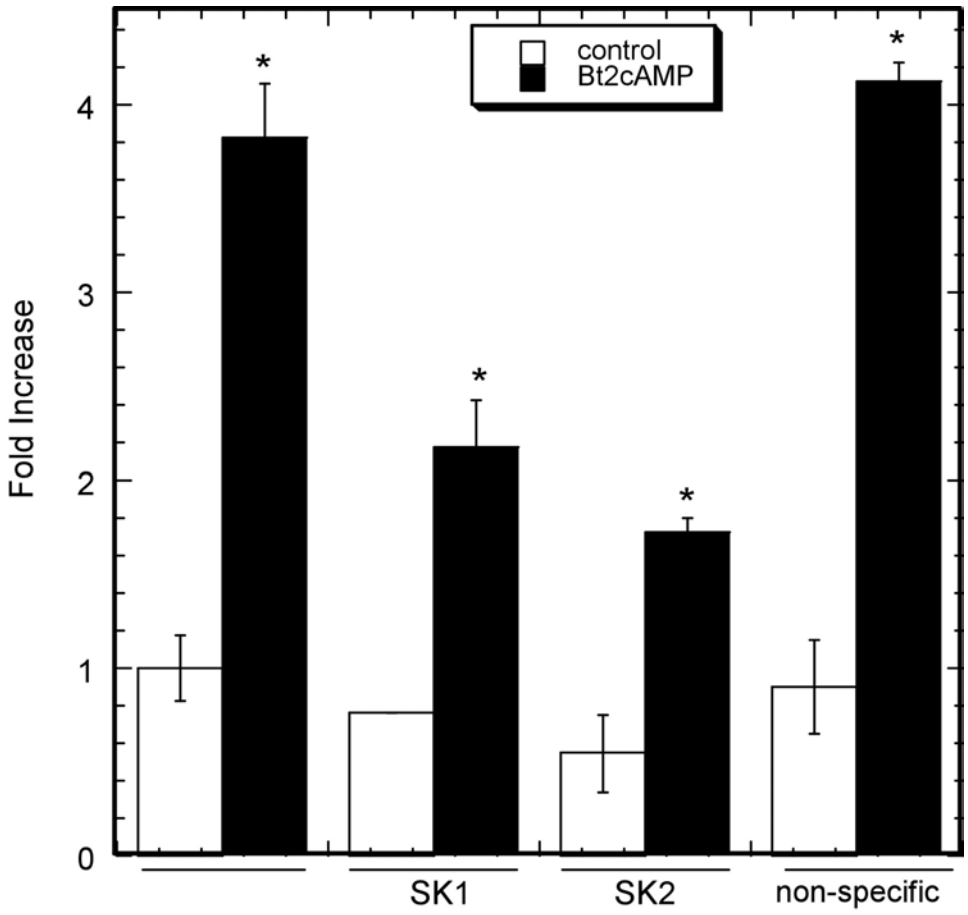


Figure 18. cAMP-dependent CYP17 mRNA expression requires SK1
H295R cells were transfected with SK1, SK2 or nonspecific siRNA as described in Materials and Methods and treated with 1 mM Bt_2cAMP. Total RNA was extracted and quantitative RT-PCR was performed by using primers for CYP17. Actin was used for normalization.

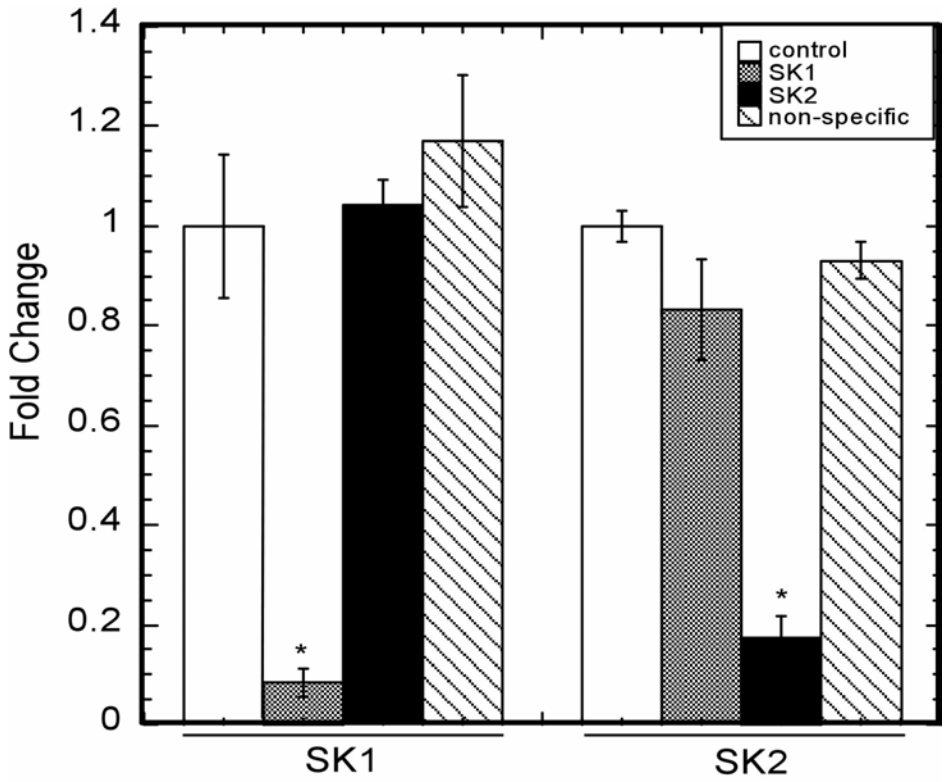


Figure 19. mRNA expression of SK1 and SK2 is decreased by siRNAs against SK1 and SK2, respectively

H295R cells were transfected with SK1, SK2 or nonspecific siRNA as described in Materials and Methods. Total RNA was extracted and quantitative RT-PCR was performed by using primers for SK1 and SK2. Actin was used for normalization.

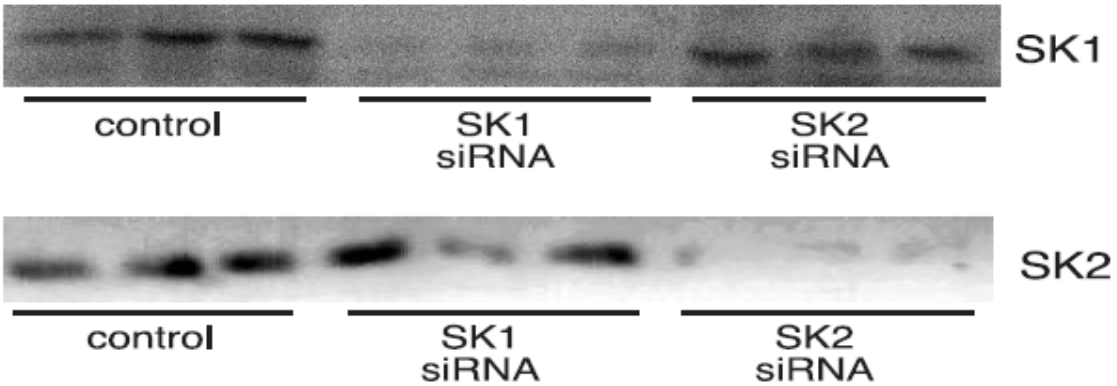


Figure 20. Effect of SK1 and SK2 siRNAs on protein expression of SK1 and SK2.

Cells were transfected with SK1, SK2 or nonspecific siRNA as described in Materials and Methods. Twenty five µg of cell lysates extracted from cells were resolved by SDS-PAGE and transferred to PVDF membranes. Blots were probed with antibodies to anti-SK1 and anti-SK2.

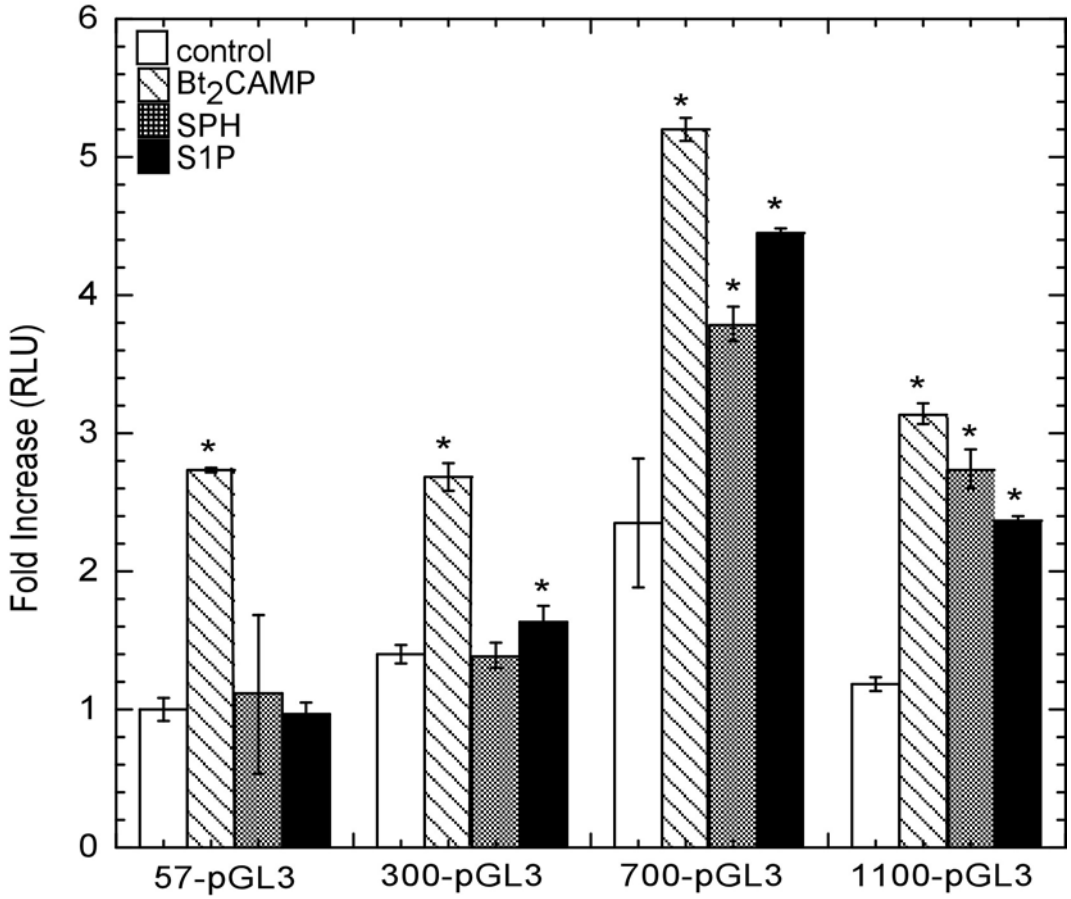


Figure 21. Transcriptional activity of CYP17 reporter plasmids
H295R cells were transfected with reporter plasmids containing 1100-, 700-, 300-, or 57 base pairs of the CYP17 promoter fused to the Firefly luciferase gene and subsequently treated with 1 mM Bt_2cAMP, 1 μM So, or 1 μM S1P for 6 hours. Data is normalized to the luciferase activity of the Renilla gene and is expressed as fold increase over the basic pGL3 plasmid. Data graphed represents the mean $\pm$ the standard error from 4 separate experiments, each performed in triplicate. *, statistically different from control, $p<.05$.

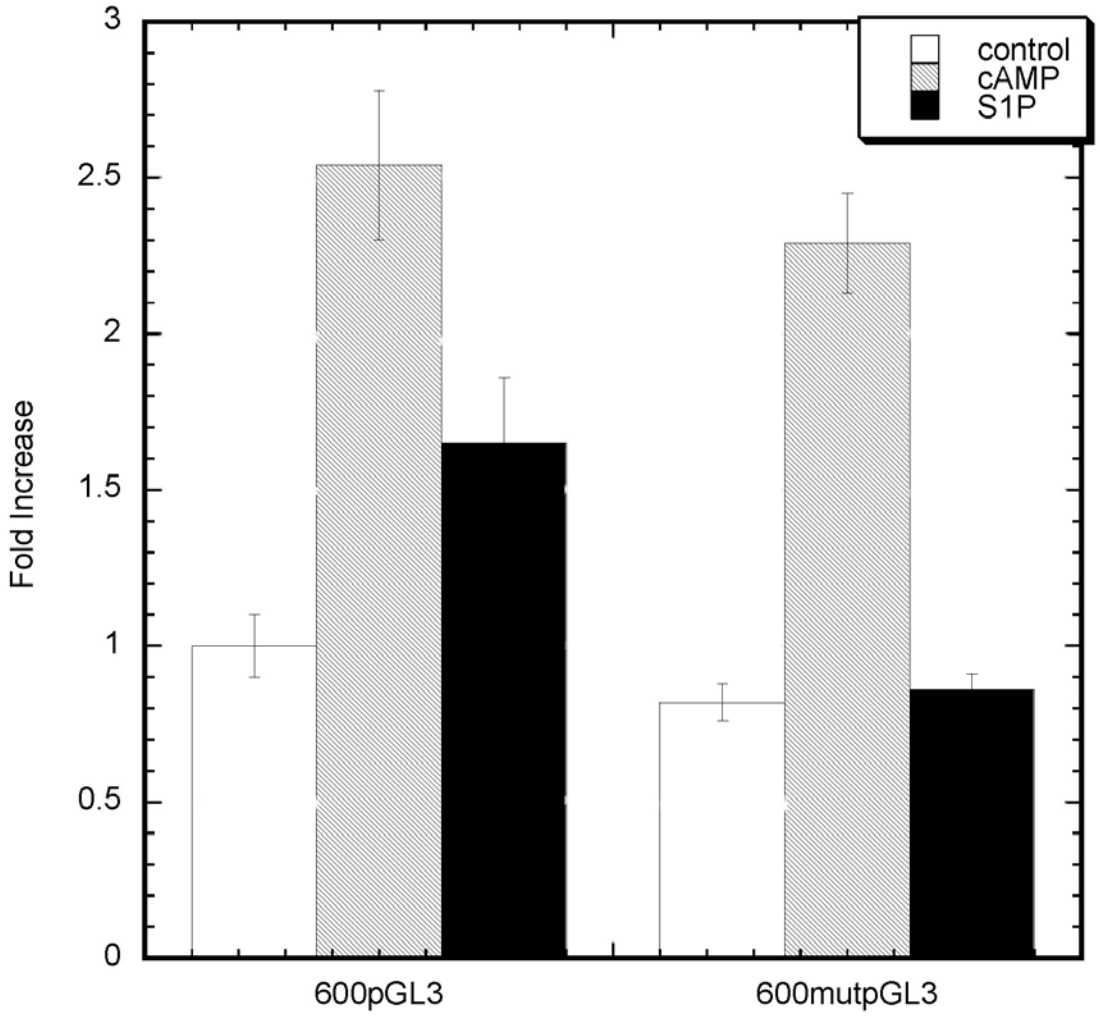


Figure 22. S1P-stimulation is lost when SRE site in CYP17 promoter is mutated H295R cells were transfected with wild type or SRE mutant reporter plasmids containing 600 base pairs of the CYP17 promoter fused to the firefly luciferase gene and subsequently treated with 1 mM Bt_2cAMP, 1 μM So, or 1 μM S1P for 12 hours. Data is normalized to the to the luciferase activity of the Renilla gene and is expressed as fold increase over the basic pGL3 plasmid.

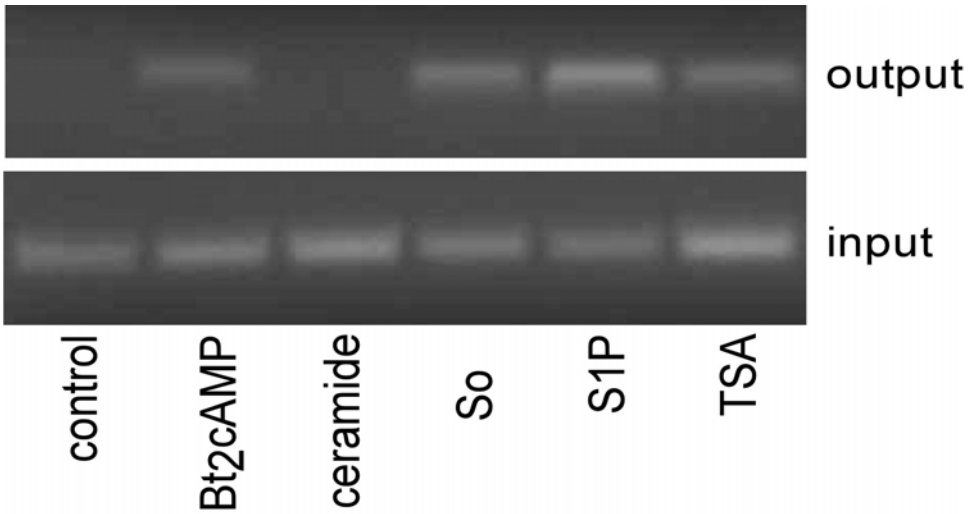


Figure 23. So and S1P increase acetylation of histone H3 at the SRE site of the CYP17 promoter

H295R cells were treated for 4 hours with 1 mM Bt_2cAMP, 1 μM C_2Ceramide, 1 μM So, 1 μM S1P or 500 nM TSA, and then incubated with 1% formaldehyde for cross-linking. Cell lysates were prepared, sonicated, and immunoprecipitated with anti-acetyl histone H3. PCR was performed using primers that amplified -520/-360 region of the CYP17 promoter.

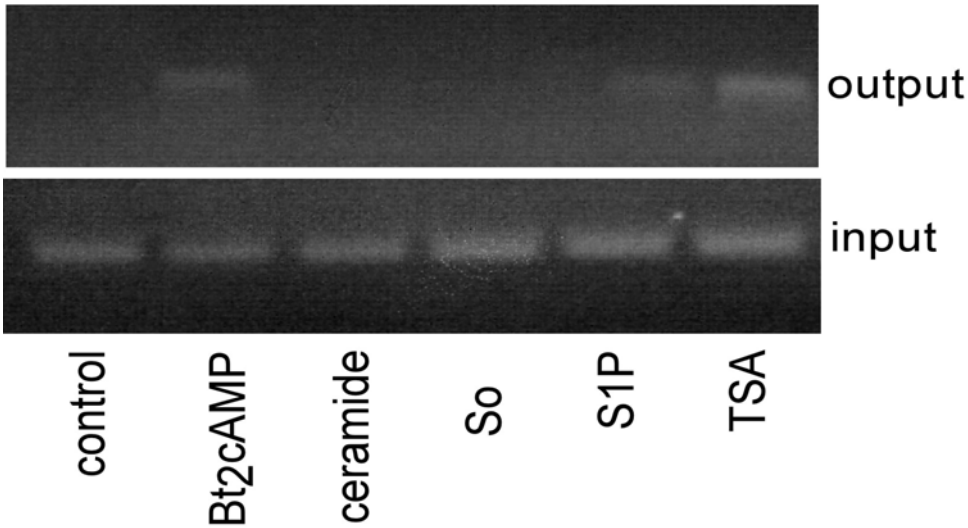


Figure 24. S1P has moderate effect on the acetylation of histone H3 at the proximal region of the CYP17 promoter

H295R cells were treated for 4 hours with 1 mM Bt_2cAMP, 1 μM C_2Ceramide, 1 μM So, 1 μM S1P or 500 nM TSA, and then incubated with 1% formaldehyde for cross-linking. Cell lysates were prepared, sonicated, and immunoprecipitated with anti-acetyl histone H3. PCR was performed using primers that amplified -142/+45 region of the CYP17 promoter.

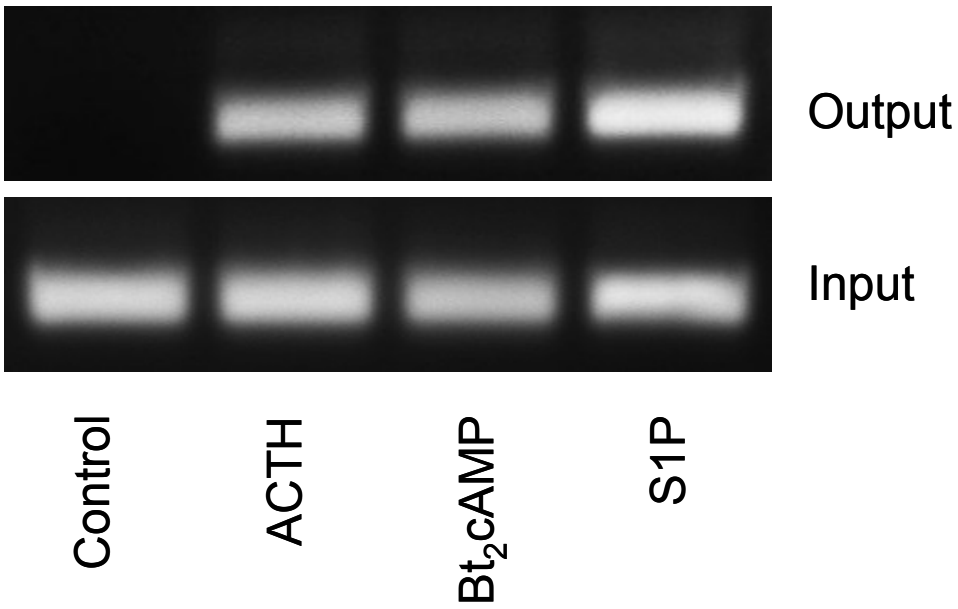


Figure 25. S1P increase SREBP1 binding to the CYP17 promoter
H295R cells were treated for 1 hour with 50 nM ACTH, 1 mM Bt_2cAMP or 1 μM S1P, and then incubated with 1% formaldehyde for cross-linking. Cell lysates were prepared, sonicated, and immunoprecipitated with anti-SREBP1. PCR was performed using primers that amplified -520/-360 region of the CYP17 promoter.

3.9 S1P induces CYP17 transcription by activating SREBP1.

As discussed before, it was previously shown that sphingolipids stimulated SREBP1 cleavage [62] and SREBP1a mediates increased transcription of StAR [63]. We next investigated the role of SREBPs in S1P-evoked CYP17 gene expression. H295R cells were transfected with SREBP1 or SREBP2 for 72 hours treated with S1P for 6 hours followed by total RNA extraction and quantitative RT-PCR.

As shown in Figure 26, SREBP1 siRNA completely attenuated S1P-stimulated transcription of CYP17, while siRNA targeted against SREBP2 decreased S1P-stimulated CYP17 mRNA expression by 31%. The effect of RNAi on SREBP1 and SREBP2 protein expression was confirmed by Western blotting (Figure 27).

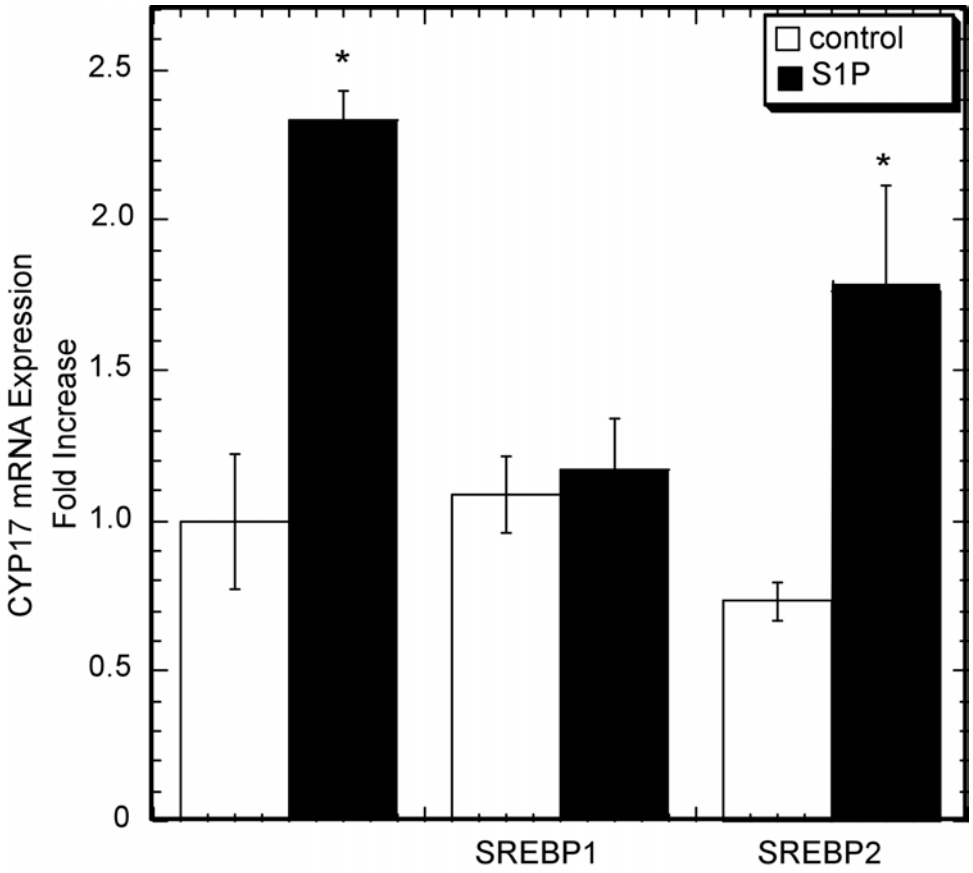


Figure 26. S1P-stimulated CYP17 mRNA expression requires SREBP1
H295R cells were transfected for 72 hours with SREBP1, SREBP2 or non-specific siRNA oligonucleotides as described in Materials and Methods and treated with 1 μM S1P. Total RNA was extracted and quantitative RT-PCR was performed to determine CYP17 mRNA expression. Actin was used for normalization. Values represent the mean ± SEM of 3 experiments, each performed in triplicate. *, $p<0.5$ statistically different from untreated control group.

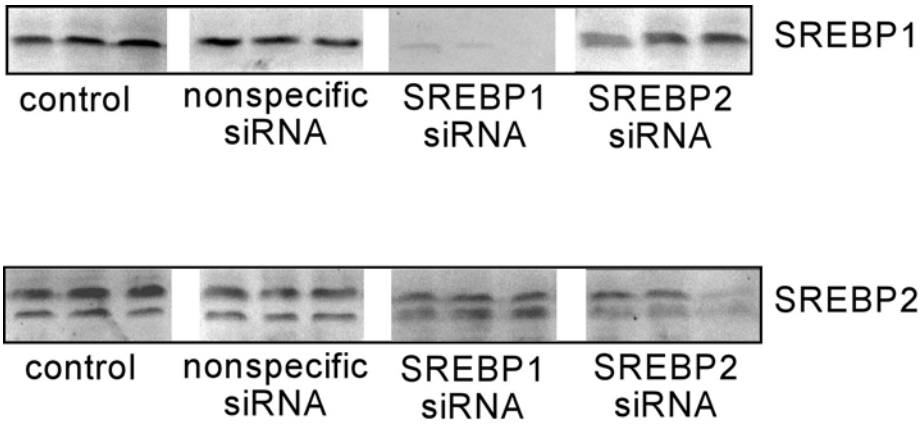


Figure 27. Protein expression of SREBP1 and SREBP2
H295R cells were transfected for 72 hours with SREBP1, SREBP2 or non-specific siRNA oligonucleotides as described in Materials and Methods. Twenty five µg of cell lysates extracted from cells were resolved by SDS-PAGE and transferred to PVDF membranes. Blots were probed with antibodies to anti-SREBP1 and anti-SREBP2.

3.10 S1P stimulates SREBP1 cleavage

The activity of SREBP1 is controlled by transport from ER to Golgi [60], [61]. SREBP1 has a bHLH domain at the N terminal that acts as DNA binding domain and two transmembrane domains that are cleaved by two membrane associated proteases in the Golgi [60], [61]. After this process the mature form is translocated to the nucleus where it activates transcription of SREBP target genes (Figure 6). To determine if S1P stimulates the processing of SREBP1, we treated H295R cells with S1P for varying time points and measured the levels of mature and precursor forms by Western blotting. Since the mature form of SREBP is subjected to ubiquitination and subsequent proteosomal degradation [76], we treated the cells with proteosome inhibitor N-acetyl-Leu-Leu-norleucinal (ALLN) as previously described by others [62], [77].

As shown in Figure 28, S1P stimulates SREBP1 maturation in a time dependent manner. Premature form also increased in a time dependent manner with S1P treatment.

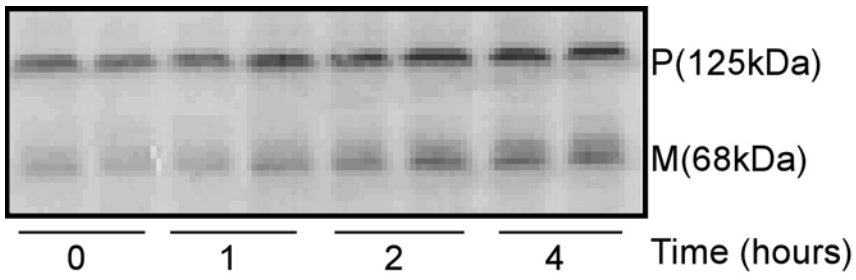


Figure 28. S1P promotes SREBP1 maturation
H295R cells were treated for 1 to 4 hours with 1 μM S1P. Two hours prior to harvesting, cells were treated with the proteosome inhibitor ALLN (25 μg/ml). The 1 hour group was first treated with ALLN followed by stimulation with S1P. Cell lysates were isolated and subjected to SDS-PAGE and Western blotting. Shown is a representative blot from three experiments, each performed in quadruplicate. S1P induce cleavage of the SREBP1 precursor (P) into the mature (M) transcription factor.

3.11 S1P stimulates nuclear translocation of SREBP1

To determine if S1P activates SREBP1 cleavage by binding to an S1P receptor or acting intracellularly, we treated H295R cells with dhS1P. Like S1P, dhS1P binds to and activates S1P receptors [78], [47]. Nuclear and membrane fractions were prepared

and SREBP1 expression in both subcellular compartments was analyzed by Western blotting.

Since both S1P and dhS1P stimulated an increase in the mature form of SREBP1 in the nucleus (Figure 29), it is plausible that dhS1P will have the same effects on CYP17 mRNA transcription. H295R cells were treated with Bt_2cAMP, S1P and dhS1P and isolated total RNA for quantitative RT-PCR. As shown in Figure 30, like Bt_2cAMP and S1P, dhS1P is able to induce CYP17 mRNA expression.

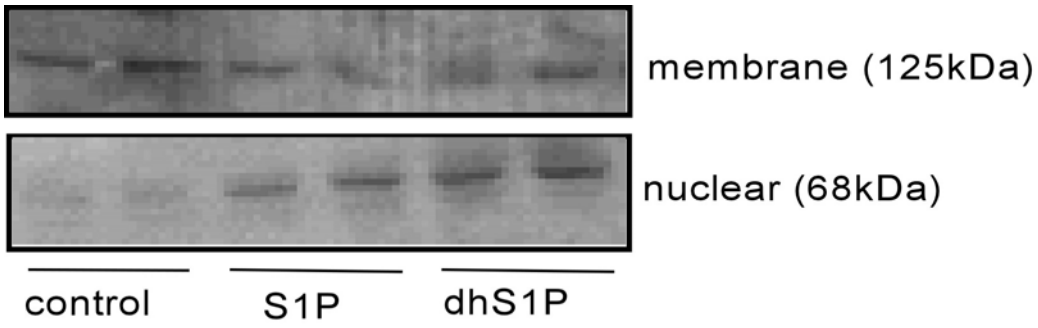


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Figure 29. S1P increases SREBP1 in the nucleus
H295R cells were treated for 4 hours with 1 μM S1P or 1 μM dhS1P and nuclear and membrane fractions isolated for analysis of SREBP1 protein expression by Western blotting as described in Materials and Methods. Shown is a representative blot from three experiments, each performed in quadruplicate.

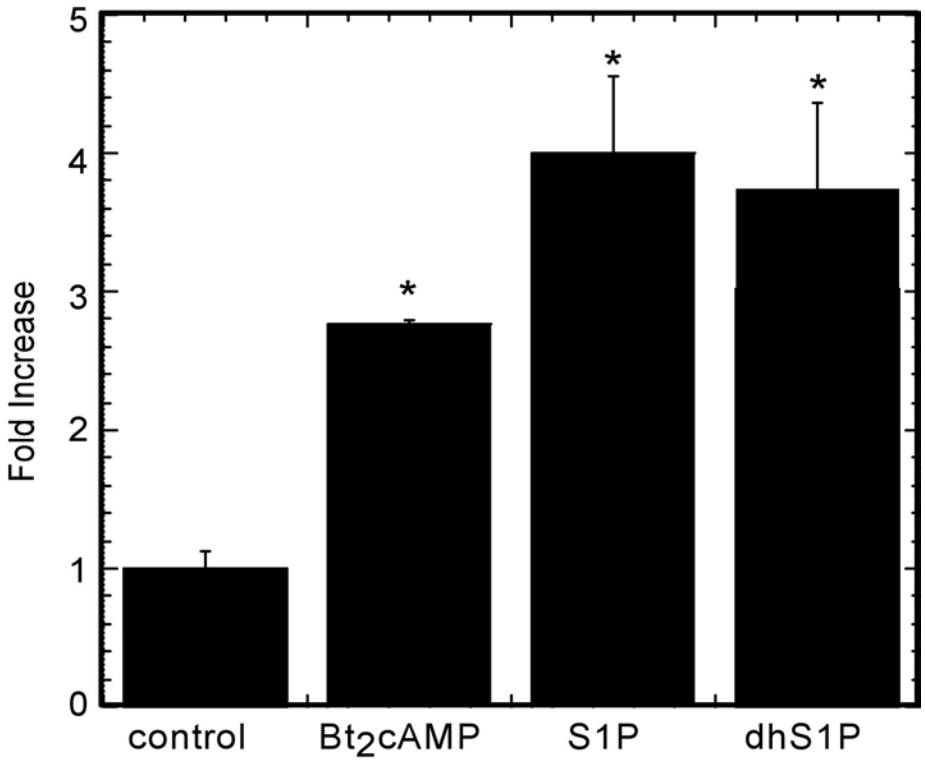

Figure 30. The effect of dhS1P on CYP17 mRNA expression
H295R cells were treated for 6 hours with 1 mM Bt_2cAMP, 1 μM S1P or 1 μM dhS1P and total RNA (100ng) was isolated and subjected to real time RT-PCR. Values represent the mean ± SEM of 3 experiments normalized to actin mRNA expression, each performed in triplicate. *, p<0.5 for cAMP-stimulated CYP17 mRNA expression versus untreated control.

3.12 S1P acts in a pararcrine/autocrine manner to induce CYP17 trancription

S1P regulates biological processes by serving as a ligand for S1P family of G-protein coupled receptors [79], [80] and by acting intracellularly [79]. To date, five S1P receptors have been identified [42], [43], [44]. These G-protein coupled receptors were initially called EDG receptors, but have been renamed as $S1P_1$ (EDG-1), $S1P_2$ (EDG-5), $S1P_3$ (EDG-3), $S1P_4$ (EDG-6) and $S1P_5$ (EDG-8) [42]. In the view of the fact that cAMP stimulates the secretion of S1P into the media (Figure 14) and dhS1P promotes

SREBP1 translocation to the nucleus (Figure 29), we hypothesized that S1P may act in an autocrine or paracrine manner to increase CYP17 mRNA expression by binding to S1P receptors on the surface of H295R cells. To identify which of the receptors are being expressed in H295R cells, we isolated total RNA and performed quantitative RT-PCR.

As shown in Figure 31 all five receptors are expressed in H295R cells. $S1P_3$ is highly expressed whereas $S1P_4$ is not as abundant as the other four receptor subtypes.

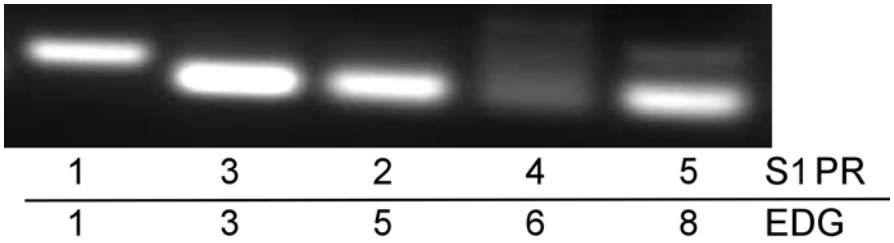

Figure 31. S1P receptors are expressed in H295R cells
The expression of S1P receptors was examined by subjecting total RNA (100ng) isolated from untreated H295R cells to real time RT-PCR using Taqman probes for EDG-1, -3, -5, -6 and -8.

CHAPTER IV
DISCUSSION

ACTH exerts its stimulatory actions on steroid hormone biosynthesis via two temporally distinct cAMP/PKA-dependent pathways. A rapid, acute response results in the transport of cholesterol into the inner mitochondrial membrane for conversion to pregnenolone by cholesterol side chain cleavage enzyme (P450scc). During the acute response, an essential site of phosphorylation by PKA is cholesterol ester hydrolase, which upon activation catalyzes the conversion of cholesterol esters to free cholesterol [81]. Key regulators of the acute response, StAR protein [82] and the peripheral benzodiazapene receptor [83], facilitate cholesterol movement in the mitochondria. The chronic effect of ACTH is to increase the transcription of steroidogenic enzymes, leading to increased steroid hydroxylase activity.

In this study, our data provide evidence for a novel signal transduction pathway initiated by ACTH. We show herein that ACTH and cAMP stimulate the metabolism of several sphingolipid species (Figure 7 to Figure 12). In addition, ACTH and cAMP rapidly and transiently activate SK catalytic activity (Figure 13). In agreement with this finding, SK has been shown to be activated by growth factors, cytokines, and hormones in various cell types [84], [32], [85].

Previous studies have demonstrated the relationship between SM and P450scc [86]. P450scc catalyzes the first step in steroid hormone biosynthesis: conversion of cholesterol to pregnenolone (Figure 1). SM inhibits the ability of cholesterol to bind to P450scc [86]. Further, the interaction between cholesterol and SM is cooperative [86], indicating that interactions between cholesterol and lipids can play a role in

steroidogenesis. In the light of these previous findings, the studies presented herein suggest that in addition to increasing availability of cholesterol for steroidogenesis, ACTH activates sphingolipid metabolism in order to maintain optimal cholesterol-lipid ratios in the membranes of adrenocortical cells.

Since decreases in levels of S1P were paralleled by increases in this bioactive molecule in the medium (Figure 14) and S1P receptors are expressed in H295R cells (Figure 31), we speculated that S1P acts in a paracrine or autocrine manner to increase steroidogenesis.

SREBPs regulate genes involved in the sterol synthesis and uptake, as well as fatty acid biosynthesis and desaturation [60], [61]. When the levels of free cholesterol in the cell are low, SREBPs undergo proteolytic processing, resulting in the release of a mature transcription factor that translocates to the nucleus for increased expression of genes involved in cholesterol biosynthesis and fatty acid metabolism [60], [61]. The activity of these transcription factors is controlled by transport from ER to Golgi by an escort protein called SREBP-cleavage activating protein (SCAP) which senses the absence of cholesterol [60], [61]. Recently sphingolipids have been shown to stimulate SREBP-1 cleavage by causing the cholesterol to be trapped in endosomes or lysosomes leading to a decrease in cellular cholesterol levels in the cells [62]. This decrease is sensed by SCAP which leads to the translocation of SREBPs to Golgi for maturation [62]. Moreover, transcription of the StAR protein (essential for acute hormone production in response to ACTH) is increased by SREBP-1 [63]. ChIP data presented in Figure 23 and Figure 25 showing increased recruitment of acetylated histone H3 and SREBP1 to the -700/-300 region of the CYP17 promoter suggest that S1P stimulates chromatin remodeling, resulting in increased accessibility to this region of the promoter and SREBP binding.

It was previously shown that the ACTH/cAMP responsive region of the human CYP17 gene lies within the first 63 base pairs upstream of the transcription initiation site [12], [87]. ACTH/cAMP increases the binding of complex containing SF-1, $p54^{nrb}$, and PSF to the -57/-37 region of the CYP17 gene by stimulating dephosphorylation of SF-1 in an MKP-1 dependent manner [12], [88]. In the present studies, transient transfection assays localized the region of the CYP17 promoter essential for S1P-dependent transcription between -700 and -300 upstream of the transcriptional initiation site (Figure 21), which is more distal from SF-1 binding site. Within this 400 base pair region there is a putative binding site for SREBPs. We confirmed the importance of this binding site for S1P-stimulated CYP17 gene transcription by constructing SRE mutant (Figure 22). Since S1P had no effect on transcriptional activity of the CYP17-57pGL3 construct (contains SF1 binding site) we have identified a novel mechanism by which cAMP/PKA activates CYP17.

Previously it was proposed by Lawler *et al*. that stimulation of human hepatocytes by TNF-α leads to the activation of neutral sphingomyelinase which in turn induces the maturation of SREBP1 [89]. This induction of SREBP1 cleavage occurred independent of cholesterol depletion. Further, it was suggested that while SRE-mediated gene transcription is decreased by the inhibition of ceramide synthesis, it is stimulated by increasing So levels [90].

According to these results we propose a model (Figure 32) for the role of S1P and SREBP1 in ACTH/cAMP-dependent CYP17 transcription and cortisol biosynthesis. Binding of ACTH to its receptor on cell surface activates adenylyl cyclase which leads to the production of cAMP and activation of PKA. PKA stimulates the hydrolysis of SM, which leads to the activation of other enzymes (such as SK) in sphingolipid pathway causing the decreased levels of several sphingolipid species. S1P produced from

cAMP-induced SK reaction is sensed by SCAP, which leads to the migration of SREBP1 to the Golgi where it will be processed to its mature form. Mature SREBP1 then translocates to the nucleus, dimerizes, and binds to the SRE site on the CYP17 promoter causing the activation of transcription of CYP17 gene. Additionally, based on the rapid increase in cortisol secretion after S1P treatment, we propose that S1P may also acutely facilitate cholesterol delivery to the mitochondria and/or increase the activity of the steroid hydroxylase P450 enzymes. Further studies are needed to determine the mechanism by which S1P acutely increases cortisol biosynthesis.

In summary, our studies demonstrate that ACTH/cAMP rapidly alter the cellular sphingolipid content in H295R cells. The actions of ACTH/cAMP on sphingolipid content are specific to individual sphingolipid species. S1P increases the transcription of human CYP17 and cortisol biosynthesis. ChIP experiments and RNA interference assays demonstrate the involvement of SREBP1 in the S1P-increased transcription of CYP17. These findings provide evidence for a role for sphingolipids in ACTH/cAMP-dependent cortisol biosynthesis and establish a novel mechanism by which S1P, acting as a paracrine or autocrine factor, can increase CYP17 gene transcription by activating SREBP1.

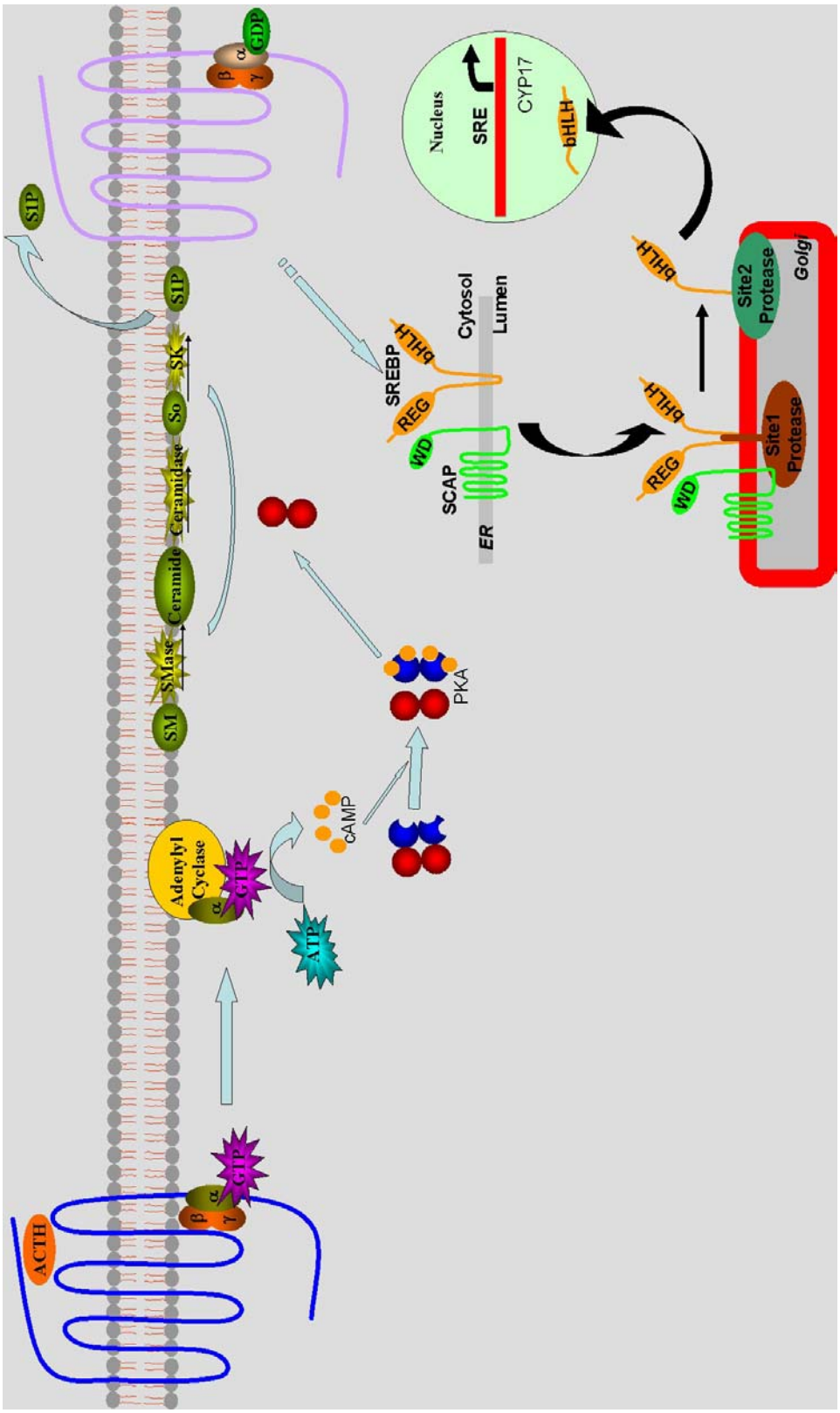

Figure 32. Model Pathway

APPENDIX-PROTOCOLS

Subculturing Cells

1. Prepare media
   Media: DMEM/F12
   To this add: 10% Nu-Serum, 1% ITS+, 1% Penicillin/Streptomycin Fungizone
2. Warm up PBS, bovine calf serum (BCS), and media.
3. Pour off media.
4. Add 2 ml trypsin. Pour off.
5. Allow to stand for 2-3 minutes.
6. Strike the flask with your hands a few times.
7. Add 1 ml serum.
8. Add 5-10 ml (~7 ml) PBS and pipet up and down to collect cells.
9. Transfer to a 15 ml tube.
10. Centrifuge 3 minutes, 1500 rpm.
11. Pour off supernatant in tube.
12. Add 6 ml media* to the pellet and pipet up and down to resuspend the pellet.
13. Add 12 ml media to each flask.
14. Add 3 ml cells to each flask.
15. Incubate at 37 ^{o}C.

* The volume of media added to the pellet depends on the final volume of cells needed for transfer.

	Media	Resuspended cells
T75 flask	12 ml	3 ml
6 well plate	2 ml	500 µl/well (3 ml/plate)
12 well plate	1 ml	250 µl/well (3 ml/plate)

Protein Isolation

6 well plate, 1 ml of media per well

1. Rinse dishes with PBS (2 x 1 ml).
2. Add 500 µl PBS to each well and scrape cells into 1.5 ml tubes.
3. Spin 5 minutes at 4000 rpm.
4. Remove supernatant.

5. Add 100 µl ice-cold RIPA buffer containing 1X protease inhibitor cocktail to each tube.
6. Pass 10 times through a 22-gauge needle.
7. Incubate cells on ice for 30 minutes.
8. Centrifuge tubes at 12K rpm, 4 ^{o}C, for 10 minutes.
9. Pipet supernatants into new tubes.
10. Store at –20 ^{o}C.

SDS-PAGE Gel

1. Resolving Gel (10 %, 10 ml):
    - 4.0 ml H_2O
    - 3.3 ml 30 % acrylamide
    - 2.5 ml 1.5 M Tris, pH 8.8
    - 0.1 ml 10 % SDS
    - 0.1 ml 10 % ammonium persulfate
    - 0.004 ml TEMED
2. Load gel.
3. Pour 30 % ethanol on top of gel
4. Stacking Gel (5%, 4 ml):
    - 2.7 ml H_2O
    - 0.67 ml 30 % acrylamide
    - 0.5 ml 1.5 M Tris, pH 6.8
    - 0.04 ml 10 % SDS
    - 0.04 ml 10 % ammonium persulfate
    - 0.004 ml TEMED
5. Remove the ethanol, load the stacking gel, and put the combs in.

Cell Lysate Preparation

1. Prepare loading buffer:
   2X SDS gel loading buffer: 1M DTT (9: 1)
2. Prepare a mixture of equal volumes of loading buffer and samples depending on the size of the wells (small—15-20 µl per well, large—30-40 µl per well)
3. Heat samples at 100 ^{o}C for 5 minutes. Put a hole on top of each tube.
4. Place tubes on ice.

Running SDS-PAGE Gel

1. Use standard markers.
2. Use 1X SDS-PAGE buffer.
3. Run the gel at 50-60 V.
4. When the bands reach the resolving gel, turn the voltage up to 100-120 V.

Transferring SDS-PAGE Gel

1. Use Western Blot Buffer :
    - 0.025 M Tris base
    - 0.192 M glycine
    - 20 % methanol
    - pH 8.3
2. Place three pre-soaked sheets of filter paper on bottom.
3. Place membrane soaked in methanol on top and then the gel.
4. Place three additional sheets of filter paper on top.
5. Transfer the gel at 100 mA for 30 minutes.
6. Place membranes in PBS-Tween.

Western Blotting

All at room temperature

1. Electrophoresis and blotting
    - Pre-treat PVDF membrane
    - In methanol for 5 second
    - In water for 5 minutes
    - In transfer buffer for 10-15 minutes
    - Separate proteins using SDS-PAGE electrophoresis and electroblotting on to PVDF membrane
2. Blocking Membrane
    - Immerse membrane in 5% (w/v) blocking agent in PBS-T for one hour on orbital shaker at room temperature
3. Washing
    - Dilute primary antibody in PBS-T
    - On orbital shaker wash twice for 2 min with large volume of PBS-T
    - On orbital shaker wash 2 times for 15 min each with large volume of PBS-T

- On orbital shaker wash 2 times for 5 min each with large volume of PBS-T

4. Incubation
    - Incubate membrane in diluted primary antibody for 1 hour on orbital shaker
5. Dilution of fluorecein-linked anti-species antibody
    - Dilute fluorescein-linked anti-mouse Ig or anti-rabbit Ig 1:2000 in PBS-T
6. Washing
    - On orbital shaker wash twice for 2 min with large volume of PBS-T
    - On orbital shaker wash 2 times for 15 min each with large volume of PBS-T
    - On orbital shaker wash 2 times for 5 min each with large volume of TBS-T or PBS-T
7. Incubation
    - Incubate membrane in diluted secondary antibody for 1 hour on orbital shaker
    - Dilute Anti-fluorescein alkaline phosphotase (AP) conjugate 1:2500 with PBS-T
8. Washing
    - On orbital shaker wash twice for 2 min with large volume of PBS-T
    - On orbital shaker wash 2 times for 15 min each with large volume of PBS-T
    - On orbital shaker wash 2 times for 5 min each with large volume of PBS-T
9. Incubation
    - Incubate membrane in diluted AP conjugate for 1 hour on orbital shaker at room temperature
10. Washing
    - On orbital shaker wash twice for 2 min with large volume of PBS-T
    - On orbital shaker wash 2 times for 15 min each with large volume of PBS-T
    - On orbital shaker wash 2 times for 5 min each with large volume of PBS-T
11. Transfer membrane onto a saran wrap (protein face up)
12. Incubation
    - Incubate membrane with ECF substrate enough to cover the membrane for 20 min
13. Incubation
    - Incubate membrane in dark place for at least 20 minutes to dry.
14. Scan

RNA Isolation

1. Wash cells 2x with PBS
2. Add 500 µl Trizol to each well and scrape into 1.5ml tubes
3. Incubate at room temperature for 5 minutes
4. Add 100 µl chloroform/ iso amy
5. Shake for 20 seconds
6. Incubate at room temperature for 3 minutes
7. Spin at 12,000 rpm for 15 minutes at 4C
8. Remove aqueous phase (clear) to new tube
9. Add 200 µl isopropanol
10. Incubate at room temperature for 10 minutes
11. Spin at 12,000 rpm 4°C for 10 minutes
12. Remove supernatant and add 500 µl 75% ETOH
13. Vortex briefly and spin at 12,000 rpm 4°C for 5 minutes
14. Remove EtOH
15. Air dry 15-30 minutes at room temperature
16. Resuspend in 25 µl DEPC-H_2O

Formaldehyde Gel

1.0 g agarose
84 ml DEPC H_2O
10 ml 10X gel-running buffer
6 ml formaldehyde

1. In an Erlenmeyer flask, heat a mixture of 1.0 g agarose, 84 ml DEPC H_2O, and 10 ml 10X gel-running buffer to boiling. Use microwave for ~3-4 minutes.
2. After cooling the mixture a few minutes, add 6 ml formaldehyde.
3. Pour into casting apparatus.

RNA Sample Preparation

1. Prepare sample dilution buffer. Will need 2X the volume of DEPC-H_2O in each tube. For example, if you have 24 samples and each RNA pellet was resuspended in 5 µl DEPC-H_20, then you will need 240 µl (10µl/sample).
   Sample dilution buffer (recipe makes 300 µl):
   a. 40 µl 10X buffer
   b. 40 µl RNA loading buffer
   c. 21 µl formaldehyde
   d. 200 µl formamide

2. Add sample dilution buffer to each tube. (If sample is 5 µl, then add 10 µl buffer).
3. Heat tubes at 65^{o}C for 15 minutes.
4. Add 1 µl ethidium bromide to each tube.
5. Cool for 10 minutes on ice.
6. Load samples onto formaldehyde gel.

Running the Gel

1. Use 1X buffer (100 ml 10X buffer and 50 ml formaldehyde diluted to 1 L).
2. Run at 70 V for about 2-3 hours.

Blotting the RNA

1. Place filter paper soaked in 20X SCC (pH 7) solution on the vacuum blotter.
2. Place membrane soaked in DEPC H_2O on top.
3. Place plastic mask on membrane so cut out region exposes membrane.
4. Place gel on top of membrane.
5. Place an additional sheet of filter paper on top.
6. Run for 30 minutes between 50 and 55.

Luciferase Assay

1. Wash cells once with PBS
2. Remove PBS completely
3. Prepare Passive Lysis Buffer by Diluting 5x stock with H_2O (1 part 5x: 4 parts Water)
4. Add 100 µl Passive Lysis Buffer to each well
5. Place plates in –80°C for 10-20 minutes
6. Place plates in 37°C water bath for approximately 1 minute (until contents in all wells have melted)
7. Repeat freeze/thaw cycle twice
8. Thaw appropriate volumes of Luciferase Assay and Stop and Glo Reagents
9. Luciferase Assay Reagent 100µl/sample
10. Stop and Glo Reagent 100 µl /sample
    - (Dilute Stop and Glo Substrate 1 part: Stop and Glo Buffer 49 parts)
11. Read RLU using Luminometer

Chromatin Immunoprecipitation (ChIP)

- Use 100 or 150 mm dishes of cells.
- Treat for appropriate time with desired agent/stimulus.
  1. After incubation time wash cells with 8 ml of PBS.
  2. Add 5 ml (for 100 mm dishes) or 12 ml (for 150 mm dishes) of 1% formaldehyde in PBS to each dish.
  3. Incubate for 10 minutes at room temperature with occasional swirling.
  4. Add 1 ml (for 100 mm dishes) or 2 ml (for 150 mm dishes) of 1M glycine (to reach a final concentration of 0.125M).
  5. Swirl and incubate for 5 minutes at room temperature.
  6. Wash cells twice with 8 ml PBS.
  7. Add 1 ml ice-cold RIPA buffer (with 1X protease inhibitors) to each dish.
  8. Scrape cells into 1.5 ml tube.
  9. Incubate on ice for 30 minutes.
  10. Sonicate 10 times for 5 sec each using Fisher 100 dismembraner set at 4.
      - KEEP SAMPLES COOL AT ALL TIMES.
  11. Spin at 12,000 rpm for 15 min at 4ºC.
  12. Add 1-10 µl antibody and incubate on tube rotator in cold room 2 h.
  13. Add 10 µl BSA (20 mg/ml), 5 µl salmon sperm DNA (5 mg), and 25 µl protein A/G agarose.
  14. Incubate overnight (o/n) at 4ºC on tube rotator.
  15. Spin 5 min at 4000 rpm.
  16. Place 50µl supernatant in clean tubes (label with ctrl input, dbc input, etc) place on ice
  17. Remove supernatant
  18. Wash beads using 0.5 ml of each buffer (IN ORDER) per wash for 5 min each wash (5 min on tube rotator followed by 2 min, 4000 rpm):
      - 3 X RIPA
      - 3 X RIPA + 500 mM NaCl
      - 3 X Washing Buffer
      - 3 X TE, pH 8
  19. Spin at 12,000 for 5 min
  20. Remove as much TE as possible without disturbing beads.
  21. Add: To each tube and to the input tubes from step 16
      - 450 µl water
      - 50 µl of 10 X Proteinase K buffer
      - 2.5 µl of 10 mg/ml RNase A (50 µg/ml final concentration)
  22. Incubate at 37°C for 1h.
  23. Add 2.5 µl of 20 mg/ml Proteinase K (100 µg/ml final concentration) and incubate o/n at 37°C.
  24. Add 90 µl of 5M NaCl and incubate at 65ºC overnight.
  25. Add
      - 300 µl Phenol
      - 300 µl Chloroform
  26. Shake vigorously
  27. Centrifuge for 5 min, 12000 rpm
  28. Transfer supernatant to clean tubes
  29. Add ethanol (volume equal to amount supernatant removed)

30. O/n in -20ºC
31. Spin 30 min at 12,000 rpm.
32. Bring up pellets in 25 µl water.

PCR Reaction:
Use 1 µl of crosslink output DNA for PCR. Also run 1 µl of uncrosslinked control input DNA.

For each reaction (total volume is 25 µl):
Using iTaq Supermix (Bio-Rad)

1 µl DNA (output or input)
1 µl Forward primer
1 µl Reverse primer
9.5 µl water
12.5 µl iTaq Supermix

Use ChIP PCR program on thermal cycler.
Program cycles:

1) 1 X 94ºC – 5 min
2) 35 X 95ºC – 1 min
   55ºC – 1 min
   72ºC –2 min
3) 72ºC – 10 min
4) cool to 4ºC

*Add protease inhibitor mix only to aliquoted amount of buffer needed just before use!!

Washing Buffer:

0.25M LiCl
1% NP40
1% sodium deoxycholate
1mM EDTA
10mM Tris-HCl, pH 8.1

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